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Sarcopenia and related body composition parameters: Assessment and protein biomarkers

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Für meine Eltern.

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List of abbreviations

ADM	adrenomedullin
ASMM	appendicular skeletal muscle mass
AUC	area under the curve
BFMI	body fat mass index
BIA	bioelectrical impedance analysis
BMI	body mass index
CCL28	C-C motif chemokine 28
CT	computed tomography
CVD	cardiovascular disease
DNER	delta and Notch-like epidermal growth factor-related receptor
DXA	dual-energy X-ray absorptiometry
EASO	European Association for the Study of Obesity
ESPEN	European Society for Clinical Nutrition and Metabolism
EWGSOP	European Working Group on Sarcopenia in Older People
FABP4	fatty acid-binding protein 4
GDF2	growth/differentiation factor 2
GH	growth hormone
HO-1	heme oxygenase 1
ICD-10-CM	International Classification of Diseases, Tenth Revision, Clinical Modification
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
KLK6	kallikrein-6
KORA	Cooperative Health Research in the Region of Augsburg
Lasso	least absolute shrinkage and selection operator
LEP	leptin
MB	myoglobin
MONICA	Monitoring of Trends and Determinants in Cardiovascular Diseases
MRI	magnetic resonance imaging
NAKO	German National Cohort (German: NAKO Gesundheitsstudie)
Notch 3	neurogenic locus notch homolog protein 3
NPX	normalized protein expression
NT-proBNP	N-terminal prohormone brain natriuretic peptide
PEA	proximity extension assay
PON3	paraoxonase
PRSS27	serine protease 27
RAGE	receptor for advanced glycosylation end products
SVM	support vector machine
TFPI	tissue factor pathway inhibitor
THBS2	thrombospondin-2
TIMP4	metalloproteinase inhibitor 4

Abstract (in English)

Background:

Detrimental alterations in body composition and strength have generally been overlooked regarding their impact on adverse health outcomes. In recent years, the recognition of disorders such as sarcopenia, a muscle disease characterized by low muscle strength and low muscle quantity/quality, amplified and observations of their associations with various chronic diseases and premature mortality accumulated. The public health and economic burden of sarcopenia and related adverse body composition such as obesity is expected to progressively increase in the future as a result of severe modifications in lifestyle, work, transportation, and demography. As these developments augment the requirement to advance medical research in terms of optimized diagnosis, targeted treatment, and more profound understanding of pathophysiological factors, this cumulative thesis aims to examine the expediency of the newest European definition for the diagnosis of sarcopenia and to identify potential biomarkers and thereby biological and pathophysiological determinants of sarcopenia-related body composition parameters.

Methods:

The data included in the present thesis was obtained from the population-based cohort studies the German National Cohort (NAKO) study (n = 200,389, aged 19 to 75 years), the Cooperative Health Research in the Region of Augsburg (KORA)-Age study (n = 1,012, aged 65 to 93 years) including 7-year mortality follow-up data, as well as the KORA S4 study (n = 1,478–1,484, aged 55 to 74 years) and its 14-year follow-up KORA FF4 (n = 608). Grip strength was measured in the KORA-Age and NAKO study by Jamar dynamometers. The body composition parameters appendicular skeletal muscle mass (ASMM), body fat mass index (BFMI), and the phase angle were assessed by bioelectrical impedance analysis in the KORA S4 and FF4 studies. The high-throughput proteomics technology proximity extension assay was implemented to measure targeted protein biomarkers in plasma samples collected during the KORA S4 study.

The statistical analyses comprised the assessment of grip strength across age of the adult life span in the data of the NAKO study using percentile curves. Cut-off points (for men and women) for the definition of probable sarcopenia, i.e. low grip strength, were derived from grip strength values of the NAKO study. Subsequent analyses in older adults of the KORA-Age study covered the comparison of the German NAKO-derived cut-off points with the cut-off points of the current sarcopenia definition of the European Working Group on Sarcopenia in Older People (EWGSOP) 2 regarding their resulting age-standardized prevalence of probable sarcopenia and time-dependent sensitivity and specificity for all-cause mortality. For the purpose of identifying potential biomarkers of sarcopenia-related body composition with KORA S4 and FF4 data, 233 protein markers were analyzed by boosting with stability selection to detect protein markers that encompassed strong associations with (low) ASMM, (high) BFMI, the coexistence of both (indicator of sarcopenic obesity), and the phase angle. The prediction accuracy of low ASMM, high BFMI, and their coexistence by protein markers besides classical risk factors was assessed with the cross-validated area under the curve. Furthermore, protein markers

selected for the phase angle were incorporated into a network and enrichment analysis to identify relevant biological factors related to the phase angle.

Results:
Grip strength over the adult life span increased until the age of ~40 years, followed by a progressive decline. The cut-off point values for probable sarcopenia derived from German NAKO data (men: 29 kg, women: 18 kg) were 2 kg higher than the EWGSOP2 values. In older adults, this difference caused a 1.5 x higher age-standardized prevalence of probable sarcopenia for the NAKO cut-off points compared to the current EWGSOP2 cut-off points. The sensitivity for all-cause mortality was higher and the specificity somewhat lower for the NAKO-derived cut-off points.

The analysis of targeted high-throughput proteomics enabled the identification of new markers of (low) muscle mass, (high) fat mass, the coexistence of a low muscle mass and a high fat mass, as well as the phase angle. Furthermore, adding protein markers to classical risk factors distinctly increased the prediction accuracy of low ASMM, high BFMI, and their coexistence. The protein profiles of ASMM and BFMI overlapped by several protein markers, whereas other protein markers were uniquely selected for either outcome. Insulin-like growth factor-binding protein 2 was strongly associated with ASMM, BFMI, and the phase angle. Myoglobin was strongly associated with ASMM and the phase angle, and adrenomedullin with BFMI and the phase angle. Another key finding entailed that the main biological processes that were related to the protein profile of the phase angle are involved in the regulation of cell mass and growth.

Conclusion:
This cumulative thesis contributes to the evaluation of the expediency of the current diagnostic criteria of sarcopenia by identifying that the grip strength cut-off points differ for reference populations, causing a large discrepancy in the prevalence of probable sarcopenia and that less conservative cut-off points could detect more patients at risk, thereby enabling an earlier intervention. Additionally, biostatistical analyses identified new potential starting points for future protein treatment targets for sarcopenia-related body composition parameters. The discovered links between their protein profiles suggest interrelationships between muscle mass, fat mass, and the phase angle on the molecular level and present jointly with the identified protein markers new insights into the pathophysiology of sarcopenia and related parameters.

Zusammenfassung (in German)

Hintergrund:
Ungünstige Veränderungen der Körperzusammensetzung und -kraft wurden häufig hinsichtlich ihrer nachteiligen gesundheitlichen Auswirkungen übersehen. In den vergangenen Jahren wurde die Bedeutung von Erkrankungen, wie z.B. der Sarkopenie, eine Muskelerkrankung, die durch eine geringe Muskelkraft und eine geringe Muskelquantität/-qualität charakterisiert ist, zunehmend erkannt sowie deren Assoziationen mit verschiedenen chronischen Erkrankungen und mit vorzeitiger Mortalität vermehrt beobachtet. Es ist davon auszugehen, dass aufgrund von starken Änderungen des Lebensstils, der Arbeitswelt, des Transportwesens und der Demografie die Belastung des öffentlichen Gesundheitswesens und die wirtschaftliche Belastung der Gesellschaft durch Sarkopenie und durch eine ungünstige Körperzusammensetzung, wie etwa die Adipositas, in der Zukunft progressiv zunehmen wird. Da diese Entwicklungen die Notwendigkeit verstärken, die medizinische Forschung durch eine optimierte Diagnostik, zielgerichtete Therapien und ein tieferes Verständnis der pathophysiologischen Faktoren voranzutreiben, zielt diese kumulative Dissertation darauf ab, die Zweckmäßigkeit der neuesten europäischen Definition zur Diagnose der Sarkopenie zu untersuchen und potentielle Biomarker sowie dadurch biologische und pathophysiologische Einflussfaktoren auf Sarkopenie-verwandte Körperzusammensetzungsparameter zu identifizieren.

Methoden:
Die vorliegende Arbeit basiert auf Daten der bevölkerungsbasierten Kohortenstudien NAKO Gesundheitsstudie (n = 200.389, im Alter von 19 bis 75 Jahren), der Kooperativen Gesundheitsforschung in der Region Augsburg (KORA)-Age Studie (n = 1.012, im Alter von 65 bis 93 Jahren) einschließlich Nachuntersuchungsdaten zur Mortalität nach sieben Jahren, sowie der KORA S4 Studie (n = 1.478–1.484, im Alter von 55 bis 74 Jahren) und dessen Nachuntersuchung nach 14 Jahren KORA FF4 (n = 608). Die Greifkraft wurde in der KORA-Age und der NAKO Studie mit Jamar Dynamometern gemessen. Die Körperzusammensetzungsparameter appendikuläre Skelettmuskelmasse (ASMM), Körperfettmassenindex (BFMI) und Phasenwinkel wurden in der KORA S4 und FF4 Studie mit der bioelektrischen Impedanzanalyse bestimmt. Die Hochdurchsatz-Proteomik Technologie Proximity Extension Assay wurde angewendet um targetierte Protein-Biomarker in Plasmaproben, die bei der KORA S4 Studie entnommen wurden, zu messen.

Die statistische Analyse umfasste die Darstellung der Greifkraft über das Alter während der Lebensspanne im Erwachsenenalter anhand von Perzentilkurven basierend auf den Daten der NAKO Studie. Zudem wurden Cut-off-Werte (für Männer und Frauen) für die Definition einer „wahrscheinlichen Sarkopenie“, d.h. einer geringen Greifkraft, von Greifkraftwerten der NAKO Studie abgeleitet. Darauffolgende Analysen von älteren Erwachsenen der KORA-Age Studie beinhalteten den Vergleich der deutschen NAKO-abgeleiteten Cut-off-Werte mit den Cut-off-Werten der aktuellen Sarkopenie Definition der European Working Group on Sarcopenia in Older People (EWGSOP) 2 hinsichtlich der daraus resultierenden altersstandardisierten Prävalenz der „wahrscheinlichen Sarkopenie“ und der zeitabhängigen Sensitivität und Spezifität für die Gesamtmortalität. Um potentielle Biomarker der

Sarkopenie-verwandten Körperzusammensetzung in KORA S4 und FF4 zu identifizieren, wurden 233 Proteinmarker durch „Boosting with Stability Selection“ analysiert, um Proteinmarker zu ermitteln, die eine starke Assoziationen mit (geringer) ASMM, (hohem) BFMI, der Koexistenz beider Faktoren (Indikator der „Sarcopenic Obesity“) und dem Phasenwinkel aufwiesen. Die Prädiktionsgenauigkeit von Proteinmarkern für eine geringe ASMM, einen hohen BFMI und deren Koexistenz zusätzlich zu klassischen Risikofaktoren wurde mit der kreuzvalidierten Fläche unter der Kurve analysiert. Die für den Phasenwinkel selektierten Proteinmarker wurden in eine „Network and Enrichment Analysis“ integriert, um relevante biologische Faktoren zu identifizieren, die mit dem Phasenwinkel zusammenhängen.

Ergebnisse:

Die Greifkraft stieg über die Lebenspanne im Erwachsenenalter bis ~40 Jahre an und fiel danach progressiv ab. Die von den deutschen NAKO Daten abgeleiteten Cut-off-Werte für eine „wahrscheinliche Sarkopenie“ (Männer: 29 kg, Frauen: 18 kg) waren 2 kg höher als die der EWGSOP2. Dieser Unterschied führte bei älteren Erwachsenen zu einer 1,5-mal höheren altersstandardisierten Prävalenz der „wahrscheinlichen Sarkopenie“ für die NAKO Cut-off-Werte im Vergleich zu den aktuellen EWGSOP2 Cut-off-Werten. Die Sensitivität für die Gesamtmortalität war bei den NAKO-abgeleiteten Cut-off-Werten höher und die Spezifität etwas niedriger.

Die Bestimmung von Proteinmarkern mittels targetierter Hochdurchsatz-Proteomik ermöglichte die Identifizierung neuer Marker der (geringen) Muskelmasse, der (hohen) Körperfettmasse, der Kombination einer geringen Muskelmasse und einer hohen Körperfettmasse sowie des Phasenwinkels. Des Weiteren erhöhte das Hinzufügen von Proteinmarkern zu den klassischen Risikofaktoren deutlich die Prädiktionsgenauigkeit für eine geringe ASMM, einen hohen BFMI und deren Koexistenz. Die Protein-Profile der ASMM und des BFMI überschnitten sich für einige Proteinmarker, während andere Proteinmarker spezifisch nur für eine der beiden Zielgrößen selektiert wurden. „Insulin-like Growth Factor-binding Protein 2“ war stark mit der ASMM, dem BFMI und dem Phasenwinkel assoziiert. Myoglobin war stark mit der ASMM und dem Phasenwinkel assoziiert, Adrenomedullin mit dem BFMI und dem Phasenwinkel. Darüber hinaus zeigten die Ergebnisse, dass die wesentlichen biologischen Prozesse, die mit dem Protein-Profil des Phasenwinkels in Verbindung standen, eine Rolle bei der Regulation von Zellmasse und Zellwachstum spielen.

Schlussfolgerung:

Diese kumulative Dissertation leistet einen Beitrag zur Bewertung der Zweckmäßigkeit der aktuellen Diagnosekriterien der Sarkopenie durch die Beobachtungen, dass Greifkraft Cut-off-Werte für verschiedene Referenz-Populationen variieren und diese Abweichungen zu einer großen Diskrepanz in der Prävalenz einer „wahrscheinlichen Sarkopenie“ führten. Zudem könnten weniger konservative Cut-off-Werte mehr Patienten als gefährdet erkennen und dadurch ein früheres Eingreifen ermöglichen. Darüber hinaus wurden mittels biostatistischer Analysen neue potentielle Ansatzpunkte für zukünftige Protein-Therapieangriffsziele für Sarkopenie-verwandte Körperzusammensetzungsparameter identifiziert. Die entdeckten Verbindungen zwischen deren Protein-Profilen implizieren

Zusammenhänge zwischen Muskelmasse, Körperfettmasse und dem Phasenwinkel auf molekularer Ebene und erlauben zusammen mit den identifizierten Proteinmarkern neue Einblicke in die Pathophysiologie der Sarkopenie und verwandter Parameter.

1 Introduction

1.1 The role of body composition and function in health and disease

Viewed from the outside the human body occurs in various sizes and forms, while the composition and detailed functions remain covered behind the exterior. The curiosity concerning the inside of the body, the body's components and their individual and interacting functions, roots already in the antiquity [1]. The early stages of body composition research developed many centuries later between the second half of the 19th and the first half of the 20th century [1] covering initially cadaver autopsy followed by in vivo methods to quantify body components [1, 2]. From the 1960s onwards, the body composition research gained increasing recognition and the awareness that body composition abnormalities are related to several disease outcomes expanded [1]. Today, we know that body composition and function disorders such as high fat mass, low lean body mass [3], and low muscle strength [4, 5] are associated next to various diseases with a higher risk of premature mortality.

Currently, disorders describing varying body size and body components that dominate medical research include predominantly obesity, i.e. excessive fat accumulation [6], and with increasing awareness also sarcopenia, a muscle disease characterized by low muscle strength and low muscle quantity and quality [7]. Although obesity [8] and sarcopenia [9] have been associated with numerous adverse health outcomes such as cardiovascular diseases (CVD) and (type 2) diabetes mellitus [8, 9], the recognition as independent diseases apart from the supplemental or secondary role to other disorders took many years. A major milestone in the recognition of sarcopenia as a disease presented the allocation of sarcopenia to the International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM) code M62.84 in 2016 [10]. Some years earlier, several official associations, including the American Medical Association in 2013, officially announced obesity as a disease [11].

In addition to the health consequences on an individual level, sarcopenia constitutes a public health burden with resulting adverse outcomes covering higher incidence of hospitalizations [12] as well as higher risk of falls [12, 13] and fractures [13]. The economic burden of healthcare [14] and hospitalization [15] costs per capita have been reported to be higher for patients with sarcopenia compared to those without sarcopenia. As the prevalence of sarcopenia increases with increasing age, factors such as the lifespan extension leading to older populations will cause a rising number of people with sarcopenia [16], thus amplifying the public health and economic burden in the future. Besides, the prevalence of obesity in the world almost tripled from 1975 to 2016, caused by for instance an increase in energy-dense nutrition and changes in work forms and transportation [6]. Moreover, obesity has been estimated to increase the average costs (including medical and non-medical costs) of different countries from 1.8 % of gross domestic product in 2019 to 3.6 % in 2060 [17].

The detrimental changes in body composition and function such as increasing fat mass as well as lower muscle mass and strength (sarcopenia) reinforced by demographic, work-related, dietary, and lifestyle

changes present a major public health and economic burden, which is expected to progressively expand in the future. Therefore, it is essential to drive medical research for prevention, diagnosis, and treatment to prepare for future medical challenges that alterations in demography and the way of life will induce on our body composition and muscle function.

1.2 Body composition

Body composition separates the human body into different parts followed by the quantification of these components. Many different compartment models subdividing the body by varying numbers of components have been developed, which commenced with the 2-compartment model consisting of fat mass and fat-free mass [2, 18]. These two components can be subdivided into further components. Moreover, components can be classified by different levels of body composition [18]. Since in 1992 Wang et al. [19] proposed the five-level model, human body composition has been commonly distinguished into the following levels: whole body, tissue-system (e.g. skeletal muscle and adipose tissue), cellular (e.g. cell mass), molecular (e.g. water and protein), and atomic (elements, e.g. hydrogen) [19]. Notably, a compartment model can incorporate components of different levels from these five levels of body composition.

The body components of the different levels can be assessed using a wide range of techniques including invasive and non-invasive [20] as well as indirect and direct methods [3]. Simple and inexpensive measurements comprise e.g. the body mass index (BMI) [20], waist circumference, waist-to-hip ratio, and calipometry to measure skin fold thickness [3]. The BMI is the most widely used parameter of obesity [3, 21] and a common concept known not only to medical professionals. A major disadvantage of the BMI is displayed in the inability to distinguish between fat mass and muscle mass [21], components that are in general oppositely associated with adverse health outcomes and that entail a tissue interconnection as fat can also infiltrate into muscle tissue [20, 22].

In body composition research, many different terms have been applied for the unofficial generic term muscle mass, e.g. fat-free mass, lean body mass, appendicular skeletal muscle mass (ASMM), and total skeletal muscle mass. Of note, appendicular refers to the upper and lower limbs [23] and is frequently assessed as these muscle regions are relevant for physical movement [24]. These various terms differ between measurement techniques and do not all refer to the same body components but are sometimes used interchangeably. For the parameter fat mass, commonly the same body component is referred to (adipose tissue consisting of, among others, adipocytes [21, 25]). However, for the generic term fat mass various different parameters have been used for research purposes, e.g. body fat percentage, body fat mass in kg, and body fat mass index (BFMI).

Measurements that are capable of assessing body fat include e.g. hydrostatic weighing and air displacement plethysmography, both premised on measuring the overall body density [20]. Muscle mass can be estimated by e.g. dilution techniques such as the D_3-creatine dilution method [26]. Other techniques include computed tomography (CT) and the commonly implemented method dual-energy X-ray absorptiometry (DXA), both based on the principle of varying X-ray attenuation of different tissues

or body components [3, 20]. Therefore, both measurements expose the body to radiation [3, 20, 23], while the radiation exposure of DXA is considerably lower [3]. Similar to CT, magnetic resonance imaging (MRI) is an imaging technique that can be applied to quantify muscle and fat mass [20]. Whole body CT and MRI are generally considered as the gold standard for body composition assessment, while the 4-compartment model that combines different methods was further described as a gold standard [18]. Common disadvantages of DXA, CT, and MRI are the high costs [3] and the restricted transportability [3, 23]. Additionally, manual image segmentation for CT and MRI requires experts [25].

A very frequently implemented method to assess body composition is the bioelectrical impedance analysis (BIA) [27]. The technology is based on the impedance of the body, i.e. the opposition of conductors to an alternating electrical current [27, 28]. The impedance depends on the resistance and reactance. The resistance reflects the resistive components of the body including fluid and electrolytes, whereas the reactance reflects the capacitive components comprising tissue interfaces and cell membranes [28]. Followed by the measurement of resistance and reactance, these values can be incorporated together with other variables such as sex, height, and weight into equations to calculate for instance skeletal muscle mass and body fat mass. The BIA is a more practical tool especially for large-scale studies compared to the methods described above, as the BIA is portable, inexpensive [3, 21, 26], does not expose to radiation [21], and is noninvasive [26, 27]. Disadvantages involve that BIA requires equations that fit to the target population (predominantly similar age and ancestry) to estimate muscle and fat mass parameters. Furthermore, the measurement values of BIA can be affected by influencing factors such as hydration [3, 26].

A unique capability of the bioelectrical impedance in contrast to other body composition methods entails the measurement of the phase angle. The phase angle is until today incompletely defined in the medical and biological setting as it is not uniquely allocated to a disease area and does not encompass a definite biological meaning. Instead, the phase angle has been related to various diseases, among these sarcopenia [29] and obesity [30]. The main application of the parameter though, has been to reflect the nutritional status of patients [31]. From a technical point of view, the phase angle is the angular transformation of reactance (Xc) to resistance (R) [28]:

$$arc\ tangent\ (Xc/R) \times (180°/\pi)$$

In the representation of the body as a circuit, the alternating electrical current that flows through the body during BIA conducts in parallel setting through the extracellular space and the cells, which consist of the intracellular fluid and the surrounding cell membranes [28]. The current progresses through the fluids continuously, whereas the cell membranes act as capacitors by storing electrical charge, which results in a delay of the current penetrating the cell membranes leading to an out-phasing of the current [28]. This delay is derived in degrees as the phase angle and thus the phase angle indicates the amount of current that flows through capacitive elements [28]. Based on these technical aspects, a higher phase angle is thought to indicate a higher body cell mass [28] with an interrelated lower ratio of extracellular to intracellular fluid volume [31]. However, the biomedical meaning of the phase angle remains to be determined beyond technical aspects of the measurement technique.

1.3 Sarcopenia

The history of the term sarcopenia leads only back a few decades to 1988, when the designation was first introduced at a meeting by Irwin H. Rosenberg described as the decline in lean body mass with aging [32]. However, Rosenberg noted that the loss of muscle mass and strength with aging was already reported earlier [33]. One of the first approaches to define sarcopenia was conducted by Baumgartner et al. published in 1998 and described as the "[…] appendicular skeletal muscle mass (kg)/height^2 (m^2) being less than two standard deviations below the mean of a young reference group" [34]. Further approaches to define sarcopenia followed, including a differentiation into sarcopenia class I and class II based on the skeletal muscle mass index (skeletal muscle mass / body mass x 100) by Janssen et al. in 2002 [35]. Several definitions and cut-off points for muscle mass parameters followed, while muscle function (or rather muscle strength) was increasingly included in the definitions of sarcopenia. Of note, several findings hinted towards an advantage of muscle strength exceeding muscle mass for the definition of sarcopenia due to stronger associations with disability and mortality [36]. A milestone in the standardization of the sarcopenia definition was achieved with the publication of an European consensus to diagnose sarcopenia by the European Working Group on Sarcopenia in Older People (EWGSOP) in 2010 [37]. The diagnosis of sarcopenia was based on low muscle mass and low muscle function (either strength or performance) with different cut-off points suggested for these parameters. The cut-off points for a low grip strength were < 30 kg for men and < 20 kg for women [37] based on the work of Lauretani et al. (2003) [38]. In 2018, the EWGSOP met again to discuss an update of the first consensus definition [7]. This new and current EWGSOP2 sarcopenia definition from 2018 recommends a pathway described as "Find-Assess-Confirm-Severity" [7], which is detailed in the following. The diagnosis of sarcopenia is performed by the parts "Assess" and "Confirm" [7].

1. Find: The first step is to "find cases" at risk for sarcopenia by clinical suspicion for symptoms that are related to sarcopenia or by implementing the self-reported SARC-F questionnaire [7], which includes five items to identify if patients hold limitations of e.g. physical function or mobility that are relevant for sarcopenia [39].
2. Assess: **Probable sarcopenia** is detected by a low muscle strength, which can be assessed by grip strength or the chair stand test [7].
3. Confirm: **Sarcopenia** is **confirmed** by low muscle quantity or quality (assessed by DXA, BIA, CT, or MRI) in addition to low muscle strength [7].
4. Severity: **Severe sarcopenia** is identified if an individual with a confirmed sarcopenia additionally exhibits low physical performance (assessed by gait speed, Short Physical Performance Battery, Timed-Up and Go test or the 400-m walk test) [7].

Distinct alterations of the sarcopenia definition from EWGSOP1 (2010) to EWGSOP2 (2018) were declared in the 2018 update involving that sarcopenia can also occur earlier in life and that the causes of sarcopenia surpass the aging process [7]. The key modification entailed that muscle strength was established as the main component of sarcopenia and that muscle mass was classified as only secondary to muscle strength [7]. Additionally, the cut-off points for low grip strength were changed to

< 27 kg for men and < 16 kg for women [7] based on a study by Dodds et al. from 2014 [40], which pooled data from 12 British studies to determine the cut-off points as 2.5 standard deviations below the peak mean. The peak mean of grip strength was identified at 32 years for both men and women [40]. Since these cut-off points are based on one limited geographical region and studies observed differences in grip strength between European regions [41, 42], verification of these cut-off points in other samples of different European countries is necessary to ensure suitability of the cut-off points for Europe beyond Great Britain. According to the EWGSOP update, a high priority for research concerns the development of validated cut-off points that are predictive for hard end-points. Additionally, further research is needed to identify if region-specific cut-off points for muscle strength improve the prediction of outcomes [7].

Beyond the updated sarcopenia definition, new developments cover the accumulation of suggestions regarding the phase angle as a potential marker of muscle quality, which may be incorporated in the definition of sarcopenia [29, 43, 44] and assessed in individuals with obesity [30]. Nevertheless, since the biomedical meaning of the phase angle is incompletely understood as depicted above, research to elucidate biological knowledge of the phase angle on a tissue, cellular, and molecular level should proceed prior to establishing the phase angle as a marker of muscle quality. In comparison, other diagnosis parameters for sarcopenia such as skeletal muscle mass and grip strength are sufficiently defined regarding their biomedical meaning.

Compared to already longer established diseases, the definition of sarcopenia underwent several alterations within the last years and is still under development. This highlights the urgent need for a sustainable definition that can be applied in a standardized manner. At the current stage, the examination of the individual components included in the definition of sarcopenia such as muscle mass, grip strength, and physical performance may facilitate the comparability of study results.

Sarcopenic obesity

Sarcopenic obesity is the coexistence of sarcopenia and obesity [45]. Following several years of research lacking a standardized definition, sarcopenic obesity was finally defined for the European population by the European Society for Clinical Nutrition and Metabolism (ESPEN) and the European Association for the Study of Obesity (EASO) in a consensus statement published early in 2022 [45]. This definition of sarcopenic obesity is based on the existence of both low skeletal muscle function (muscle strength) and altered body composition that is indicated by increased fat mass and reduced muscle mass [45].

The concept of sarcopenic obesity was based on the observation that a low muscle mass combined with a high fat mass encompasses a more detrimental effect on health outcomes compared with the diseases sarcopenia and obesity alone. Participants with a low ASMM/stature^2 and a coexisting high body fat percentage at baseline were observed to report 2 to 3 x more onset of instrumental activities of daily living disability compared to participants with only sarcopenia or obesity after a follow-up of up to eight years [46]. Individuals with sarcopenic obesity (sarcopenia based on EWGSOP1 criteria and

BMI ≥ 30.0 kg/m^2) were observed to have a higher fall risk and a lower health-related quality of life compared to participants with only sarcopenia [47]. Additionally, prior studies suggest a higher cardiovascular risk and a higher mortality in sarcopenic obesity (defined by varying criteria) compared to solely sarcopenia or obesity [48, 49]. The more detrimental effect of sarcopenic obesity might result from the similar biological and pathophysiological factors that influence both sarcopenia and obesity. These similar factors include among others aging, lower physical activity and other unfavorable lifestyle changes, decreased metabolic rate, and alterations in hormone levels [49]. Furthermore, the causes of sarcopenic obesity are considered to cover among other factors the fat infiltration into muscle, leptin resistance, and muscle inflammation resulting from higher secretion of adipokines and cytokines [47]. Additionally, in obesity, the fat accumulation in skeletal muscle augments, which results in increased insulin resistance [22]. However, the established mechanisms that connect skeletal muscle and fat mass on a molecular level are limited. The identification of similar molecular markers for (low) muscle and (high) fat mass may support the explanation of the pathophysiology of sarcopenic obesity.

1.4 Biomarkers of sarcopenia and related body composition parameters

Biomarkers can be described as quantitative indicators of biological and pathophysiological processes [50], which are applied to several aspects of diseases such as diagnosis, progression, monitoring, differentiation for severity, identification of drug targets, and understanding of pathogenic mechanisms [50, 51]. As detailed in the chapter above, the main diagnostic markers of sarcopenia and sarcopenic obesity (muscle strength, skeletal muscle mass, and body fat mass) have already been defined, whereas the use of molecular biomarkers in diagnosis and personalized prevention/treatment of sarcopenia and sarcopenic obesity is still in its infancy. General strategies to decrease the progression of sarcopenia [52, 53] and sarcopenic obesity [53] are dietary interventions and physical exercise. These treatments are rather unspecific as they are employed to counteract various adverse health outcomes. More specific intervention strategies based on molecular mechanisms, as for instance the approach of targeting senescent cells that produce among others proinflammatory cytokines [54], are rare but necessary to complement the general intervention strategies. Besides the implementation as potential targets for intervention, molecular markers measured in for instance serum, plasma or urine enable the identification of the underling pathophysiological factors and mechanisms of diseases. Thus, identifying specific markers for sarcopenia and sarcopenic obesity is an important future aim. However, considering the still developing definitions of these disorders, which already changed several times and might therefore be altered again in the future, additionally identifying potential biomarkers of the individual components of the disease entities (such as muscle mass) may be more sustainable.

Protein biomarkers

Proteins are a common target of medical research as they play a role in nearly all biological activities providing a comprehensive opportunity to understand the biology of diseases [55] and because they represent the majority of drug targets [50]. Prior research attempting to determine potential protein

biomarkers of sarcopenia and related body composition primarily focused on single or only a few markers. For instance inflammation markers such as interleukin-6 and C-reactive protein have been frequently associated with muscle mass and muscle strength in inverse direction and with fat mass in positive direction [54]. However, a single biomarker is insufficient to understand the pathophysiology of sarcopenia as the disease is multidimensional [56] and caused by a combination of factors [53]. An advancement to identify several potential protein biomarkers at once presents the simultaneous measurement of many proteins, termed proteomics. In this context, proteomics describes the quantitative assessment of proteins that are encompassed in a cell, tissue or an organism [57].

Various techniques have been developed to measure proteins and the progression of research in this area has accelerated over the last few years. Next to conventional techniques, which are limited to the measurement of a few specific proteins such as enzyme-linked immunosorbent assays or western blotting, more advanced techniques to perform high-throughput proteomics (measuring many proteins for many samples simultaneously) such as protein microarray or mass spectrometry have been developed [57]. High-throughput proteomics present a promising opportunity to accelerate biomarker discovery, to identify many potential biomarkers at once, and to receive a more comprehensive picture of the underlying biology of the investigated outcome.

1.5 Aims of the cumulative thesis

Figure 1 illustrates the interfaces of the individual topics of the three manuscripts incorporated in this cumulative thesis.

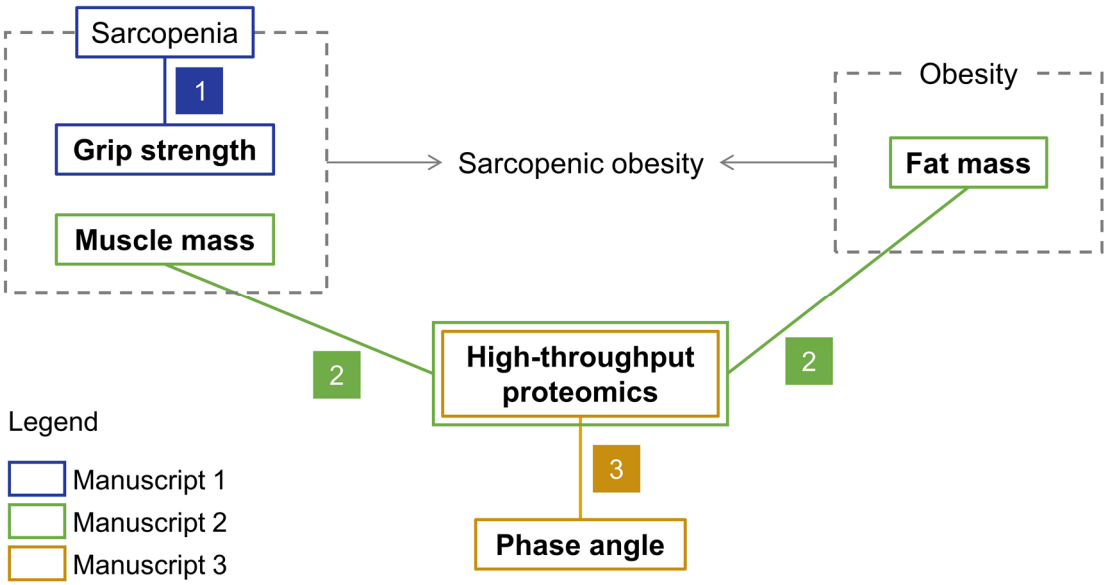

Figure 1: Overview of the general topics of the manuscripts reported in this cumulative thesis

With regard to the progressively increasing public health burden of sarcopenia and related adverse body composition, reinforced by demographic and detrimental work and lifestyle changes, requiring effective diagnosis and treatment, this cumulative thesis aims to examine the expediency of the newest sarcopenia definition and to identify potential biomarkers and thereby biological/pathophysiological determinants of related body composition. Specifically, the objectives of the manuscripts of this cumulative thesis comprise:

1. The comparison of grip strength cut-off points for probable sarcopenia from the EWGSOP2 with cut-off points derived from a large German population-based sample through resulting prevalence of probable sarcopenia and by prediction of all-cause mortality in older people (Manuscript 1) [58].
2. The identification of new protein markers for (low) muscle mass, (high) fat mass, the coexistence of a low muscle and a high fat mass, and their change over 14 years using a proteomics approach as well as the evaluation of the importance of protein markers beyond classical risk factors for the prediction of these body composition parameters (Manuscript 2) [59].
3. The detection of protein markers strongly associated with the phase angle and thereby related biological factors through proteomics, contributing to a more comprehensive understanding of the biomedical meaning of the phase angle (Manuscript 3) [60].

2 Manuscripts: Overview and contributions

This cumulative thesis incorporates three manuscripts. This chapter entails a short description of the manuscripts' key data and my contribution to the manuscripts.

2.1 Manuscript 1

Manuscript 1 entitled "Grip strength values and cut-off points based on over 200,000 adults of the German National Cohort - a comparison to the EWGSOP2 cut-off points" was published in *Age and Ageing* in 2023 [58]. We analyzed data from 200,389 participants aged 19 to 75 years obtained from the German National Cohort (NAKO, German: NAKO Gesundheitsstudie) and data from 1,012 participants aged 65 to 93 years derived from the Cooperative Health Research in the Region of Augsburg (KORA)-Age study. With the NAKO data, we deduced cut-off points for probable sarcopenia, i.e. low grip strength. In the KORA-Age data, we compared the prevalence of probable sarcopenia as well as the time-dependent sensitivity and specificity for all-cause mortality of the NAKO-derived to the EWGSOP2 cut-off points. We observed that the German NAKO-derived cut-off points were 2 kg higher than the ones of the EWGSOP2 for both men and women, which yielded a relatively large discrepancy in the resulting prevalence of probable sarcopenia and a higher sensitivity as well as somewhat lower specificity for all-cause mortality of the NAKO-derived cut-off points [58].

Contribution to Manuscript 1

As the first author of Manuscript 1, I initiated the project through selecting the research topic and developing the research questions. I wrote the draft of the proposal for the data application of the NAKO data and subsequently applied for the NAKO and KORA-Age data. Furthermore, I performed some parts of the literature research, developed all parts of the statistical analysis plan and performed all following statistical analyses. I wrote the entire drafts of the manuscript and supplement and created all tables and figures. After distribution of the manuscript to all coauthors, I incorporated the comments of the coauthors, prepared the paper for submission, and subsequently submitted it to the journal. I further wrote the draft for the revision of the manuscript in response to the peer reviewers' comments.

2.2 Manuscript 2

Manuscript 2 entitled "Proteomic profiling of low muscle and high fat mass: a machine learning approach in the KORA S4/FF4 study" was published in 2021 in the *Journal of Cachexia, Sarcopenia and Muscle* [59]. We derived data from the KORA S4 and FF4 studies encompassing 1,478 participants aged 55 to 74 years in the cross-sectional and 608 participants in the longitudinal analysis. We implemented several machine learning methods to identify protein markers strongly associated with (low) muscle mass, (high) fat mass, and their coexistence and to identify the relevancy of protein markers beyond classical risk factors for the prediction of these outcomes. We identified several previously unknown protein markers for (low) muscle mass, (high) fat mass, and their coexistence and further observed that

the protein profiles of muscle and fat mass partially overlapped. Adding protein markers to classical risk factors increased the prediction accuracy of all three binary outcomes, i.e. low muscle mass, high fat mass, and their coexistence [59].

Contribution to Manuscript 2

My contribution as the first author of Manuscript 2 encompassed the process from finalizing the research question to the publication of the manuscript. To initiate the process, I conducted extensive literature research regarding the central topic and suitable statistical analysis methods. I contributed to the research question by redefining the main outcomes of the project. Furthermore, I performed and documented some parts of the general quality control of the proteomics data before the data was transferred to the KORA database for subsequent use by data applicants including myself. I applied for all data, conceived the largest part of the statistical analysis plan and performed all statistical analyses. Moreover, I drafted all sections of the manuscript as well as the supplement and produced all corresponding figures and tables. I implemented the suggestions by the coauthors and prepared the submission of the paper to the journal. After submitting the paper for publication, I revised the manuscript according to the peer reviewers' comments.

2.3 Manuscript 3

Manuscript 3 is entitled "Proteomics of the phase angle: Results from the population-based KORA S4 study" and was published in 2022 in *Clinical Nutrition* [60]. We analyzed data of 1,484 participants aged 55 to 74 years from the KORA S4 study. We investigated the proteomic profile of the phase angle as well as related gene ontology terms to contribute to the elucidation of the biomedical meaning of the phase angle. We identified seven protein markers that were strongly associated with the phase angle of which six markers were previously unknown and identified that the key biological processes related to the phase angle's protein profile are linked to the amount and growth of cells [60].

Contribution to Manuscript 3

I am also the first author of Manuscript 3 and initiated the project by selecting the research topic and substantially developing the research question. I further conducted all literature research and applied for all required data. Besides constructing the complete statistical analysis plan and executing all of the corresponding analyses, I drafted the manuscript as well as the supplement and created all figures and tables. I implemented the coauthors' comments, prepared the paper for submission, and submitted the paper to the journal. Thereafter, I incorporated the suggestions of the peer reviewers into the manuscript.

3 Methods

3.1 Study populations

This cumulative thesis presents the results based on data from two prospective population-based cohort studies from Germany, the KORA study and the NAKO study. Manuscript 1 encompasses data from the NAKO baseline assessment as well as the KORA-Age study [58]. Manuscripts 2 [59] and 3 [60] entail data from the KORA S4 study and Manuscript 2 additionally includes data from the KORA FF4 study, a follow-up of KORA S4. The studies and corresponding key measurements that are incorporated in this thesis are illustrated in Figure 2.

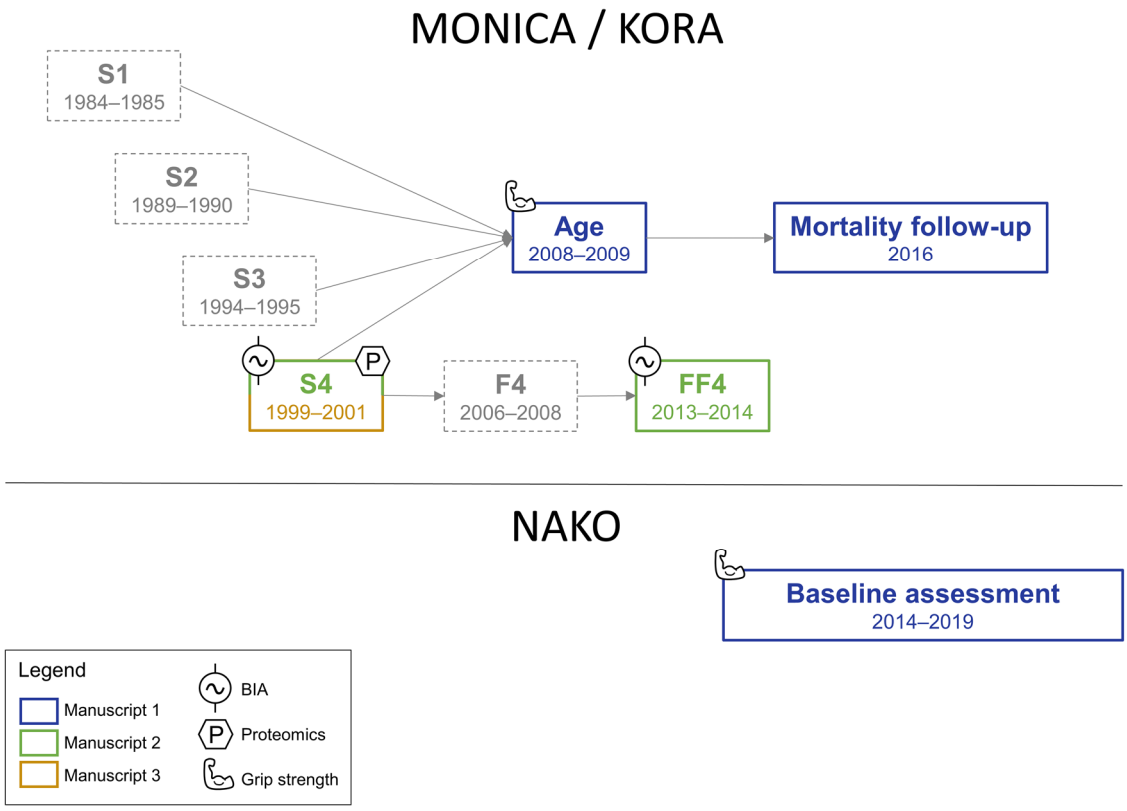

Figure 2: Studies and corresponding measurements included in this thesis

Grey font and dashed lines indicate that the data of these studies was not incorporated in the cumulative thesis. The depiction of these studies only serves to provide a comprehensive picture.

BIA: bioelectrical impedance analysis, KORA: Cooperative Health Research in the Region of Augsburg, MONICA: Monitoring of Trends and Determinants in Cardiovascular Diseases, NAKO: German National Cohort.

From 1984 onwards, starting with the Monitoring of Trends and Determinants in Cardiovascular Diseases (MONICA) Augsburg survey S1, examinations have been conducted every five years (MONICA Augsburg S2, MONICA Augsburg S3, and KORA S4). The study samples of the four surveys encompassed participants randomly drawn from the residents of Augsburg and adjacent counties [61].

The KORA-Age study was performed in 2008 to 2009 and included participants from the MONICA Augsburg S1, S2, S3, and KORA S4 studies that were born in 1943 or earlier. Within the KORA-Age study, 1,079 participants received physical examinations [62]. The study sample of KORA-Age participants analyzed in Manuscript 1 encompassed 1,012 individuals aged 65 to 93 years after exclusions. Mortality follow-up data until 2016 was further used for analysis [58]. Manuscript 1 additionally includes data of the NAKO baseline assessment in 2014 to 2019, which comprises 18 study centers across Germany with over 205,000 participants [63]. The final sample size for analysis after exclusions comprised 200,389 participants aged 19 to 75 years [58].

The KORA S4 study was conducted in 1999 to 2001 and comprised 4,261 participants aged 25 to 74 years [61]. In 2013 to 2014, examinations of 2,279 participants of the S4 study were performed in the second follow-up, the KORA FF4 study [59]. In the analyses of Manuscript 2 and Manuscript 3, only S4 participants aged 55 to 74 years (n = 1,653) were included because proteomics were only measured in this age group [59, 60]. After exclusions, Manuscript 2 encompassed a final sample of 1,478 participants [59] and Manuscript 3 of 1,484 participants [60] for the cross-sectional analysis of the KORA S4 data. The additional longitudinal analysis of Manuscript 2 entailed 608 participants after exclusions due to incomplete follow-up data [59].

3.2 Grip strength

Grip strength data was obtained from the NAKO and KORA-Age study for the analysis in Manuscript 1. Jamar hand dynamometers were used to assess the grip strength in both the NAKO [64] and the KORA-Age study [5]. The maximum value of three trials of both hands in the NAKO [64] and of the dominant hand in KORA-Age [5] was incorporated in the analysis [58].

3.3 Body composition

The body composition parameters ASMM, BFMI (Manuscript 2), and the phase angle (Manuscript 3) were derived using the BIA 2000-S (DATA-INPUT GmbH, Frankfurt, Germany) in the KORA S4 and FF4 study [59, 60]. For Manuscript 2, ASMM and BFMI were calculated using the equation of Sergi et al. [65] and the equation of Kyle et al. [66, 67], respectively [59]. For the cross-sectional analysis, we calculated based on the continuous variables of ASMM and BFMI the binary variables low ASMM (ASMM < 25^{th} sex-specific percentile), high BFMI (BFMI > 75^{th} sex-specific percentile), and the combined outcome low ASMM and high BFMI (overlap of ASMM < 40^{th} sex-specific percentile and BFMI > 60^{th} sex-specific percentile) [59]. For the longitudinal analysis, we derived the relative change (%) in ASMM and BFMI between S4 and FF4 and subsequently calculated the binary outcomes using the same cut-off points as described above for the cross-sectional analysis [59]. The phase angle was analyzed as a continuous variable in a cross-sectional analysis in Manuscript 3. The BIA device immediately calculates the phase angle from resistance and reactance during the measurement [60].

3.4 Proteomics

Targeted high-throughput proteomics measured with proximity extension assay (PEA) technology in plasma blood samples that were collected during the KORA S4 study examinations were analyzed in Manuscript 2 [59] and Manuscript 3 [60]. Measurements included the three proteomics panels Olink® CVDII, CVDIII, and Inflammation (Olink Proteomics, Uppsala, Sweden) each including 92 protein markers. We performed the analysis with the protein markers as log2-normalized protein expression (NPX) values divided by their respective standard deviation derived before any exclusions [59, 60]. We incorporated the same 233 protein markers into the analysis of Manuscript 2 and Manuscript 3 after exclusions from the original proteomics data set (n = 276) [59, 60].

3.5 Statistical methods

Manuscript 1 encompasses two analysis parts. The first part involves data from the NAKO study to calculate the grip strength distribution over age as well as cut-off points for probable sarcopenia and the second part entails KORA-Age data to compare the EWGSOP2 and NAKO-derived cut-off points for probable sarcopenia [58]. In the first part, the LMST method (lambda, mu, and sigma, with Box-Cox-t) using the Box-Cox-t-orig. distribution [68] was implemented to illustrate the distribution of grip strength across age by compiling percentile curves from the NAKO grip strength data [58]. Furthermore, the mean and standard deviation of grip strength were derived for each individual age as well as age groups. Based on the sex-specific highest (i.e. peak) mean of all ages (considering the individual ages) and its respective standard deviation, the cut-off points for probable sarcopenia were calculated stratified for sex with the following equation as implemented by Dodds et al. (2014) [40]: peak mean - 2.5 x standard deviation [58]. In the second analysis part using the KORA-Age data, the cut-off points determined based on the NAKO data and the EWGSOP2 definition were applied to calculate the (age-standardized) prevalence of probable sarcopenia. Subsequently, time-dependent sensitivity and specificity for the prediction of all-cause mortality were calculated for both cut-off points. In addition, Cox proportional hazards regression models were applied to investigate the association between grip strength (cut-off points) and all-cause mortality. The shape of this association was investigated by depicting covariate-adjusted Cox regression models with penalized splines. Covariates for the Cox regression analyses were selected based on stepwise backward model selection by Akaike Information Criterion [58].

In order to analyze the proteomics data and thus a large number of variables that could be associated with the outcomes, we applied various machine learning methods in Manuscript 2 and Manuscript 3. In both manuscripts, boosting with stability selection, a method that allows controlling for false positives [69], was implemented to identify protein markers that were independent of covariates and strongly associated with ASMM and BFMI in Manuscript 2 [59] and the phase angle in Manuscript 3 [60].

Besides this association analysis in Manuscript 2 applied to identify new protein markers of (low) ASMM, (high) BFMI, and their coexistence, we further performed a prediction analysis to investigate the importance of protein markers for these outcomes in comparison to classical risk factors [59]. The

prediction analysis included the application of group least absolute shrinkage and selection operator (lasso) with 100x bootstrapping to determine a ranking of the protein markers together with classical risk factors based on their selection frequency. Prediction models of the outcomes low ASMM, high BFMI, and their combination with protein markers that were selected in ≥ 90 % of the lasso bootstrap iterations were then evaluated by calculating the cross-validated area under the curve (AUC) to assess the prediction accuracy of these protein markers in addition to classical risk factors. We additionally performed the methods random forest and support vector machine (SVM) with linear Kernel to determine a ranking of the variables (protein markers and classical risk factors) by importance for each outcome. Afterwards, we compared the top ten ranking of the variables from the group lasso to the top ten ranking by random forest and SVM in a sensitivity analysis. We then identified which variables (proteins or classical risk factors) were ranked in the top ten by all three methods (group lasso, random forest, and SVM) [59]. The cross-sectional and longitudinal analysis were performed with the same analysis procedure but with different outcomes [59] as described in the chapter 3.3 Body composition.

In Manuscript 3, the protein markers strongly associated with the phase angle selected by boosting with stability selection were incorporated into a network and enrichment analysis to identify relevant biological factors related to the phase angle [60]. In this analysis, gene ontology terms of the sources gene ontology biological process, cellular component, and molecular function were selected if the protein markers associated with the phase angle were significantly overrepresented for the gene ontology terms [60]. To perform the enrichment analysis and for the construction of a functionally grouped network of the phase angle protein marker set with its gene ontology terms, we used the software Cytoscape v3.8.2 [70] with the plugins ClueGo v2.5.8 [71] and Cluepedia v1.5.8 [72]. All other analyses of the manuscripts within this cumulative thesis were performed using R [58-60].

4 Main results

The key findings reported in this cumulative thesis comprising of the individual results of the three manuscripts are listed in Table 1.

Table 1: Key findings of the manuscripts included in this cumulative thesis

<table>
<tr><th>Grip strength in the sarcopenia definition
(Manuscript 1 [58])</th><th>Proteomic profiling of muscle and fat mass
(Manuscript 2 [59])</th><th>Proteomic profiling of the phase angle
(Manuscript 3 [60])</th></tr>
<tr><td rowspan="2">Probable sarcopenia cut-off points from German data (NAKO) were 2 kg higher compared to the EWGSOP2 cut-off points.
In older adults, the prevalence of probable sarcopenia and the sensitivity for all-cause mortality were higher, while the specificity was somewhat lower for NAKO-derived compared to EWGSOP2 cut-off points.</td><td>Proteomics enabled the identification of novel markers of (low) muscle mass, (high) fat mass, and their combination.
The protein profiles of muscle and fat mass overlapped partially.
Protein markers are an important addition to classical risk factors for the prediction of low muscle mass, high fat mass, and their coexistence.</td><td>Proteomics enabled the identification of novel markers of the phase angle.
The main biological processes related to the protein profile of the phase angle are involved in cell mass and growth.</td></tr>
<tr><td colspan="2">Few proteins were strongly associated with muscle mass/fat mass and additionally with the phase angle.</td></tr>
</table>

Grip strength across the adult life span

In the NAKO data, grip strength of men increased from around the age of 20 years to approximately the age of 40 years, followed by a consistent decrease after a small plateau around the age of 40 years. The grip strength percentile curves of women demonstrated a comparable course across age but with an overall less pronounced increase and a decrease starting at an older age than in men [58]. We identified that grip strength in the NAKO study peaked at age 38 years in men and at age 39 years in women (peak mean ± standard deviation: 52.1 ± 9.2 kg in men and 32.5 ± 5.7 kg in women) [58].

Grip strength as a dichotomized disease marker

Based on the peak mean of grip strength and its corresponding standard deviation, we determined the cut-off points for probable sarcopenia at 29 kg for men and at 18 kg for women [58].

In the KORA-Age cohort including older people (65 to 93 years), the prevalence of probable sarcopenia increased with a higher age for both men and women. The overall age-standardized prevalence of probable sarcopenia was 1.5 x higher for the NAKO-derived (17.7 %) than the EWGSOP2 (11.7 %) cut-off points [58].

Time-dependent (3-year and 6-year survival) sensitivity for predicting all-cause mortality was higher and specificity was somewhat lower for the NAKO-derived than EWGSOP2 cut-off points for probable sarcopenia. The increase in sensitivity was higher than the decrease in specificity. For a 6-year survival, probable sarcopenia based on NAKO-derived cut-off points yielded a 1.3 x higher sensitivity for women and a 1.5 x higher sensitivity for men than EWGSOP2-defined probable sarcopenia [58].

The graphical representation of the association between grip strength and all-cause mortality depicted a nearly linear, inverse relation with no apparent cut-off point for both men and women individually [58].

New markers of muscle mass, fat mass, and their combination

Through exploring the proteomics data in a cross-sectional design using boosting with stability selection, we detected novel protein markers independent of covariates and strongly associated with (low) ASMM, (high) BFMI, and the combination of low ASMM and high BFMI (Figure 3).

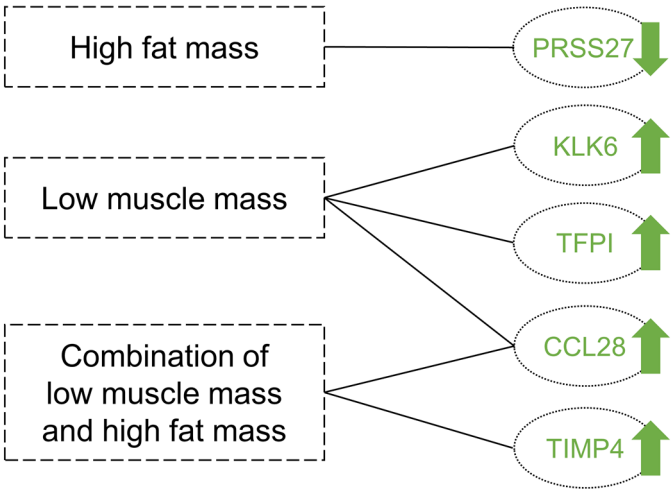

Figure 3: Newly identified protein markers of (low) muscle mass, (high) fat mass, and their combination

Adapted from the image of the poster of an online conference presentation of Huemer et al. (2021) [73]. The poster was published in the online conference system. The results illustrated in this figure are from Manuscript 2 [59].

Green arrows next to the proteins indicate the direction of association with the outcomes, which are illustrated in the rectangles.

CCL28: C-C motif chemokine 28, KLK6: kallikrein-6, PRSS27: serine protease 27, TFPI: tissue factor pathway inhibitor, TIMP4: metalloproteinase inhibitor 4.

The overlap of protein profiles from muscle and fat mass

The boosting with stability selection analysis of cross-sectional data further revealed that the protein profiles of (low) ASMM and (high) BFMI overlapped by several similar proteins (Figure 4). The coexistence of low ASMM and high BFMI was strongly associated with the proteins leptin, C-C motif chemokine 28 (CCL28), and metalloproteinase inhibitor 4 [59].

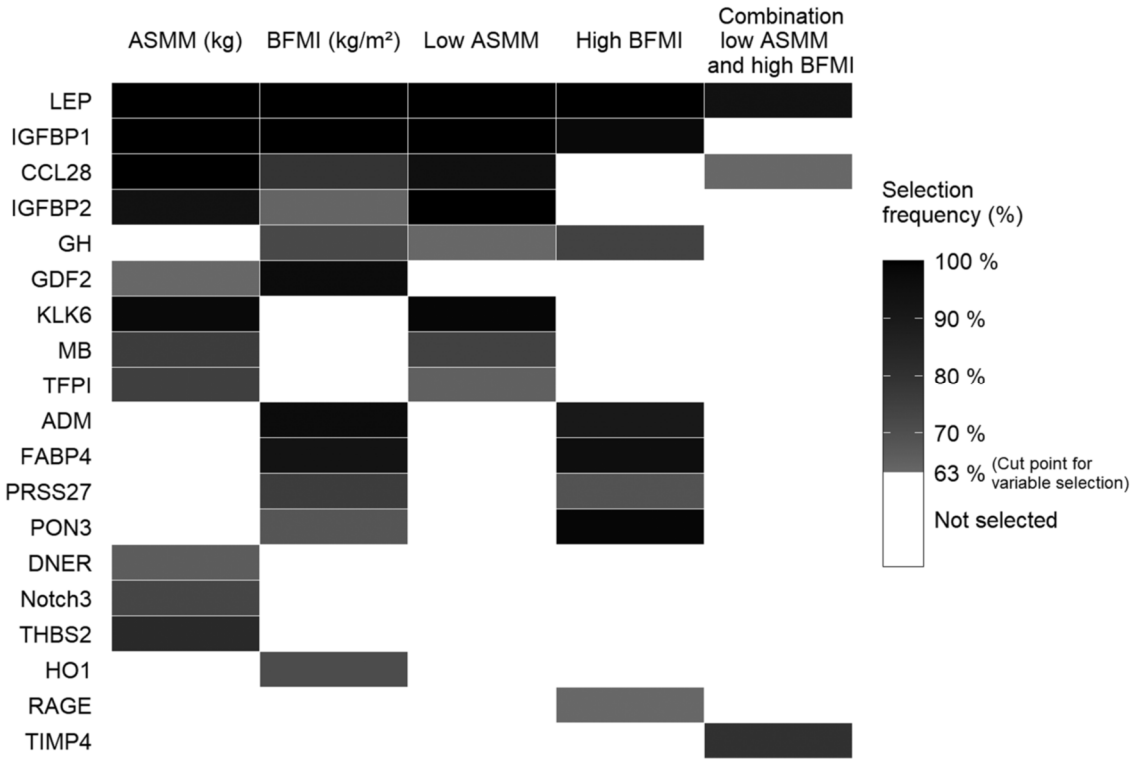

Figure 4: The overlap of protein profiles of ASMM, BFMI, and their binary parameters

This figure is an image from the manuscript “Proteomic profiling of low muscle and high fat mass: a machine learning approach in the KORA S4/FF4 study” by Huemer et al. (2021) [59], published in the *Journal of Cachexia, Sarcopenia and Muscle*, available under the following link: https://onlinelibrary.wiley.com/doi/10.1002/jcsm.12733 and licensed under the Creative Commons Attribution License “Attribution 4.0 International (CC BY 4.0)” (https://creativecommons.org/licenses/by/4.0/). No changes have been made to the original published version of the image.

Original title and original description of the image: “Figure 2 Association analysis — boosting with stability selection — comparison of protein biomarker selection between the outcomes. Protein biomarkers are primarily ordered according to the number of outcomes the biomarkers were selected for and secondary according to their selection for the outcomes in the table from left to right. Only protein biomarkers are included that were selected for at least one outcome. The cut point for variable selection was a selection frequency of 63%, which was determined by the algorithm based on the number of variables available for selection, the number of selected variables per iteration, and the maximum number of tolerable false positives. ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.” [59]

ADM: adrenomedullin, CCL28: C-C motif chemokine 28, DNER: delta and Notch-like epidermal growth factor-related receptor, FABP4: fatty acid-binding protein 4, GDF2: growth/differentiation factor 2, GH: growth hormone, HO-1: heme oxygenase 1, IGFBP: insulin-like growth factor-binding protein, KLK6: kallikrein-6, LEP: leptin, MB: myoglobin, Notch 3: neurogenic locus notch homolog protein 3, PON3: paraoxonase, PRSS27: serine protease 27, RAGE: receptor for advanced glycosylation end products, TFPI: tissue factor pathway inhibitor, THBS2: thrombospondin-2, TIMP4: metalloproteinase inhibitor 4.

A single protein marker, N-terminal prohormone brain natriuretic peptide (NT-proBNP), was selected for the two outcomes strong decrease in ASMM and the combination of a strong decrease in ASMM and a strong increase in BFMI in the longitudinal analysis [59].

The importance of protein markers next to classical risk factors for the prediction of muscle and fat mass parameters

The cross-validated AUC increased for all three binary outcomes (low ASMM, high BFMI, and their combination) after selected protein markers were additionally included into the model of the classical risk factors. Further findings of the prediction analysis with cross-sectional data (using group lasso with bootstrapping, random forest, and SVM) entailed that the ranking of some protein markers were similar to or at a higher rank compared to the classical risk factors. In the sensitivity analysis investigating the overlap of variables in the top ten variable rankings of the three methods group lasso with bootstrapping, random forest, and SVM, we observed that some protein markers overlapped more frequently between the methods than the classical risk factors [59].

New markers of the phase angle

Similar to Manuscript 2, we implemented the combined method boosting with stability selection to identify protein markers that have strong associations with the phase angle independent of covariates. In this respect, NT-proBNP, insulin-like growth factor-binding protein (IGFBP) 2, adrenomedullin, myoglobin, matrix metalloproteinase-9, protein-glutamine gamma-glutamyltransferase 2, and fractalkine were selected. To our knowledge, all markers except NT-proBNP have not been observed in an association with the phase angle before [60].

Biological factors related to the protein marker set of the phase angle

For the protein marker set that was strongly associated with the phase angle (listed above), the enrichment analysis identified 20 significantly overrepresented gene ontology terms. The top five of these included, in descending order based on the p-value corrected with Bonferroni step down, positive regulation of cell population proliferation, extracellular space, anatomical structure formation involved in morphogenesis, regulation of multicellular organismal development, and metal ion homeostasis [60].

Similar protein markers of muscle mass, fat mass, and the phase angle

Figure 5 illustrates the overlapping protein markers that were selected by boosting with stability selection for the continuous outcomes ASMM, BFMI, and the phase angle in the KORA S4 data.

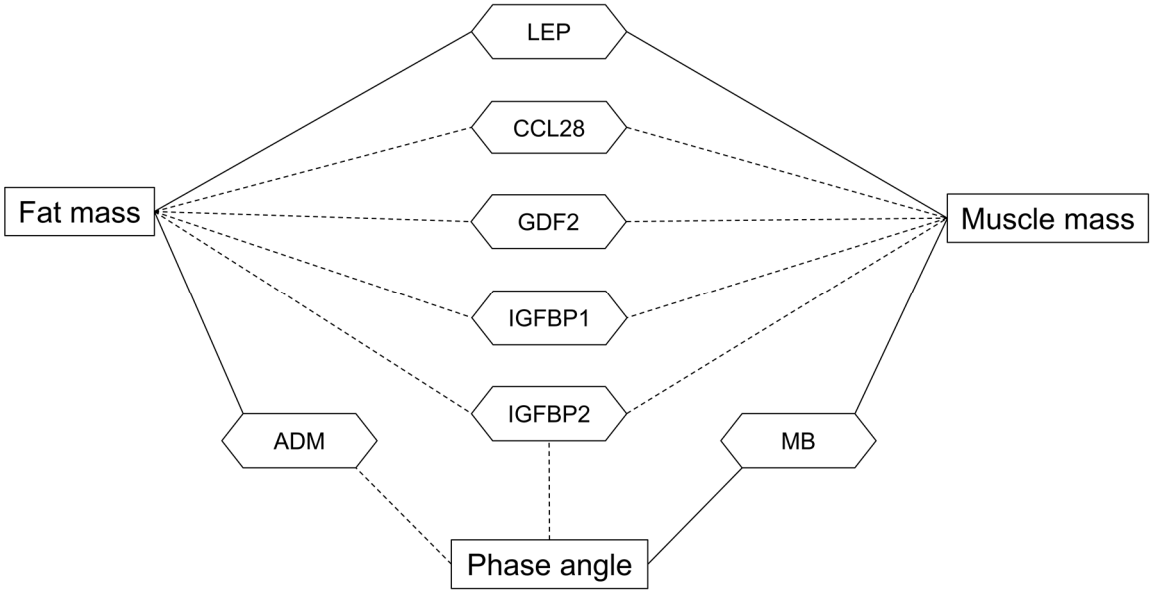

Figure 5: Protein markers simultaneously associated with the continuous parameters fat mass (BFMI), muscle mass (ASMM), and/or the phase angle

Dashed lines between the body composition parameters and protein markers indicate inverse association; solid lines indicate positive association.

The results regarding muscle and fat mass are from Manuscript 2 [59] and the results regarding the phase angle from Manuscript 3 [60].

ADM: adrenomedullin, CCL28: C-C motif chemokine 28, GDF2: growth/differentiation factor 2, IGFBP: insulin-like growth factor-binding protein, LEP: leptin, MB: myoglobin.

5 Discussion

The main findings of this cumulative thesis entailed that the grip strength cut-off points for probable sarcopenia derived from a large German study were 2 kg higher than the EWGSOP2 cut-off points, which emerged into a considerable discrepancy in the prevalence of probable sarcopenia in older people. The higher cut-off points resulted in a higher sensitivity for all-cause mortality, thus suggesting prevention and treatment for more patients at risk for premature death, whereas other patients may receive intervention without immediate reason due to the concurrent decrease in specificity [58]. High-throughput proteomics facilitated the discovery of new potential biomarkers and confirmed previously identified markers of sarcopenia-related body composition parameters muscle mass, fat mass, the combination of a low muscle and a high fat mass [59], as well as the phase angle [60]. Protein markers might be a relevant addition to other known risk factors for the prediction of body composition outcomes [59]. The overlapping protein profiles of muscle mass, fat mass, and the phase angle support a linkage of those tissues on the molecular level. Furthermore, the biostatistical analysis strengthened the technical-based consensus that the phase angle reflects body cell mass since the most significant biological processes that were related to the protein profile of the phase angle are linked to the amount and growth of cells [60].

5.1 The interrelationships of muscle mass, fat mass, and the phase angle based on proteomic profiling

Understanding the interrelationships of the body composition parameters muscle mass, fat mass, and the phase angle can help to explain adverse body composition alterations occurring in related disorders such as sarcopenia and sarcopenic obesity. The phase angle has been positively associated with muscle mass parameters such as muscle mass percentage [74] and muscle mass/height^2 assessed by BIA [75] as well as muscle cross-sectional area/height^2 measured by ultrasound (of the quadriceps rectus femoris) [76]. In contrast, inverse correlations/associations of body fat mass percentage with the phase angle have been observed [77, 78] but these results have not always been consistent [74, 75, 77]. A positive correlation has been reported between muscle and fat mass parameters assessed by BIA [59] and DXA [79] after adjustment for age and sex. Beyond the direct relationships between body composition parameters, underlying biological or pathophysiological links can be explored by overlapping proteomic profiles as conducted in the present thesis as well as other observational studies [80, 81]. The findings of the present thesis displayed that several protein markers were strongly associated with more than one of the examined continuous body composition parameters, muscle mass, fat mass, and the phase angle. The following section will particularly focus on these protein markers and discuss how they can provide indications to explain the links between the body composition parameters on a molecular level.

IGFBP2 was the only protein marker that was strongly (and inversely) associated with all three body composition parameters. IGFBPs bind to insulin-like growth factor (IGF)-1 and IGF-2 with high affinity

[82] and function as transporters of IGFs [83]. Next to the IGF-binding actions, IGFBPs encompass IGF-independent functions such as inhibition of cell proliferation [82]. Thus, the inverse association of IGFBP2 with the phase angle and ASMM may be explained by the characteristic of IGFBP2 to bind to and thereby mainly inhibit IGFs, resulting in reduced cell proliferation, as well as by IGF-independent functions of IGFBP2 resulting in the inhibition of proliferative processes [82]. In accordance with this, ASMM/height^2 has been inversely correlated with IGFBP2 in men and women and positively with IGF-1 and IGF-2 in men after adjustment for age and physical activity in an US-American study [84]. The inverse association with BFMI may root in the frequent observations that IGFBP2 inhibits adipogenesis [85]. The varying functions of IGFBP2 may ground on its different domains. The N-domain of IGFBP2 binds to IGF [85], whereas peptides containing the sequences of heparin-binding domain 1 and 2 incorporated in the linker region and C-terminal region of IGFBP2, respectively, have been demonstrated to inhibit adipogenesis in male mice [86]. The different functions of IGFBP2 affecting muscle and fat mass both in inverse direction may indicate why IGFBP2 was not selected as a marker for the combination of low muscle and high fat mass. IGFBP2 could therefore indicate a phenotype of concurrently low muscle and low fat mass rather than sarcopenic obesity. Furthermore, we observed that IGFBP1 was inversely associated with ASMM and BFMI [59]. Similar to IGFBP2, IGFBP1 also inhibits adipogenesis and is decreased in obesity [85]. Concerning muscle mass, IGFBP1 has been reported to inhibit IGF1-stimulated protein synthesis in the muscle [85].

Leptin might be an indicator of sarcopenic obesity or more specifically, coexistence of low muscle mass and high fat mass, as we observed that out of the 233 markers (increased) leptin was selected as the most important marker for this coexistence [59]. Notably, leptin was positively associated with both continuous parameters (ASMM and BFMI) individually. However, after adjusting the association between leptin and ASMM for BFMI, the direction of association changed from positive to inverse [59], suggesting that fat mass is a crucial factor for the link between leptin and muscle mass. On a molecular level though, leptin can reduce fatty acid accumulation in muscle and fat tissue, while in obesity in the presence of leptin resistance, this process may be diminished due to reduced fatty acid oxidation [87]. Therefore, sarcopenic obesity may be related to leptin resistance and fat infiltration into muscle as suggested before [47]. Of note, in a study published only a few days after Manuscript 2 [59], serum leptin was positively associated with sarcopenic obesity (defined based on the parameters body mass index and skeletal muscle mass divided by body weight) [88].

CCL28 and growth/differentiation factor 2 (GDF2) were inversely associated with muscle and fat mass [59]. In line with these results, CCL28 was inversely associated with BMI and waist circumference in a recent study also using PEA proteomics [89]. The expression of CCL28 is induced by pro-inflammatory processes [90], potentially explaining the link to lower muscle mass. However, sufficient research able to explain the mechanisms linking CCL28 and GDF2 to muscle and fat mass is currently lacking.

Myoglobin was strongly and positively associated with both ASMM [59] and the phase angle [60]. Myoglobin is mainly expressed in oxidative skeletal muscle fibers and cardiomyocytes [91]. Recent results from rat muscle conjecture that myoglobin is located in skeletal muscle mitochondria and might

be involved in mitochondrial respiration [92], beyond well-known functions such as oxygen storage [91]. Concluding from the positive link between myoglobin and muscle mass, the strong association of myoglobin with the phase angle might carefully support (or at least not argue against) the assumption that the phase angle reflects muscle quality as suggested before [29, 30, 43, 44].

Adrenomedullin was inversely associated with the phase angle [60] and positively with BFMI [59]. Inflammatory processes may be a potential link that might connect the phase angle and fat mass through adrenomedullin. As suggested in Manuscript 3, adrenomedullin synthesis is triggered by inflammation markers, which may lead to cell damage (and thus lower phase angle) [60] and inflammation is associated with obesity, or more specifically increased fat mass. Besides, adrenomedullin is increased in obesity and secreted by fat cells [93].

Figure 6 depicts Figure 5 of the results chapter with the addition of suggested biological and pathophysiological links between the protein markers and the body composition parameters based on prior literature as described within this chapter or in Manuscript 2 and Manuscript 3.

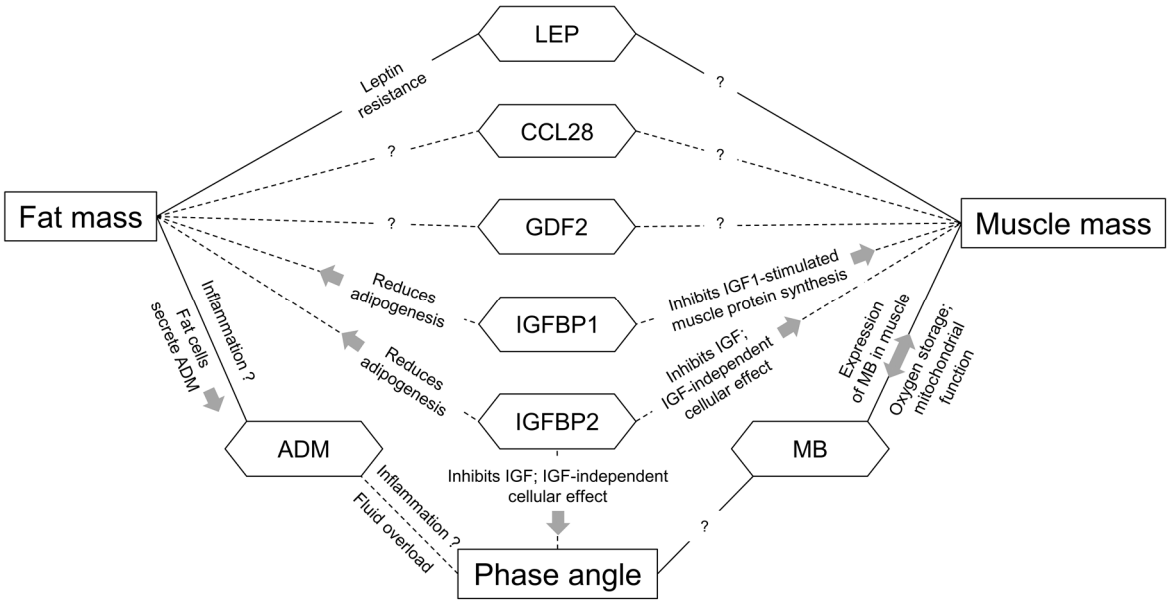

Figure 6: Suggested biological and pathophysiological links that may connect the protein markers to fat mass, muscle mass, and the phase angle. The links indicated between the protein markers and the body composition parameters fat mass, muscle mass, and the phase angle do not reflect all processes between these parameters and are suggestions based on information from the literature described in this section of the discussion or in Manuscript 2 and Manuscript 3.

Dashed lines between the body composition parameters and protein markers indicate inverse association; solid lines indicate positive association.

The results regarding muscle and fat mass are from Manuscript 2 [59] and the results regarding the phase angle from Manuscript 3 [60].

ADM: adrenomedullin, CCL28: C-C motif chemokine 28, GDF2: growth/differentiation factor 2, IGFBP: insulin-like growth factor-binding protein, LEP: leptin, MB: myoglobin.

5.2 Implications for sarcopenia and sarcopenic obesity

Grip strength

In line with other studies that observed differences in grip strength data between European regions [41, 42], we reported discrepancies in grip strength peak values and distributions across age between the German NAKO data and other European reference populations of various articles [58]. Additionally, grip strength cut-off points to define probable sarcopenia were 2 kg higher derived from German NAKO data [58] than the EWGSOP2 cut-off points derived from British data [40]. This comparatively minor difference in cut-off point values led to a large difference in the prevalence of probable sarcopenia in older people [58]. This may also be considered in the process of comparing studies using the first (men: 30 kg, women: 20 kg) [37] and the second (men: 27 kg, women: 16 kg) [7] EWGSOP sarcopenia definitions as these grip strength cut-off points differ even more, potentially causing larger discrepancies of the prevalence of probable sarcopenia. A considerable difference has already been reported by a recent systematic review comparing the complete sarcopenia definitions of EWGSOP1 and EWGSOP2 [94]. Considering the discrepancies in grip strength values between different regions as well as in the derived cut-off points and the resulting prevalence of probable sarcopenia, harmonization of grip strength data from a wide range of European countries would facilitate the derivation of suitable cut-off points for Europe that are tailored to not only one geographical region. As opposed to assembling data from various populations, implementation of different cut-off points for different countries would complicate research comparability, while patients may be misclassified if the standardized cut-off point deviates from the target population in a large amount [58]. Thus, the harmonization of grip strength data would additionally allow to investigate whether and to what extent grip strength values differ between different regions or if some discrepancies observed between studies might have resulted from different study protocols such as measurement device and number of measurement trials.

Besides the differences between European regions, the expediency of the cut-off points concerning the prediction of adverse outcomes should further be considered in the process of generating and validating cut-off points. As concluded in the EWGSOP2 consensus paper, generating cut-off points that are predictive for hard end-points should be the priority of future research [7]. Since a distinct cut-off point for grip strength did not emerge due to the nearly linear shape of the association between grip strength and all-cause mortality in Manuscript 1 [58] as well as in other European studies in men [4, 95] and women [4], clear dichotomized risk stratification by grip strength may be limited. However, generating cut-off points is necessary for clinical practice and whether the linear association is also present for other end-points such as multi-morbidity or CVD needs to be investigated in future research. Additionally, we observed that the sensitivity for all-cause mortality was higher for a higher grip strength cut-off point in men and women, which was in line with the observed linear relationship of grip strength with all-cause mortality [58]. In clinical practice a high priority constitutes prevention and treatment for patients at risk. Therefore, from a clinical perspective choosing less conservative cut-off points for probable sarcopenia suggests earlier intervention for more patients that are at risk for premature death based on their low grip strength. On the other hand, due to the lower specificity for all-cause mortality

resulting from higher cut-off points, some patients would receive intervention or more diagnostic measures even though there is no immediate reason. Of note, the decrease in specificity for the higher cut-off points (NAKO-derived vs. EWGSOP2) was lower compared to the increase in sensitivity. However, an alteration of the cut-off points should be preceded by studies investigating whether intervention is effective for patients with a grip strength above the current cut-off points [58]. The evaluation of higher grip strength cut-off points within the complete definition of sarcopenia (including muscle quantity/quality [7]) should also be prioritized before establishing new cut-off points.

Even though changing the current grip strength cut-off points seems plausible based on the above described issues, we should consider that the more frequently the definition changes the more difficult the comparison will be over time. Therefore, concluding from the observations of varying grip strength values between European regions and the linear relationship between grip strength and all-cause mortality, the sarcopenia definition may rather be changed only if new cut-off points emerge from harmonized European data sets comprising various regions and have been thoroughly tested for effectiveness regarding prevention and treatment.

Muscle and fat mass

As we found similar protein markers associated with muscle and fat mass [59], this reinforces the assumption of physiological/pathophysiological links on the molecular level between the two tissues. However, we only identified three markers for the coexistence of low muscle and high fat mass, yet more markers that were associated with both continuous outcomes in the same direction [59]. This may be explained by the fact that the overlap between low ASMM and high BFMI in terms of number of participants was rather low resulting in a low prevalence of “sarcopenic obesity” [59]. Likewise, other European studies also reported a low prevalence of sarcopenic obesity for different definitions [96, 97].

In the longitudinal analysis, NT-proBNP, a known marker of heart failure, was associated with a strong decrease in ASMM combined with a strong increase in BFMI over 14 years of follow-up [59]. NT-proBNP was further an important marker of the phase angle, though in cross-sectional analysis [60]. Of note, body composition abnormalities such as low lean mass entail a high prevalence in patients with heart failure, potentially linked through a reduction in cardiorespiratory fitness, which can also be lowered by elevated intermuscular fat [98].

As the definition of sarcopenic obesity including grip strength, muscle mass, and fat mass was published in 2022 [45] after Manuscript 2 was published in 2021 [59], future research may explore the protein profile of (low) grip strength with subsequent linkage to muscle and fat mass to enhance the understanding of the protein profile and thus the underlying pathophysiology of sarcopenic obesity.

Phase angle

The findings presented in this thesis suggest that the phase angle reflects cell mass amount and growth and reinforce the prior implementation of the phase angle as a risk factor for many rather than one single disorder [60]. In previous research, the phase angle has been repeatedly suggested as a marker

of muscle quality for potential integration into the sarcopenia definition [29, 43, 44]. Notably, a suitable marker of muscle quality has been characterized by "[…] ratios of muscle strength and/or muscle power per unit of muscle mass […]" [99]. Moreover, a review suggested that the phase angle might further be implemented as a measure of muscle quality in obesity, though subsequent to further research regarding connections to muscle parameters and metabolism [30]. Additionally, a recent study from Japan observed that the phase angle was positively associated with muscle quality (grip strength / upper limbs muscle mass) and accurately predicted sarcopenia [43]. However, the phase angle displayed only a small-to-moderate correlation with muscle quality (total strength / appendicular lean soft tissue) in Brazilian older women [100].

Furthermore, we only identified two proteins (myoglobin and IGFBP2) that were strongly associated with both ASMM and the phase angle. As detailed above, myoglobin seems to be a marker for muscle tissue, tentatively hinting to the concept that the phase angle could reflect muscle quality.

Another attribute that would characterize the phase angle as an indicator of sarcopenia is the age-related decline. The phase angle increases until the age of 18 years, plateaus from 19 to 48 years and decreases progressively afterwards in men and women of various studies [101]. We observed that the grip strength shows an overall comparable course over aging but there appeared an increase after the age of 20 years until approximately the age of 40 years and the decline started somewhat earlier [58] than the decline in the phase angle.

If future research can verify that the phase angle measured by BIA reflects muscle quality accurately and represents a suitable parameter for the assessment of sarcopenia, muscle quality could be assessed by a simple, portable, and comparably inexpensive measurement method. DXA is not capable of determining the muscle quality in terms of fat infiltration into the muscle [23] and methods that can be used to assess muscle quality (through fat infiltration into the muscle) depend on operator skills (ultrasound) [23, 26] or are not portable and expensive (CT and MRI) [3, 23]. Furthermore, the phase angle is a raw BIA parameter since it is directly calculated from the reactance and resistance without the need for additional variables such as weight, height, and sex. Thus, prediction equations as required for muscle mass are not necessary, further simplifying the process. However, until the phase angle can be considered for representing a diagnostic parameter of sarcopenia, further extensive research is required to assess the links of the phase angle with specific muscle quality parameters including image-based quantification.

5.3 A new era of protein biomarkers

The present thesis as well as other recent studies [60, 102-104] exemplify that protein biomarkers and in particular proteomics can help to elucidate which underlying biological factors and pathophysiological mechanisms could be related to the examined health outcomes. This was more difficult before the development of these technologies, such as proteomics, that can simultaneously measure hundreds of markers since assessing single markers in different studies can only provide limited insight into the underlying biological system. Next to proteomics, other omics technologies can complement the

understanding of disease biology including for instance genomics (e.g. single nucleotide polymorphisms), transcriptomics (e.g. non-coding RNA), and metabolomics (e.g. amino acids) [105, 106]. Additionally, the progressively increasing advancement of statistical techniques, software, and publicly available pooled data of e.g. gene ontology and pathways offers the opportunity to extract various information out of high-throughput data from a biostatistical standpoint. Recent developments enable the analysis of multi-omics integration to combine different single omics (e.g. proteomics and genomics) using machine learning methods [105]. Multi-omics can increase the information output as the information of the original causes of the disease such as genetic or environmental causes can be combined with the resulting effects on a functional level such as protein expression [106].

Next to the insights into pathophysiology and biological mechanisms, protein markers appear to be an important addition to classical risk factors in predicting body composition parameters of sarcopenia and sarcopenic obesity [60, 107]. Furthermore, the simultaneous analysis of numerous markers enables to rank the markers by importance for an outcome, which is not possible for single marker measurements as assessing only a few markers in different studies cannot provide clear evidence regarding the relevance of one marker compared to others. Additionally, the discovery of new potential biomarkers can be accelerated by high-throughput proteomics as exemplified in the manuscripts of this thesis and other studies [59, 60, 108, 109].

Apart from sole research purposes, precision medicine can also benefit from recent advancements in omics to provide specific treatment for individual patients [105]. In aging research, biomarker sets of for instance transcriptomics, genomics, and proteomics can display specific profiles for the different progressing stages of aging. Since the aging progression varies between individuals, biomarkers from all types can be employed to identify the personalized aging progression [110]. Also specifically for sarcopenia and frailty, omics can be implemented to identify next to biomarkers for diagnosis, biomarkers to monitor the progression of the disorders by investigating phenotypic groups [111]. Another focus of research may be the identification of mechanistic links of muscle changes to performance outcomes as well as mechanistic biomarkers to connect diagnostics to precision interventions to enable personalized treatment [111]. However, the research area of omics in precision medicine of sarcopenia is in the early stages and these concepts still have to be transferred into practice.

5.4 Strengths and limitations

Strengths include that the analyzed data from all three manuscripts was derived from population-based studies [58-60]. Further strengths entail the large sample size (> 200,000 participants) as well as the standardized measurements and collective quality control for the different study centers of the NAKO data included in Manuscript 1 [58]. Strengths of Manuscript 2 and Manuscript 3 entail that a large number of protein markers was incorporated and that the statistical approach included boosting with stability selection, which restricts the number of false positive markers selected for the body composition

parameters [59, 60]. The additional longitudinal approach and the employment of multiple machine learning methods are further strengths of Manuscript 2 [59].

Limitations entail that the generalizability of the findings of all three manuscripts is restricted as only data from the German population with limited age ranges was analyzed [58-60]. Additionally, the NAKO study included in Manuscript 1 currently lacks follow-up examinations and data of very old people relevant for the assessment of sarcopenia [58]. Limitations of Manuscript 2 and Manuscript 3 comprise the relative and not absolute protein marker values. Furthermore, the targeted approach of the proteomics data set could have limited the selection of other important protein markers not included in the data set [59, 60]. Further limitations of the thesis include the lack of data on the proteomics of grip strength.

5.5 Outlook

Harmonization of grip strength data from different European countries could help to assemble reference values and cut-off points for probable sarcopenia that fit to various regions in Europe. Future approaches should further focus on evaluating the effectiveness of intervention measures for different cut-off points of probable sarcopenia. Subsequent research is also needed to externally validate the protein markers of the body composition parameters that were newly identified in this cumulative thesis and the proteomics that were analyzed in this thesis may be explored for grip strength to complement the picture of proteomics of sarcopenia- and sarcopenic obesity-related parameters. Moreover, multi-omics studies of sarcopenia components could identify genetic causes of sarcopenia and their resulting functional consequences of the disease.

5.6 Conclusion

Adverse alterations in body composition and muscle strength present a major and progressively advancing burden on an individual's health and quality of life as well as the public health system. Thus, the effective and standardized diagnosis of diseases such as sarcopenia and sarcopenic obesity is essential. This thesis points out disparities in grip strength values and cut-off points between European study populations and suggests that harmonization of grip strength values may help to overcome these differences. Higher grip strength cut-off points as suggested by this thesis would identify more patients at risk of premature death, thereby enabling the access to intervention for more patients at risk in clinical practice. Next to the diagnosis, understanding the disease determinants and mechanisms in order to develop treatment strategies is needed to counteract the increasing medical challenge of detrimental body composition. In this respect, this thesis identified new potential starting points for future treatment in the form of relevant proteins as well as underlying pathophysiological processes of sarcopenia-related body composition on the molecular level.

Manuscript 1

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RESEARCH PAPER

Grip strength values and cut-off points based on over 200,000 adults of the German National Cohort - a comparison to the EWGSOP2 cut-off points

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Abstract

Background: The European Working Group on Sarcopenia in Older People (EWGSOP) updated in 2018 the cut-off points for low grip strength to assess sarcopenia based on pooled data from 12 British studies.
Objective: Comparison of the EWGSOP2 cut-off points for low grip strength to those derived from a large German sample.
Methods: We assessed the grip strength distribution across age and derived low grip strength cut-off points for men and women (peak mean -2.5 × SD) based on 200,389 German National Cohort (NAKO) participants aged 19–75 years. In 1,012 Cooperative Health Research in the Region of Augsburg (KORA)-Age participants aged 65–93 years, we calculated the age-standardised prevalence of low grip strength and time-dependent sensitivity and specificity for all-cause mortality.
Results: Grip strength increased in the third and fourth decade of life and declined afterwards. Calculated cut-off points for low grip strength were 29 kg for men and 18 kg for women. In KORA-Age, the age-standardised prevalence of low grip strength was 1.5× higher for NAKO-derived (17.7%) compared to EWGSOP2 (11.7%) cut-off points. NAKO-derived cut-off points yielded a higher sensitivity and lower specificity for all-cause mortality.
Conclusions: Cut-off points for low grip strength from German population-based data were 2 kg higher than the EWGSOP2 cut-off points. Higher cut-off points increase the sensitivity, thereby suggesting an intervention for more patients at risk, while other individuals might receive additional diagnostics/treatment without the urgent need. Research on the effectiveness of intervention in patients with low grip strength defined by different cut-off points is needed.

Keywords: grip strength, probable sarcopenia, European Working Group on Sarcopenia in Older People (EWGSOP), mortality, cut-off points

Key Points

- Cut-off points for low grip strength from German population-based data (NAKO) were 2 kg higher than the EWGSOP2 cut-off points.
- A relatively small difference between the cut-off points resulted in a large difference in the prevalence of low grip strength.
- Higher cut-off points may propose intervention for more patients at risk, while others may receive intervention without the need.
- Research on the effectiveness of intervention in patients with low grip strength defined by various cut-off points is needed.

Introduction

The severe loss of muscle strength with aging constitutes a detrimental factor for the health of older people. To determine the strength of an individual, handgrip strength measured with dynamometers has been established as it is suitable to indicate overall muscle strength [1, 2]. Handgrip strength has been reported to predict a multifaceted decline in various health parameters necessary to maintain daily activities such as cognition, mobility, and functional status in older people [3]. Besides functional deterioration, low handgrip strength has further been associated with an increased risk of premature death [1, 3, 4] and longer hospital stays [1, 5]. As an indicator of disease, handgrip strength represents the main component of sarcopenia [6]. Current cut-off points to identify low grip strength, which defines probable sarcopenia, as part of the sarcopenia definition for European populations were suggested by the European Working Group on Sarcopenia in Older People (EWGSOP) in 2018 (i.e. EWGSOP2) [6] based on pooled data of 12 British studies [7]. Premised on reported comparability of normative grip strength values of the British data with other more developed regions, Dodds *et al*. [8] suggested that these cut-off points for low grip strength could be employed across Europe, Northern America, Australia,

and Japan. Other studies reported discrepancies in grip strength between European regions [9, 10], encouraging the verification of the current cut-off points in other European countries. Most articles presenting European grip strength values and/or cut-off points for low grip strength though, reported data based on a small number of participants (because of the necessary multiple stratification by age and sex) [11–23] and/or did not include data of young adults [9, 10, 18, 19, 24]. However, young adults were recommended as the reference group for the derivation of low grip strength cut-off points [6].

In this article, we analyse the data of a large German population-based sample encompassing younger adults. Similar to other European studies, the majority of prior studies that reported German adult grip strength data either encompassed, relative to other available European data, a small number of participants [25–33] and/or were based on older individuals [28–31]. Only one previous German study calculated low grip strength cut-off points based on a younger study population (11,790 participants, aged 17–90 years) [27].

Therefore, we aimed to analyse grip strength and its distribution across age in 200,389 adults of the German National Cohort (NAKO, German: NAKO Gesundheitsstudie) aged 19–75 years and to derive cut-off points for low grip strength based on data from younger adults of the NAKO. As these cut-off points are mainly intended to define low grip strength in older people, we further aimed to compare the NAKO-derived cut-off points to the ones of the EWGSOP2 in an independent German cohort of older individuals aged 65–93 years from the Cooperative Health Research in the Region of Augsburg (KORA)-Age study using all-cause mortality since the EWGSOP recommended validation of cut-off points by their prediction of hard end-points [6].

Methods

Study sample

The NAKO is a population-based cohort study including 18 study centres across Germany. Over 205,000 men and women randomly invited from the German general population participated in the baseline examination between 2014 and 2019 [34]. General information regarding the NAKO study design and methods are described elsewhere [34–36]. We analysed grip strength data of the NAKO baseline examination after measurements for all baseline participants were completed. From the available data set of 204,916 participants, we excluded 4,527 participants due to missing, outside the measurement range or implausible ($\leq$ 0 kg and $\geq$ 90 kg) grip strength values. The data set for analysis included the remaining 200,389 participants aged 19–75 years encompassing 100,640 women and 99,749 men. We did not exclude participants with diseases, as we aimed to calculate values for a general population in line with previous studies [7, 27]. Data on mortality are not yet available for the NAKO sample.

The KORA-Age study consisted of 1,079 individuals aged $\geq$ 65 years, who participated in the physical examination between 2008 and 2009 [4]. From the 1,079 KORA-Age participants, we excluded 10 participants with missing maximum grip strength values and 57 participants with missing values for any covariate leading to a final sample size of 1,012 participants (499 women and 513 men). Further details regarding the study sample are included in the Supplementary data.

Grip strength measurement

In the NAKO study, three grip strength measurement trials were conducted at each hand. We used the maximum grip strength value if at least two measurement values were available for at least one hand [37]. For analyses with the KORA-Age data, the maximum grip strength value of three trials of the dominant hand was used. We analysed the maximum grip strength value to ensure comparability to Dodds *et al*. [7] and, therefore, the EWGSOP2 low grip strength cut-off points [6]. Jamar dynamometers were used for both, NAKO and KORA-Age measurements. Details regarding the measurement procedures and devices are included in the Supplementary data.

All-cause mortality – KORA-Age

All-cause mortality was determined between the enrolment into the KORA-Age study and the end of the follow-up in 2016. Population registries inside and outside of the KORA study area were asked for the vital status of the participants. Local health authorities provided the death certificates [4].

Covariates

Sociodemographic variables, anthropometry, lifestyle, diseases, blood markers, and details regarding their data acquisition are described in the Supplementary data.

Statistical analysis

With the NAKO data, percentile curves of grip strength across age stratified for sex were created with the LMST (i.e. lambda, mu, and sigma, with Box-Cox-t) method using Box-Cox-t-orig. (BCTo) distribution [38]. Percentiles, means, and standard deviations (SD) given in the tables were calculated based on original data and not based on estimated percentile curves. Low grip strength cut-off points for men and women were calculated with the sex-specific peak mean of all ages and corresponding SD from the NAKO data using the *T*-score calculation (peak mean -2.5 $\times$ SD) as described by Dodds *et al*. [7]. We used the values rounded to the nearest integer as cut-off points in accordance with the EWGSOP2 consensus [6].

In an independent sample of older people, the KORA-Age study, we calculated the prevalence and the directly age-standardised prevalence of low grip strength (grip strength < cut-off point) for both cut-off point definitions (NAKO-derived and EWGSOP2) for the whole sample

and stratified for men and women. We standardised the prevalence with the age groups (65–69, 70–74, 75–79, 80–84, 85– women: –90, men: –93) of the German population on 31 December 2008 [39]. We further calculated the rate ratio and corresponding 95% confidence interval of the NAKO-derived to EWGSOP2 prevalence of low grip strength. In a sensitivity analysis, we further calculated several *T*-scores (peak mean -1 × SD; -1.5 × SD; -2 × SD; -3 × SD) based on the NAKO data and the resulting prevalence of low grip strength in the KORA-Age sample.

We investigated the shape of the association of grip strength with all-cause mortality in Cox proportional hazards regression models with penalised splines stratified for men and women and fully adjusted for covariates in model 4 as detailed below. To check for potential discontinuity of the grip strength distribution in the section between the two cut-off points (EWGSOP2 and NAKO-derived), we created density plots for men and women. The association of grip strength (continuous variable) and low grip strength defined based on NAKO-derived and EWGSOP2 cut-off points with all-cause mortality was analysed using Cox proportional hazards regression models. To account for potentially confounding variables, models were adjusted as follows: model 1 was unadjusted, model 2 was adjusted for age (and sex only in the models with all participants), model 3 was additionally adjusted for physical activity, smoking, education, and body mass index (as a penalised spline term due to non-linear association with mortality), and model 4 was further adjusted for lung disease, cancer within the last three years, diabetes mellitus, heart problems or disease, neurological disease, estimated glomerular filtration rate, and albumin. Covariates for all Cox regression analyses were chosen based on stepwise backward model selection by Akaike information criterion. Variables that were available for selection and a detailed description are listed in the Supplementary data. The proportional hazards assumption was checked for all Cox proportional hazards regression models using scaled Schoenfeld residuals. There were no violations of the assumption.

We further calculated time-dependent (3-year and 6-year survival) sensitivity and specificity for all-cause mortality of EWGSOP2 and NAKO-derived cut-off points as well as the differences in sensitivity and specificity between the two cut-off points (EWGSOP2 and NAKO-derived).

All statistical analyses were performed using R, V. 4.0.5 [40]. The R packages that were used for the analyses and further details are described in the Supplementary data.

Results

Distribution of grip strength across age in the NAKO sample

Descriptive statistics of grip strength stratified by sex are listed for age groups in Table 1 and for every age individually in Supplementary Table S1, available in *Age and Ageing* online. The mean and SD of grip strength across age (Supplementary Table S1, available in *Age and Ageing* online) are illustrated for men and women separately in Supplementary Figure S1, available in *Age and Ageing* online.

The peak mean was 52.1 kg (SD: 9.2 kg) at age 38 years and 32.5 kg (SD: 5.7 kg) at age 39 years in men and women, respectively. Considering one decimal place, the peak mean of women appeared at ages 37–40 years (Supplementary Table S1, available in *Age and Ageing* online). The second decimal place revealed the highest peak mean at age 39 years (32.53 kg).

The percentile curves demonstrated an increase in grip strength in the third and fourth decade of life, which appeared more pronounced in men than in women. After plateauing in the later years of the fourth decade, grip strength decreased continuously in men. The grip strength curves of women were overall flatter, the plateau was more prominent around age 40, and the decline started slightly later (Figure 1).

Cut-off points for low grip strength in the NAKO sample

Low grip strength cut-off points (peak mean -2.5 × SD) based on NAKO data were 29 kg (not rounded: 29.1 kg) for men and 18 kg (not rounded: 18.25 kg) for women.

Prevalence of low grip strength based on NAKO-derived and EWGSOP2 cut-off points in the KORA-Age sample

Study population characteristics of KORA-Age participants (n = 1,012) are listed in Supplementary Table S2, available in *Age and Ageing* online.

The prevalence of low grip strength was higher for the NAKO-derived compared to the EWGSOP2 cut-off points for all age groups in both men and women (Supplementary Figure S2, available in *Age and Ageing* online). After age-standardisation, the prevalence of low grip strength decreased for both NAKO-derived and EWGSOP2 cut-off points but the rate ratio between both definitions remained similar (Table 2).

The T-scores of peak mean -2 × SD and -3 × SD yielded a prevalence of low grip strength of 43.0% and 11.1%, respectively compared to 20.7% with the main *T*-score (-2.5 × SD) (Supplementary Table S3, available in *Age and Ageing* online).

Association of (low) grip strength with all-cause mortality in the KORA-Age sample

The shape of the association of grip strength with all-cause mortality was nearly linear, inverse for men and women (Figure 2).

In density plots, no discontinuity of the grip strength distribution appeared in the section between the two cut-off points (EWGSOP2 and NAKO-derived) in men and women (Supplementary Figure S3, available in *Age and Ageing* online).

Grip strength values and cut-off points of the German National Cohort

Table 1. Grip strength stratified by age groups and sex in the NAKO sample

Age	*n*	Grip strength (kg)							
		Percentiles							
		5th	10th	25th	50th	75th	90th	95th	Mean (SD)
Men									
19–24	3,140	32.6	35.8	41.5	47.3	53.6	59.5	63.8	47.6 (9.4)
25–29	6,615	34.5	38.0	43.2	49.0	55.5	61.6	65.2	49.4 (9.4)
30–34	5,507	35.8	39.4	45.0	50.8	56.8	62.5	65.9	50.9 (9.3)
35–39	**5,041**	37.0	40.3	45.7	51.7	57.7	63.1	66.7	**51.7 (9.1)**
40–44	10,670	36.9	40.4	45.7	51.3	57.3	62.9	66.3	51.5 (9.0)
45–49	15,338	36.4	39.8	45.2	50.8	56.4	61.5	64.9	50.7 (8.7)
50–54	14,356	35.3	38.7	44.0	49.3	54.9	60.1	63.2	49.4 (8.6)
55–59	12,167	33.5	36.9	42.2	47.6	52.8	57.8	60.7	47.4 (8.4)
60–64	12,598	32.2	35.2	40.0	45.3	50.5	55.3	58.3	45.2 (8.1)
65–69	12,106	30.8	33.6	38.2	43.3	48.1	52.8	55.8	43.2 (7.7)
70–75	2,211	29.2	31.9	36.3	41.3	46.3	50.7	53.6	41.3 (7.4)
All	99,749	33.6	36.9	42.4	48.1	54.1	59.8	63.3	48.3 (9.1)
Women									
19–24	3,476	21.2	23.4	26.8	30.4	34.1	37.6	39.6	30.5 (5.6)
25–29	6,512	22.1	24.2	27.4	31.0	34.8	38.4	40.6	31.2 (5.7)
30–34	5,572	22.5	24.7	28.1	31.9	35.6	39.0	41.2	31.9 (5.7)
35–39	5,200	22.9	25.1	28.6	32.5	36.1	39.5	41.5	32.3 (5.7)
40–44	**10,435**	23.1	25.3	28.6	32.3	36.1	39.6	41.7	**32.4 (5.7)**
45–49	15,706	22.8	24.9	28.4	31.9	35.5	39.0	41.2	31.9 (5.7)
50–54	14,746	21.3	23.4	27.1	30.6	34.3	37.5	39.7	30.6 (5.7)
55–59	12,401	20.6	22.7	25.9	29.3	32.6	35.8	37.6	29.2 (5.3)
60–64	12,964	19.9	21.8	24.8	28.0	31.3	34.3	36.3	28.0 (5.1)
65–69	11,630	18.8	20.7	23.7	26.8	30.0	32.9	34.8	26.8 (4.9)
70–74	1,998	18.2	19.9	23.0	25.9	28.8	31.4	33.1	25.8 (4.6)
All	100,640	20.9	23.0	26.3	30.1	33.9	37.5	39.7	30.1 (5.8)

Bold font indicates the highest mean of all age groups. n: number of participants, SD: standard deviation.

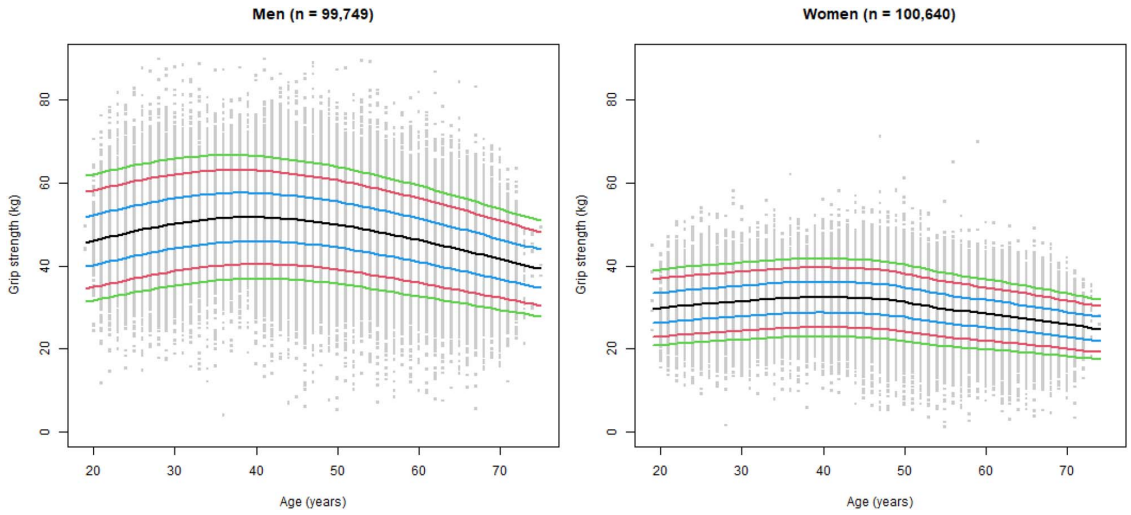

Figure 1. Percentile curves of grip strength across age for men and women in the NAKO sample. The 5th (green), 10th (red), 25th (blue), 50th (black), 75th (blue), 90th (red) and 95th (green) percentiles of grip strength (kg) across age (years) are presented for men (left) and women (right). *n*: number of participants.

In Cox regression models, the fully adjusted (model 4) hazard ratio (95% confidence interval) for all-cause mortality was 0.96 (0.92, 1.01) in women and 0.97 (0.94, 0.99) in men for a 1-kg increase in grip strength (Supplementary Table S4, available in *Age and Ageing* online). Correspondingly, the estimated decrease in all-cause

Table 2. Prevalence comparison between the NAKO-derived and EWGSOP2 cut-off points for low grip strength in the KORA-Age sample

		Low grip strength		Prevalence of low grip strength (%)	Rate ratio of NAKO-derived to EWGSOP2 prevalence (95% CI)[a]	Age-standardised prevalence of low grip strength (%)[b]	Rate ratio of NAKO-derived to EWGSOP2 age-standardised prevalence (95% CI)[a]
		Yes (*n*)	No (*n*)				
All	**EWGSOP2**	139	873	13.7	1.5 (1.3, 1.7)	11.7	1.5 (1.3, 1.7)
(*n* = 1,012)	**NAKO**	209	803	20.7		17.7	
Men	**EWGSOP2**	73	440	14.2	1.6 (1.3, 1.9)	10.6	1.6 (1.3, 1.9)
(*n* = 513)	**NAKO**	115	398	22.4		17.0	
Women	**EWGSOP2**	66	433	13.2	1.4 (1.1, 1.7)	12.1	1.4 (1.1, 1.8)
(*n* = 499)	**NAKO**	94	405	18.8		17.4	

Low grip strength defined based on NAKO-derived cut-off points: <29 kg for men and <18 kg for women. Low grip strength defined based on EWGSOP2 cut-off points: <27 kg for men and <16 kg for women [6]. CI: confidence interval, EWGSOP2: European Working Group on Sarcopenia in Older People 2, *n*: number of participants, NAKO: German National Cohort. [a]EWGSOP2 is the reference group for comparison. [b]Age-standardisation was performed with the German population on 31 December 2008 [39].

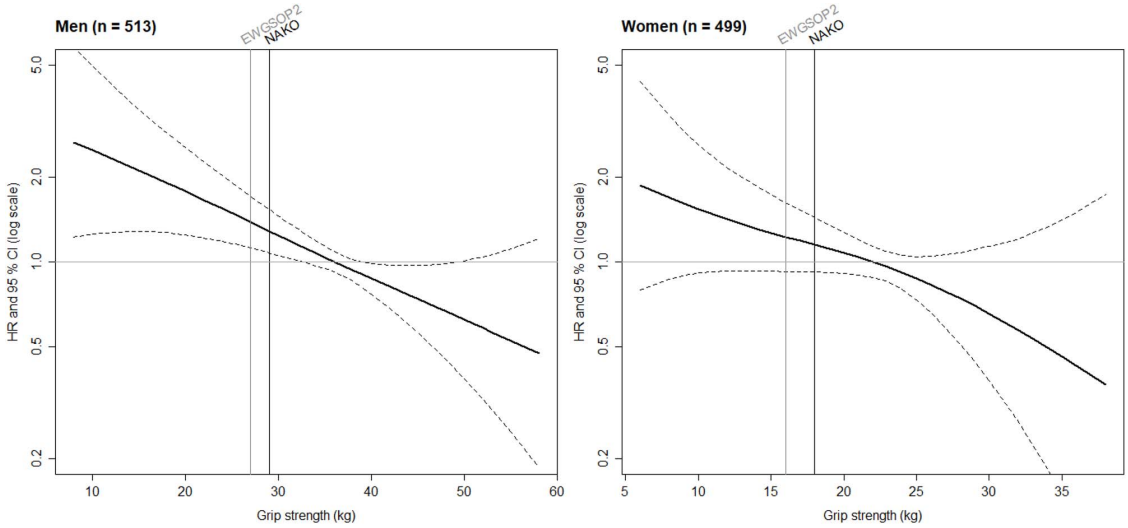

Figure 2. Association of grip strength with all-cause mortality by Cox regression with penalised splines in the KORA-Age sample. Solid black curve indicates the hazard ratio for all-cause mortality and dashed black curves depict the corresponding 95% confidence intervals. The reference (hazard ratio = 1) was represented by the median of the grip strength (men: 36 kg, women: 22 kg). Grey vertical line shows the cut-off point of the EWGSOP2 sarcopenia definition for low grip strength (men: 27 kg and women: 16 kg [6]) and black vertical line represents the cut-off point for low grip strength calculated based on the NAKO data (men: 29 kg and women: 18 kg). The *y*-axis is presented as a log scale. Cox regression models with grip strength as a penalised spline term were adjusted for body mass index (penalised spline term), age, physical activity scale for the elderly: total score, smoking, education, estimated glomerular filtration rate, albumin, lung disease (asthma, emphysema, COPD), cancer within the last three years, diabetes mellitus, heart problems or disease, and neurological disease (without stroke). CI: confidence interval, COPD: chronic obstructive pulmonary disease, EWGSOP2: European Working Group on Sarcopenia in Older People 2, HR: hazard ratio, *n*: number of participants, NAKO: German National Cohort.

mortality for a 2-kg increase in grip strength was -6% for men and -8% for women.

Out of all 1,012 participants, 23% (*n* = 233) died during the approximate seven years of follow-up. Employing the NAKO cut-off points, 209 individuals in the KORA-Age sample had low grip strength and 95 of them (45.5%) died. Based on the EWGSOP2 cut-off points, a total of 139 participants had low grip strength and 66 of them (47.5%) died. Due to the higher NAKO cut-off points, 70 additional participants were classified as having low grip strength as compared to the EWGSOP2 cut-off points and 29 of these 70 participants (41.4%) died. Hazard ratios of all-cause mortality were slightly (but not significantly) higher for low grip strength based on NAKO-derived compared to EWGSOP2 cut-off points (Supplementary Table S5, available in *Age and Ageing* online).

Low grip strength defined based on NAKO-derived cut-off points showed consistently higher sensitivity and lower

Table 3. Time-dependent sensitivity and specificity of EWGSOP2 and NAKO-derived cut-off points for all-cause mortality in the KORA-Age sample

	3-year survival		6-year survival	
	Sensitivity (95% CI) in %	Specificity (95% CI) in %	Sensitivity (95% CI) in %	Specificity (95% CI) in %
Men (*n* = 513)				
EWGSOP2 low grip strength cut-off point	32.7 (20.2, 45.4)	87.9 (85.3, 90.3)	30.1 (22.5, 37.2)	90.8 (88.0, 93.1)
NAKO-derived low grip strength cut-off point	51.9 (38.7, 63.1)	80.9 (78.0, 84.5)	45.5 (35.6, 52.4)	84.9 (81.1, 87.9)
Difference (EWGSOP2 - NAKO-derived cut-off point)	-19.2 (-29.9, -8.5)	6.9 (4.6, 9.3)	-15.4 (-21.8, -9.1)	5.9 (3.6, 8.2)
Women (*n* = 499)				
EWGSOP2 low grip strength cut-off point	37.5 (22.5, 53.0)	88.0 (84.7, 90.9)	27.3 (18.9, 40.6)	88.9 (85.3, 91.6)
NAKO-derived low grip strength cut-off point	54.2 (37.1, 72.1)	82.9 (79.4, 86.2)	36.4 (28.2, 52.6)	83.8 (80.2, 87.4)
Difference (EWGSOP2 - NAKO-derived cut-off point)	-16.7 (-31.6, -1.8)	5.1 (3.1, 7.0)	-9.1 (-16, -2.2)	5.1 (3.0, 7.1)

CI: confidence interval, EWGSOP2: European Working Group on Sarcopenia in Older People 2, *n*: number of participants, NAKO: German National Cohort.

specificity compared to EWGSOP2 cut-off points for all-cause mortality while the difference in sensitivity was larger than the difference in specificity (Table 3).

Discussion

Analysing data of 200,389 adults from the German population-based study NAKO, we observed that grip strength increased in the third and fourth decade of life and declined after the fourth decade. Derived cut-off points for low grip strength were 29 kg for men and 18 kg for women, each 2 kg higher than the EWGSOP2 cut-off points. In KORA-Age, the age-standardised prevalence of low grip strength was 1.5 (95% confidence interval: 1.3, 1.7) times higher for the NAKO-derived compared to the EWGSOP2 cut-off points. The shape of the association between grip strength and all-cause mortality was nearly linear, inverse, without an indication of a clear cut-off point. The sensitivity for all-cause mortality was higher and the specificity lower for the NAKO-derived compared to the EWGSOP2 cut-off points. These findings were similar for the two investigated time points.

Distribution of grip strength across age in the NAKO sample

In line with the percentile curves of British data reported by Dodds *et al*. [7], we observed that grip strength increased in early adulthood and decreased progressively after the fourth decade. Irish [20] and Italian [21] percentile curves did not display such a distinct increase in grip strength in early adulthood. Comparable to our results, another German study that analysed data from the German Socio-Economic Panel (SOEP) observed an increase of the mean grip strength during the third and fourth decade of life and a decline starting in the mid-forties [27].

The age at peak mean was considerably higher for German NAKO data (men: 38 years, women: 39 years) compared to the British data (men and women: 32 years) [7]. Additionally, the peak mean was somewhat higher and the SD lower in the NAKO data (men: 52.1 ± 9.2 kg, women: 32.5 ± 5.7 kg) compared to the British sample (men: 51.9 ± 9.9 kg, women: 31.4 ± 6.1 kg) [7]. The higher SD in the British data might have resulted from the pooling of 12 different studies with various measurement protocols [7]. Presumably due to smaller sample sizes, most previous studies did not report the mean for each age, but only for age groups. The Irish study reported a peak mean of grip strength (average of the highest scores of two measurements from each hand) in men of 51.3 ± 8.5 kg (30–39 years) and in women of 32.3 ± 5.2 kg (30–39 years) [20], which were close to the NAKO results (men: 35–39 years, 51.7 ± 9.1 kg; women: 40–44 years, 32.4 ± 5.7 kg). As opposed to this, the peak mean of the Italian sample, with grip strength based on the maximum value of both hands, was distinctly lower (minimum ∼4 kg) [21] compared to the present and other studies [7, 20, 25, 27]. The peak mean of Danish grip strength data (maximum of three trials of the dominant hand) [22] was ∼1 kg higher in men and 2 kg higher in women than our results. Results of the German SOEP study displayed the peak mean (weighted) of the maximum value of two measurements at each hand at ages 40–44 for men (53.8 ± 9.3 kg) and women (34.5 ± 6.3 kg) [27]. These peak means were ∼2 kg higher than our results and those of other European studies [7, 20]. Another German study with a small sample size (*n* = 769, age range 20–95 years) reported a similar peak mean for men as the SOEP data, however, only based on the right hand [25].

The NAKO-derived low grip strength cut-off points for men and women were each 2 kg higher than the EWGSOP2 cut-off points [6]. As opposed to our study and the EWGSOP2 cut-off points, other studies calculated cut-off

points as 2 instead of 2.5 SDs below the sex-specific peak mean [20, 27]. Since the NAKO low grip strength cut-off points were derived within a German sample, this may imply that these might be more suitable for a German population. However, our peak mean values were closer to an Irish [20] and a British [7] study than to other German studies [25,27].

Of note, the willingness to participate was lower in younger people after the halftime of the NAKO baseline measurements [34]. It is however unlikely that this would affect the cut-off points derived from younger participants, as non-participation due to health reasons is rather unlikely for younger participants.

Prevalence of low grip strength in the KORA-Age sample

As discussed above, we identified disparities between the grip strength of different European studies and further demonstrated that relatively small changes in cut-off points led to relatively large differences in the prevalence of low grip strength. The implementation of different cut-off points in different populations would, however, decrease comparability between studies, while in clinical practice, the use of cut-off points that do not fit to the patient population could lead to misclassification. Thus, harmonisation and pooling of grip strength data from European countries may support to find suitable cut-off points for Europe.

Association of (low) grip strength with all-cause mortality in the KORA-Age sample

We observed that the shape of the association between grip strength and all-cause mortality was nearly linear, inverse. In line with our findings, other European studies of older people with larger sample sizes also observed linear inverse associations for men [41, 42] and women [41]. However, data of older Norwegians indicated that the association might have only been present below the mean of *z*-standardised grip strength for women [42]. The observed nearly linear association of grip strength with all-cause mortality may indicate that there is no clear cut-off point. However, cut-off points for low grip strength are necessary and reasonable for clinical practice. Of note, a linear association between disease marker and hard end-points has also been observed for other diseases with established cut-off points such as blood pressure (hypertension), which is linearly related to cardiovascular and renal diseases [43].

We observed higher sensitivity and lower specificity of low grip strength for all-cause mortality for the NAKO-derived compared to the EWGSOP2 cut-off points. However, the difference in sensitivity was larger than in specificity. Thus, higher cut-off points may be more suitable in clinical practice to increase the sensitivity, i.e. to identify more patients at risk for premature death, suggesting an earlier start of intervention, while other individuals could concurrently receive additional diagnostics/treatment without the urgent need. Due to the nearly linear, inverse association between grip strength and all-cause mortality, changing the cut-off point to a lower value may easily lead to misclassification of persons at risk. According to the EWGSOP2 algorithm, in clinical practice, low grip strength '[. . .] is enough to trigger assessment of causes and start intervention' [6]. Through the subsequent steps (i.e. assessment of muscle quality/quantity), sarcopenia can be confirmed, but if we exclude a high number of patients at the preceding step (low grip strength), then the prevalence of confirmed sarcopenia may decrease even more. Of note, the cut-off point of blood pressure to define hypertension, which also had a linear association to hard end-points, was changed after a first definition to a lower value classifying more patients into the disease group [43]. This approach though, may be conducted after evaluation if prevention and treatment programs are effective for people that have a higher grip strength than the current EWGSOP2 cut-off points. Additionally, the higher costs should be considered as intervention for more patients would increase overall health care costs.

Strengths and limitations

Strengths include foremost the sample size of the NAKO sample and its population-based origin. Furthermore, the data of the NAKO is homogeneous as all 18 study centres performed measurements according to the same protocol and with the same dynamometer type as well as combined quality control of data. Limitations include the not yet available follow-up data in the NAKO regarding outcomes and data of older age groups, prohibiting an internal assessment of the association between low grip strength and mortality. For this purpose, a different study was used, but this study had a much smaller sample size. Furthermore, the generalizability of the NAKO data could be limited due to the low response proportion [36] especially for younger people [34].

Conclusion

Cut-off points for low grip strength from German population-based data (NAKO) were 2 kg higher than the EWGSOP2 cut-off points. The relatively small difference between the cut-off points resulted in a large difference in the prevalence of low grip strength and a higher sensitivity but lower specificity for all-cause mortality of the NAKO-derived cut-off points. A higher cut-off point as suggested by the NAKO data could detect more patients at risk of premature death and thereby propose an earlier intervention, while other individuals could concurrently receive additional diagnostics/treatment without the urgent need. Future research on the effectiveness of intervention regarding hard end-points in patients with low grip strength defined by different cut-off points is crucial.

Supplementary Data: Supplementary data mentioned in the text are available to subscribers in *Age and Ageing* online.

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Declaration of Conflicts of Interest: None.

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Ethical Statement: Local ethics committees of all study centres approved the German National Cohort (NAKO) study. The ethics committee of the Bavarian Medical Association approved the KORA study. The NAKO and the KORA study were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from NAKO and KORA participants included in the studies.

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. Data from the German National Cohort (NAKO) are available upon reasonable request.

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- Supplementary data -
Grip strength values and cut-off points based on over 200,000 adults of the German National Cohort - a comparison to the EWGSOP2 cut-off points

Contents

Details regarding the study sample

German National Cohort (NAKO)

The response proportion was 17 % for the whole NAKO sample (9 %–32 % for the individual study centers) [1]. To ensure standardized measurement procedures, examiners from all study centers were trained and certified before and during the study in joint sessions. Additionally, monitoring of examiners was conducted during the study in the individual study centers by internal and external quality control [2].

Cooperative Health Research in the Region of Augsburg (KORA)-Age

The KORA-Age participants were recruited from the group of participants born in 1943 or earlier that took part in one of the three Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) Augsburg studies S1, S2, and S3 conducted between 1984–1995 or the KORA S4 study conducted between 1999–2001 [3].

Grip strength measurement

NAKO

Details regarding grip strength measurements during the NAKO examinations including calibration and quality control have been reported before [4]. Briefly, the maximum isometric handgrip strength was measured using Jamar Plus+ hand dynamometer (Sammons Preston, Rolyon, Bolingbrook, IL, USA). Every study center used several devices of exactly the same device type. The manufacturer calibrated the hand dynamometers every two years. Additionally, the measurement accuracy was tested in the study centers using standard weights every six weeks. Data collection protocols were the same for all study centers. Influences of examiners and devices on results were tested frequently and in case of any irregularities, examiners received further training, while measurement devices were tested for measurement accuracy and if applicable calibrated or replaced. During handgrip measurement, participants were in seating position on a chair without armrests, placed their feet on the ground and remained their shoulders and forearm in neutral position, while the elbow was flexed approximately 90°. The hand dynamometer was set to the grip width 2 for all participants. The measurement was performed three times with each hand, alternating. Participants were excluded from performing the handgrip measurement if they had acute injury or operation at both hands or amputation or paralysis of both arms. For this article, we used the maximum grip strength value of all available measurements (both hands) if minimum two measurement values were available for at least one hand [4].

KORA-Age

In KORA-Age, the handgrip strength was measured with a Jamar dynamometer (SAEHAN Corporation, Masan, Korea). The maximum value of three measurements of the dominant hand with short breaks in between [5] was used for analysis. The dominant hand was identified as the hand that is used to cut with a scissor or to hold a knife cutting bread. The measurement was conducted in standing position (if impossible in upright seating position) and the participants were instructed to hold their upper arm against their body, while holding their elbow approximately 90° flexed. The handle of the dynamometer was adjusted to fit the hand of the participants.

Description of the covariates

Covariates for the cut-off point calculation with the NAKO data comprised sex (women / men) and age (years) at the examination date.

Covariates for the Cox regression analyses with KORA-Age data included age at reference date (December 31, 2008) (years), sex (women / men), physical activity scale for the elderly (PASE): total score, smoking (never / former / current), education (> 10 years / ≤ 10 years), body mass index (BMI) (kg/m^2), estimated glomerular filtration rate (eGFR) (ml/min/1.73 m^2), albumin (g/dl), lung disease (asthma, emphysema, COPD) (no / yes), cancer within the last three years (no / yes), diabetes mellitus (no / yes), heart problems or disease (e.g. angina, congestive heart disease, coronary heart disease, myocardial infarction, bypass, stent) (no / yes), and neurological disease (without stroke) (no / yes).

The continuous score PASE: total score was calculated based on the publication of Washburn et al. from 1993 [6] using interview questions regarding leisure, household, and work physical activities [7]. Smoking status was assessed based on interview questions. Smoking status “never” was defined as having smoked ≤ 100 cigarettes in life (yes or no question) and smoking status “former” as having smoked > 100 cigarettes in life and not smoking cigarettes currently. Smoking status “current” was defined as smoking regularly and occasionally [8]. For assessing the education, the highest level of vocational training and the highest level of school graduation were combined and assessed based on prior data from the studies S1, S2, S3, and S4 [8]. We categorized the variable education into > 10 years and ≤ 10 years.

Self-reported diseases (lung disease, cancer within the last three years, diabetes mellitus, heart problems or disease, and neurological disease) were assessed during a telephone interview in the KORA-Age study [8].

In KORA-Age, Albumin was measured in serum with modified bromocresol purple (BCP) dye-binding method using Dimension® Flex® reagent cartridge ALB (Siemens Healthcare

Diagnostics Inc.). eGFR was calculated with serum creatinine according to Inker et al. (2012) [9]. Creatinine was measured in serum with modified kinetic Jaffe reaction using Dimension® Flex® reagent cartridge CREA (Siemens Healthcare Diagnostics Inc.).

Statistical analysis

Description of the selection of covariates in the KORA-Age sample

Covariates for Cox regression models were chosen based on stepwise backward model selection by Akaike information criterion (AIC) in a preliminary data set of 1,003 participants (smaller sample size compared to the final data set due to more exclusions based on missing values for any of the tested variables). The variables that were available for selection included: age, sex, PASE: total score, smoking, education, BMI, eGFR, albumin, lung disease, cancer within the last three years, diabetes mellitus, heart problems or disease, neurological disease without stroke (all chosen by the model selection with grip strength as a continuous variable and low grip strength defined by European Working Group on Sarcopenia in Older People (EWGSOP) 2 cut-off points and NAKO-derived cut-off points), nutrition score, number of medication without plant-based and homeopathic, high sensitivity C-reactive protein (mg/l) (transformed by natural logarithm), alcohol intake (0 g/day / men 0.1–39.9 g/day and women 0.1–19.9 g/day / men ≥ 40 g/day and women ≥ 20 g/day), arthritis or rheumatic disease or arthrosis (no / yes), and stroke (no / yes) (all not chosen by the model selection).

All continuous covariates were tested prior to the model selection individually for linear and non-linear association with all-cause mortality using Cox regression models adjusted for age and sex with the tested variables as a penalized spline term. To identify non-linearity of the associations, we used graphical representations of the Cox regression models and in a second step significance of the p-values for the linear and non-linear terms in the Cox regression models. If both (linear and non-linear) p-values were significant, we chose the term with the higher portion (lower p-value). Based on these analyses, only BMI showed a non-linear association with all-cause mortality. Therefore, the only covariate included as a penalized spline term for all following Cox regression models was BMI.

After testing for linear and non-linear association of all continuous covariates with all-cause mortality, we calculated the stepwise backward model selection by AIC to choose the covariates. We calculated three Cox regression models (function “coxph” of R package “survival” [10, 11]) with grip strength as a continuous variable or low grip strength defined by EWGSOP2 cut-off points or NAKO-derived cut-off points plus all variables available for selection including BMI as a penalized spline term with function “pspline” of R package “survival” [10, 11]. Number of degrees of freedom for the penalized spline term was chosen by AIC corrected with the method of Hurvich et al. [12]. A stepwise backward model selection

by AIC was performed for each of the three models (with either of the three grip strength outcomes) using the "stepAIC" function from the R package "MASS" [13]. The variables listed in the section "Description of the covariates" are the covariates that were selected by this method for all three models.

R packages used in the statistical analysis

Figure 1: Percentile curves were created using the function "lms" from the R package "gamlss" [14]. For both graphs (men and women), the "BCTo" method with fitting method Rigby and Stasinopoulos algorithm was chosen by the "lms" function to fit the curves. The percentiles were plotted using the "centiles" function of the "gamlss" R package [14].

Table 2: We calculated the directly age-standardized prevalence of low grip strength with the R function "dsr" and the rate ratio of NAKO-derived to EWGSOP2 prevalence (EWGSOP2 as the reference) with the function "dsrr" of the R package "dsr" [15].

Figure 2: We calculated the Cox models with grip strength as a penalized spline term, stratified for sex and adjusted for BMI (penalized spline term) and all other covariates chosen by the model selection as described above. We therefore used the functions "coxph" and "pspline" of the R package "survival" [10, 11]. We used the function "dfmacox" of the R package "smoothHR" [16, 17] to calculate the number of degrees of freedom by AIC corrected with the method of Hurvich et al. [12]. The "dfmacox" function enabled the calculation of the optimal degrees of freedom for a model with multiple nonlinear covariate effects [16] (here: grip strength and BMI). The function "termplot" from the R package "stats" [18] was used to calculate the data from the Cox regression models for plotting. We centered the plot to the median of the grip strength (women: 22 kg, men: 36 kg) as the reference (hazard ratio = 1). Curves were plotted with the function "matplot" from the R package "graphics" [18].

Table 3: Time-dependent sensitivity and specificity of the EWGSOP2 and NAKO-derived cut-off points for the time points 3-year (1,095 days) and 6-year (2,190 days) survival were calculated using the R package "tdROC" [19] with the function "tdROC". (The confidence intervals of sensitivity and specificity were calculated within the same function with 100x bootstrapping.) The differences (and corresponding confidence intervals) in sensitivity and specificity between the two cut-off points were calculated with the R package "DTComPair" [20] using the function "sesp.diff.ci".

Supplementary Table S4 and S5: Cox proportional hazards regression models were calculated using the function "coxph" of the R package "survival" [10, 11]. BMI was included as a penalized spline term using the function "pspline" of the R package "survival" [10, 11]. To calculate the spline term's number of degrees of freedom by AIC corrected with the method of Hurvich et al. [12], we used the "caic = T" specification within the "pspline" function.

Supplementary Table S1: Grip strength stratified by age and sex in the NAKO sample

Age	n	Grip strength (kg)							
		Percentiles							
		5th	10th	25th	50th	75th	90th	95th	Mean (SD)
Men									
19*	3	-	-	-	-	-	-	-	-
20	118	33.7	36.4	40.4	46.2	52.3	57.7	60.7	46.5 (8.5)
21	468	31.2	36.2	41.5	46.5	51.9	59.1	62.1	46.7 (9.2)
22	733	32.1	34.8	40.5	46.6	53.7	59.3	63.6	47.1 (9.7)
23	879	32.6	35.8	42.0	47.6	53.4	58.5	63.1	47.7 (9.1)
24	939	33.9	36.5	42.2	48.1	54.7	60.8	65.1	48.5 (9.5)
25	1,055	34.3	37.3	42.2	48.0	54.3	60.4	64.0	48.5 (9.2)
26	1,150	34.2	37.9	42.3	48.3	55.3	61.5	64.8	49.0 (9.5)
27	1,331	34.7	38.3	43.5	49.4	55.7	62.0	66.0	49.7 (9.4)
28	1,537	35.2	38.0	43.3	49.1	55.6	61.6	64.9	49.5 (9.4)
29	1,542	34.4	38.6	44.2	49.7	56.2	62.0	65.5	50.1 (9.4)
30	1,336	35.7	38.8	44.7	50.2	56.2	61.7	65.3	50.4 (9.0)
31	1,129	34.4	38.6	44.5	50.6	56.7	62.8	65.7	50.5 (9.5)
32	1,036	35.8	39.0	44.8	51.1	56.8	63.3	66.8	51.0 (9.6)
33	1,006	37.1	40.7	45.9	51.2	56.8	62.2	65.9	51.3 (9.0)
34	1,000	36.1	40.1	45.4	51.5	57.4	62.7	65.8	51.4 (9.2)
35	1,011	36.6	39.5	45.1	51.6	57.5	62.7	66.5	51.3 (9.2)
36	1,047	36.5	40.0	45.1	51.6	57.7	63.1	65.9	51.5 (9.2)
37	1,008	36.1	40.4	46.0	51.8	57.9	62.9	66.5	51.8 (9.1)
38	**984**	37.3	40.4	46.2	51.8	58.2	63.8	68.1	**52.1 (9.2)**
39	991	37.9	40.6	45.8	51.7	57.6	63.0	66.6	51.7 (8.9)
40	1,435	37.6	41.2	46.0	51.6	57.7	63.5	66.4	51.9 (8.9)
41	2,022	36.1	40.5	45.7	51.5	57.6	62.9	66.2	51.6 (9.1)
42	2,378	36.8	40.1	45.6	51.5	57.3	63.0	66.7	51.4 (9.2)
43	2,382	37.4	40.4	45.6	51.0	57.0	62.8	66.0	51.3 (8.8)
44	2,453	36.8	40.5	45.8	51.3	57.2	62.6	65.9	51.4 (9.0)
45	2,677	36.4	40.4	45.9	51.5	57.3	62.4	66.0	51.5 (9.0)
46	2,878	36.5	40.4	45.5	50.8	56.2	61.4	64.8	50.8 (8.7)
47	3,032	36.2	39.7	45.1	50.6	56.4	61.2	64.7	50.6 (8.7)
48	3,290	36.4	39.5	44.9	50.9	56.5	61.7	65.2	50.7 (8.7)
49	3,461	36.4	39.5	44.8	50.2	55.7	60.8	63.8	50.2 (8.6)
50	3,257	36.4	39.5	44.6	50.0	55.6	60.6	64.1	50.1 (8.6)
51	2,999	35.3	39.1	44.3	49.7	55.4	60.5	63.7	49.7 (8.6)
52	2,808	35.6	39.0	44.0	49.2	54.7	60.2	63.3	49.3 (8.6)
53	2,752	35.0	37.9	43.4	48.8	54.3	59.7	62.4	48.8 (8.5)
54	2,540	34.7	37.7	43.5	48.6	54.2	59.3	61.8	48.7 (8.5)
55	2,536	34.0	37.3	42.8	48.1	53.3	58.4	61.7	48.0 (8.5)
56	2,454	34.2	37.5	42.6	48.2	53.4	58.0	60.4	47.9 (8.2)
57	2,466	33.0	36.6	42.0	47.6	53.1	57.9	60.5	47.4 (8.5)
58	2,387	34.2	37.0	42.0	47.2	52.4	57.6	60.2	47.2 (8.2)
59	2,324	32.4	35.7	41.4	46.5	51.8	56.7	60.4	46.4 (8.4)
60	2,233	32.6	35.8	40.5	45.8	51.5	56.7	59.7	46.0 (8.3)
61	2,373	33.4	36.4	40.9	46.2	51.6	56.4	59.5	46.3 (8.2)
62	2,515	31.4	34.7	39.8	45.2	50.0	54.6	57.0	44.9 (8.0)
63	2,784	32.2	34.9	39.7	44.9	50.1	54.9	58.0	44.9 (8.0)
64	2,693	31.5	34.5	39.3	44.4	49.7	54.0	57.1	44.4 (7.8)
65	2,739	31.9	34.6	39.1	44.3	49.1	54.1	57.3	44.2 (7.9)
66	2,602	31.5	34.2	38.7	43.7	48.5	53.4	56.1	43.6 (7.6)
67	2,438	30.5	33.4	38.1	43.2	48.1	52.5	55.3	43.1 (7.7)
68	2,308	29.9	32.9	37.4	42.7	47.7	52.0	54.9	42.5 (7.7)

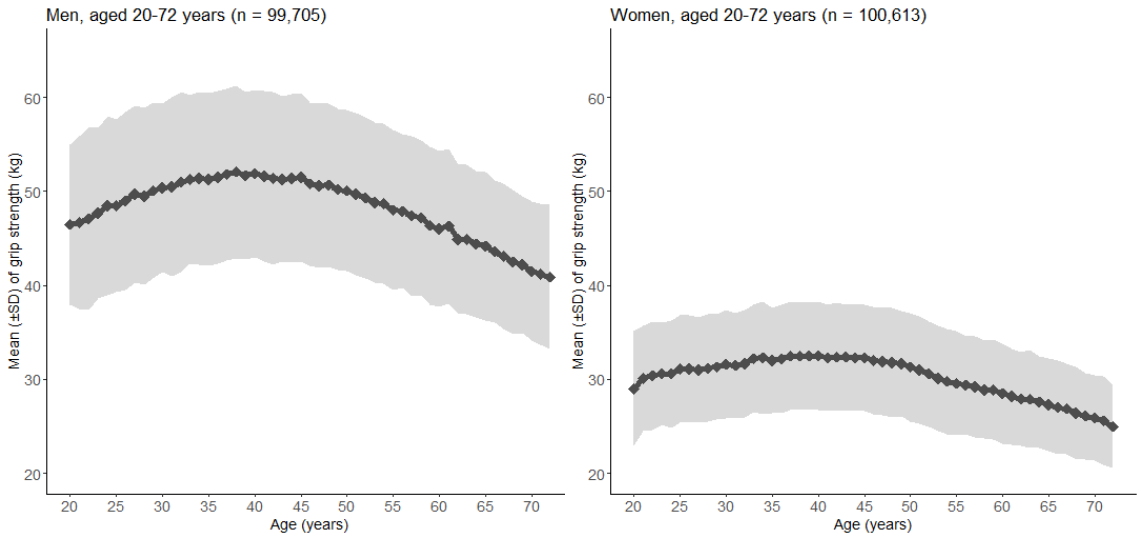
69	2,019	30.0	32.9	37.4	42.1	47.0	51.5	54.0	42.2 (7.3)
70	1,358	29.6	32.2	36.6	41.5	46.6	50.6	53.6	41.5 (7.4)
71	629	29.0	31.8	36.0	41.4	46.2	50.9	53.5	41.2 (7.5)
72	183	28.5	31.3	35.9	40.6	46.0	51.3	54.0	40.9 (7.7)
73*	29	-	-	-	-	-	-	-	-
74*	9	-	-	-	-	-	-	-	-
75*	3	-	-	-	-	-	-	-	-
Women									
19*	6	-	-	-	-	-	-	-	-
20	126	18.8	21.1	24.3	28.8	33.1	37.0	39.2	29.0 (6.1)
21	466	20.8	23.3	26.7	29.8	33.5	37.2	39.4	30.1 (5.6)
22	862	21.2	23.4	26.6	30.4	34.3	37.7	40.0	30.4 (5.8)
23	1,008	22.2	23.5	27.1	30.8	34.0	37.5	39.6	30.6 (5.4)
24	1,008	21.0	23.4	27.2	30.7	34.2	37.6	39.5	30.6 (5.7)
25	1,174	22.0	24.0	27.3	31.0	34.5	38.2	40.8	31.1 (5.7)
26	1,186	21.7	24.4	27.6	30.9	34.7	38.6	40.9	31.1 (5.7)
27	1,379	22.1	24.4	27.4	30.7	34.7	38.3	40.5	31.0 (5.6)
28	1,421	22.1	24.1	27.5	31.0	34.8	38.5	40.7	31.2 (5.7)
29	1,352	22.3	24.3	27.4	31.3	35.1	38.4	40.6	31.3 (5.6)
30	1,301	22.2	24.4	28.1	31.5	35.5	39.1	41.2	31.6 (5.8)
31	1,105	22.6	24.6	27.8	31.7	35.2	38.5	40.4	31.5 (5.6)
32	1,051	22.2	24.5	28.0	31.8	35.5	38.8	41.0	31.7 (5.7)
33	1,043	23.1	25.4	28.5	32.0	35.8	39.2	41.8	32.2 (5.7)
34	1,072	22.5	25.0	28.3	32.3	36.2	39.5	42.2	32.3 (6.0)
35	1,105	22.3	24.6	28.3	32.3	35.9	38.9	40.4	32.0 (5.6)
36	1,024	22.7	24.9	28.4	32.2	35.8	39.4	41.5	32.2 (5.8)
37	1,045	23.3	25.4	28.8	32.6	36.0	39.7	41.9	32.5 (5.8)
38	1,012	23.3	25.3	28.8	32.6	36.3	39.9	41.6	32.5 (5.7)
39	**1,014**	23.3	25.2	28.8	32.5	36.3	39.7	41.8	**32.5 (5.7)**
40	1,393	23.2	25.4	28.8	32.2	36.0	39.6	41.8	32.5 (5.8)
41	1,777	23.1	25.2	28.4	32.2	36.3	39.7	41.4	32.3 (5.7)
42	2,249	23.0	25.3	28.5	32.3	36.1	39.8	42.0	32.4 (5.8)
43	2,450	23.2	25.3	28.7	32.5	36.0	39.4	41.6	32.4 (5.6)
44	2,566	23.1	25.3	28.6	32.3	36.1	39.6	41.5	32.3 (5.7)
45	2,638	23.2	25.4	28.6	32.2	35.9	39.4	41.6	32.3 (5.7)
46	2,991	22.5	24.7	28.4	32.0	35.6	39.1	41.2	32.0 (5.7)
47	3,180	22.9	25.0	28.4	31.9	35.4	39.0	41.1	31.9 (5.7)
48	3,370	22.4	24.6	28.2	31.9	35.6	39.0	41.0	31.8 (5.8)
49	3,527	22.7	24.9	28.1	31.6	35.2	38.6	40.8	31.7 (5.6)
50	3,251	22.1	24.1	27.6	31.3	35.0	38.3	40.8	31.3 (5.8)
51	2,944	21.6	23.6	27.5	31.0	34.7	37.9	40.0	31.0 (5.7)
52	2,848	21.4	23.4	27.2	30.6	34.3	37.5	39.7	30.6 (5.6)
53	2,986	20.8	23.2	26.7	30.3	33.7	37.0	39.1	30.1 (5.6)
54	2,717	20.7	22.8	26.2	29.9	33.4	36.6	38.8	29.8 (5.6)
55	2,731	20.9	22.9	26.3	29.7	33.1	36.2	38.0	29.6 (5.5)
56	2,513	21.0	23.2	26.3	29.5	32.7	35.9	37.8	29.4 (5.2)
57	2,505	20.5	22.6	26.0	29.3	32.5	35.8	37.8	29.2 (5.4)
58	2,352	20.6	22.6	25.7	28.9	32.3	35.4	37.3	28.9 (5.2)
59	2,300	20.4	22.3	25.4	28.9	32.3	35.4	37.2	28.9 (5.3)
60	2,332	20.0	22.0	25.2	28.5	31.9	35.1	37.0	28.5 (5.3)
61	2,470	19.9	22.0	25.1	28.2	31.6	34.4	36.5	28.2 (5.1)
62	2,586	19.9	21.7	24.7	28.0	31.2	34.2	35.9	27.9 (5.0)
63	2,792	19.8	21.8	24.7	27.8	31.1	34.3	36.4	27.9 (5.2)
64	2,784	19.8	21.5	24.3	27.7	30.9	33.9	35.6	27.6 (4.9)
65	2,725	19.3	21.3	24.1	27.4	30.4	33.4	35.2	27.3 (4.9)
66	2,580	18.8	20.8	23.9	26.9	30.1	33.1	35.2	27.0 (5.0)
67	2,446	19.2	20.9	23.8	27.1	30.1	32.8	34.8	26.9 (4.8)

68	2,175	18.3	20.4	23.2	26.4	29.7	32.4	34.1	26.4 (4.9)
69	1,704	18.4	20.2	23.1	26.1	29.1	32.3	33.8	26.1 (4.6)
70	1,231	18.2	19.8	23.2	26	28.9	31.4	33.0	25.9 (4.5)
71	574	18.3	20.1	22.7	25.5	28.8	31.2	33.3	25.6 (4.7)
72	172	18.4	19.4	21.8	24.9	28.0	30.6	32.3	25.0 (4.4)
73*	19	-	-	-	-	-	-	-	-
74*	2	-	-	-	-	-	-	-	-

Bold font indicates the highest mean of all ages.

* Not enough participants to calculate valid descriptive statistics.

n: number of participants, SD: standard deviation.



Supplementary Figure S1: Mean (± standard deviation) of grip strength across age in the NAKO sample

Dark grey line indicates the mean and surrounding light grey area indicates the standard deviation.

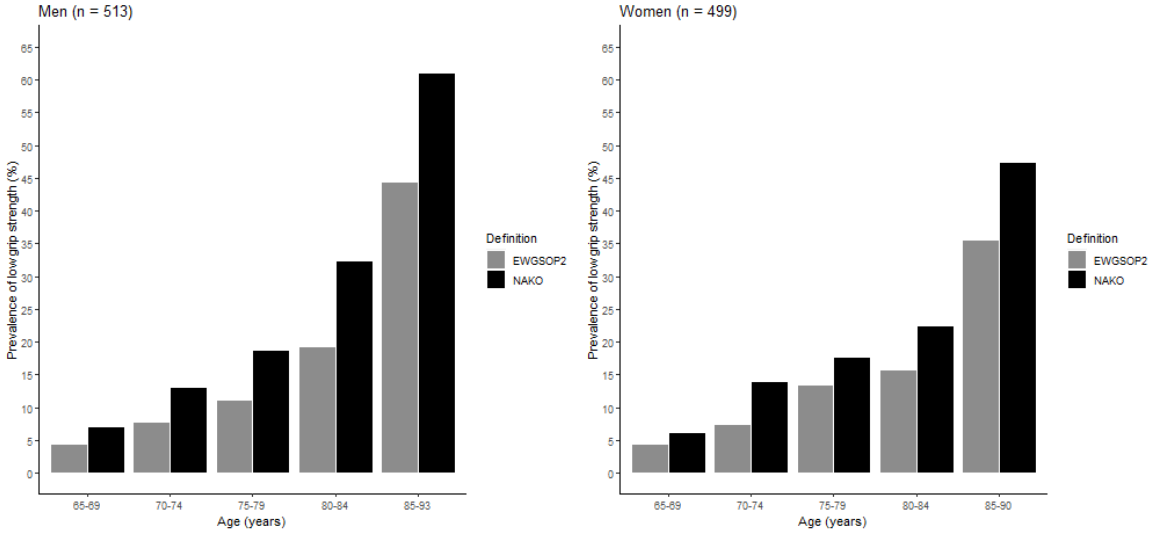
n: number of participants, SD: standard deviation.

Supplementary Table S2: Characteristics of the study participants in the KORA-Age sample

Characteristic	**N = 1,012**
Age (years) [a]	75.7 ± 6.5
Sex, n (%)	
Female	499 (49.3)
Male	513 (50.7)
Physical activity scale for the elderly: total score [a]	117.9 ± 56.0
Smoking, n (%)	
Never	577 (57.0)
Former	388 (38.3)
Current	47 (4.6)
Education, n (%)	
> 10 years	372 (36.8)
≤ 10 years	640 (63.2)
Body mass index (kg/m^2) [a]	28.5 ± 4.3
eGFR (ml/min/1.73 m^2) [a]	67.7 ± 17.2
Albumin (g/dl) [a]	3.8 ± 0.3
Lung disease (asthma, emphysema, COPD), n (%)	
No	907 (89.6)
Yes	105 (10.4)
Cancer within the last three years, n (%)	
No	972 (96.0)
Yes	40 (4.0)
Diabetes mellitus, n (%)	
No	834 (82.4)
Yes	178 (17.6)
Heart problems or disease, n (%)	
No	700 (69.2)
Yes	312 (30.8)
Neurological disease (without stroke), n (%)	
No	978 (96.6)
Yes	34 (3.4)
Maximum grip strength (kg) [a]	28.3 ± 9.8

[a] Continuous variables are listed as arithmetic mean ± standard deviation.

COPD: chronic obstructive pulmonary disease, eGFR: estimated glomerular filtration rate, n: number of participants.



Supplementary Figure S2: Prevalence of low grip strength based on the NAKO-derived and EWGSOP2 cut-off points stratified for age groups in the KORA-Age sample

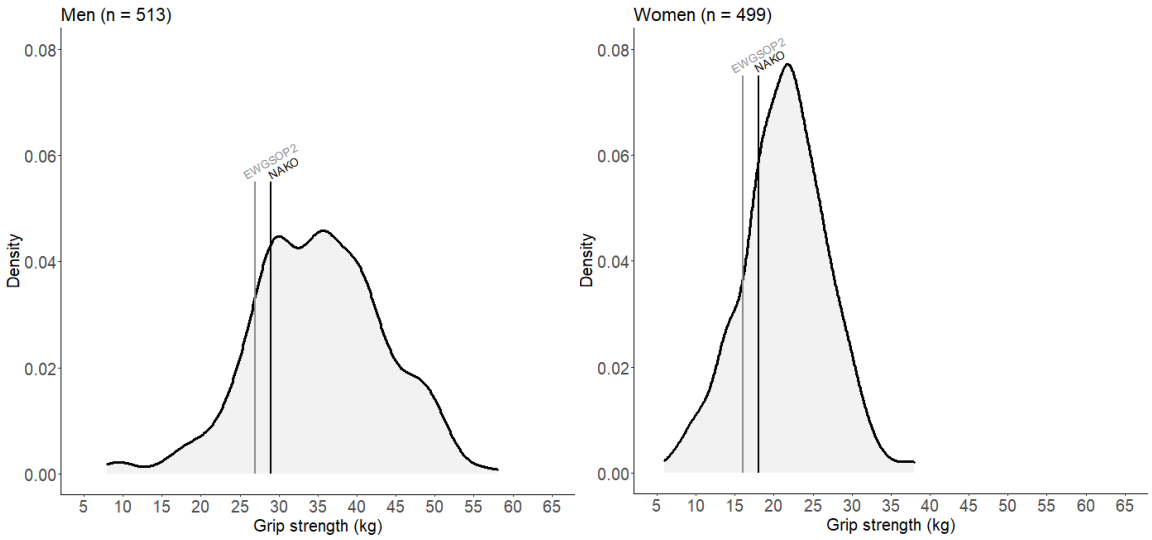
Low grip strength defined based on NAKO-derived cut-off points: < 29 kg for men and < 18 kg for women.
Low grip strength defined based on EWGSOP2 cut-off points: < 27 kg for men and < 16 kg for women [21].

EWGSOP2: European Working Group on Sarcopenia in Older People 2, n: number of participants, NAKO: German National Cohort.

Supplementary Table S3: T-scores based on data from the NAKO sample and resulting prevalence of low grip strength in the KORA-Age sample

T-score calculation	Men (n = 513)		Women (n = 499)		All (n = 1,012)
	T-score low grip strength (kg)	Prevalence of low grip strength (%)	T-score low grip strength (kg)	Prevalence of low grip strength (%)	Prevalence of low grip strength (%)
EWGSOP2 low grip strength cut-off points					
peak mean - 2.5 x SD	27	14.2	16	13.2	13.7
NAKO-derived low grip strength cut-off points					
peak mean - 1 x SD	43	83.0	27	85.2	84.1
peak mean - 1.5 x SD	38	60.0	24	62.5	61.3
peak mean - 2 x SD	34	40.7	21	45.3	43.0
peak mean - 2.5 x SD	29	22.4	18	18.8	20.7
peak mean - 3 x SD	25	9.4	15	12.8	11.1

EWGSOP2: European Working Group on Sarcopenia in Older People 2, n: number of participants, NAKO: German National Cohort.



Supplementary Figure S3: Density plot of grip strength in the KORA-Age sample

Grey vertical line shows the cut-off point of the EWGSOP2 sarcopenia definition for low grip strength (men: 27 kg and women: 16 kg [21]) and black vertical line represents the cut-off point for low grip strength calculated based on the NAKO data (men: 29 kg and women: 18 kg).

EWGSOP2: European Working Group on Sarcopenia in Older People 2, n: number of participants, NAKO: German National Cohort.

Supplementary Table S4: Hazard ratios for the association of grip strength (continuous variable) with all-cause mortality in the KORA-Age sample

	HR (95 % CI)
Men (n = 513)	
Model 1	0.93 (0.91, 0.95)
Model 2	0.95 (0.93, 0.98)
Model 3	0.96 (0.94, 0.99)
Model 4	0.97 (0.94, 0.99)
Women (n = 499)	
Model 1	0.89 (0.86, 0.93)
Model 2	0.95 (0.91, 0.99)
Model 3	0.95 (0.90, 0.99)
Model 4	0.96 (0.92, 1.01)

HRs are shown for a 1-kg increase in grip strength.

Model adjustments:
Model 1: crude model
Model 2: age
Model 3: model 2 + physical activity scale for the elderly: total score, smoking, education, and body mass index (as penalized spline term)
Model 4: model 3 + estimated glomerular filtration rate, albumin, lung disease (asthma, emphysema, COPD), cancer within the last three years, diabetes mellitus, heart problems or disease, and neurological disease (without stroke)

CI: confidence interval, COPD: chronic obstructive pulmonary disease, HR: hazard ratio, n: number of participants.

Supplementary Table S5: Hazard ratios for the association of low grip strength with all-cause mortality in the KORA-Age sample

	EWGSOP2 low grip strength cut-off points	**NAKO-derived low grip strength cut-off points**
	HR (95 % CI)	HR (95 % CI)
All (n = 1,012)		
Model 1	3.07 (2.31, 4.08)	3.33 (2.56, 4.33)
Model 2	1.85 (1.37, 2.50)	2.03 (1.53, 2.68)
Model 3	1.65 (1.22, 2.24)	1.85 (1.40, 2.45)
Model 4	1.40 (1.02, 1.92)	1.72 (1.29, 2.30)
Men (n = 513)		
Model 1	3.05 (2.11, 4.40)	3.24 (2.31, 4.53)
Model 2	1.96 (1.33, 2.88)	2.10 (1.47, 3.00)
Model 3	1.67 (1.13, 2.49)	1.86 (1.29, 2.67)
Model 4	1.64 (1.08, 2.47)	1.93 (1.32, 2.82)
Women (n = 499)		
Model 1	3.06 (1.94, 4.83)	3.32 (2.18, 5.06)
Model 2	1.63 (1.01, 2.64)	1.86 (1.19, 2.91)
Model 3	1.76 (1.08, 2.88)	2.02 (1.28, 3.18)
Model 4	1.44 (0.86, 2.41)	1.87 (1.17, 3.02)

Model adjustments:

Model 1: crude model

Model 2: sex (only in the models with all participants), age

Model 3: model 2 + physical activity scale for the elderly: total score, smoking, education, and body mass index (as penalized spline term)

Model 4: model 3 + estimated glomerular filtration rate, albumin, lung disease (asthma, emphysema, COPD), cancer within the last three years, diabetes mellitus, heart problems or disease, and neurological disease (without stroke)

CI: confidence interval, COPD: chronic obstructive pulmonary disease, EWGSOP2: European Working Group on Sarcopenia in Older People 2, HR: hazard ratio, n: number of participants, NAKO: German National Cohort.

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Manuscript 2

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Proteomic profiling of low muscle and high fat mass: a machine learning approach in the KORA S4/FF4 study

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Abstract

Background The coexistence of low muscle mass and high fat mass, two interrelated conditions strongly associated with declining health status, has been characterized by only a few protein biomarkers. High-throughput proteomics enable concurrent measurement of numerous proteins, facilitating the discovery of potentially new biomarkers.

Methods Data derived from the prospective population-based Cooperative Health Research in the Region of Augsburg S4/FF4 cohort study (median follow-up time: 13.5 years) included 1478 participants (756 men and 722 women) aged 55–74 years in the cross-sectional and 608 participants (315 men and 293 women) in the longitudinal analysis. Appendicular skeletal muscle mass (ASMM) and body fat mass index (BFMI) were determined through bioelectrical impedance analysis at baseline and follow-up. At baseline, 233 plasma proteins were measured using proximity extension assay. We implemented boosting with stability selection to enable false positives-controlled variable selection to identify new protein biomarkers of low muscle mass, high fat mass, and their combination. We evaluated prediction models developed based on group least absolute shrinkage and selection operator (lasso) with 100× bootstrapping by cross-validated area under the curve (AUC) to investigate if proteins increase the prediction accuracy on top of classical risk factors.

Results In the cross-sectional analysis, we identified kallikrein-6, C-C motif chemokine 28 (CCL28), and tissue factor pathway inhibitor as previously unknown biomarkers for muscle mass [association with low ASMM: odds ratio (OR) per 1-SD increase in log2 normalized protein expression values (95% confidence interval (CI)): 1.63 (1.37–1.95), 1.31 (1.14–1.51), 1.24 (1.06–1.45), respectively] and serine protease 27 for fat mass [association with high BFMI: OR (95% CI): 0.73 (0.61–0.86)]. CCL28 and metalloproteinase inhibitor 4 (TIMP4) constituted new biomarkers for the combination of low muscle and high fat mass [association with low ASMM combined with high BFMI: OR (95% CI): 1.32 (1.08–1.61), 1.28 (1.03–1.59), respectively]. Including protein biomarkers selected in ≥90% of group lasso bootstrap iterations on top of classical risk factors improved the performance of models predicting low ASMM, high BFMI, and their combination [delta AUC (95% CI): 0.16 (0.13–0.20), 0.22 (0.18–0.25), 0.12 (0.08–0.17), respectively]. In the longitudinal analysis, N-terminal prohormone brain natriuretic peptide (NT-proBNP) was the only protein selected for loss in ASMM and loss in ASMM combined with gain in BFMI over 14 years [OR (95% CI): 1.40 (1.10–1.77), 1.60 (1.15–2.24), respectively].

Conclusions Proteomic profiling revealed CCL28 and TIMP4 as new biomarkers of low muscle mass combined with high fat mass and NT-proBNP as a key biomarker of loss in muscle mass combined with gain in fat mass. Proteomics enable us to accelerate biomarker discoveries in muscle research.

Keywords Appendicular skeletal muscle mass; Body fat mass index; Fat mass; Muscle mass; Machine learning; Proteomics

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Introduction

For several decades, the disorder of low muscle mass was not recognized as a severe condition, although it is associated with various pathological conditions such as non-alcoholic fatty liver disease, type 2 diabetes,[1] hypertension,[2] and cardiovascular mortality.[3] A milestone turn in perception of condition severity constituted the assignment of an ICD-10-CM code for the term sarcopenia in 2016. Besides the increasing awareness of low muscle mass, early decisive research observed that the combination of low muscle and high fat mass had a more detrimental effect on disability in daily living activity,[4] multi-morbidity,[5] and an increased 10 year cardiovascular disease (CVD) risk[6] in comparison with participants solely experiencing low muscle mass.

Previous studies investigated the association of different muscle mass and fat mass parameters with a low number of biomarkers. Most studies focused on classical inflammatory biomarkers, predominantly C-reactive protein (CRP) and interleukin (IL)-6, and found contradictory results regarding the relation to muscle mass (fat free mass index[7] and loss of appendicular skeletal muscle mass (ASMM)[8,9]). The combination of low muscle mass (appendicular lean mass) and high body fat has been investigated and observed to be independently associated with CRP and fibrinogen.[10] However, there has been the concern that a low number of biomarkers might be insufficient in describing disease development. The principle of parsimony, that is, only selecting a small set of biomarkers as predictors for the outcome, could provide incomplete results as few biomarkers only reflect the most prominent proteins related to general processes.[11] As a response, multiplex measurements including a high number of proteins, that is, proteomics, have been established over the last years. Several recently published cross-sectional and longitudinal studies used different proteomics measurements to investigate various body composition parameters including body mass index (BMI), waist circumference (WC), waist-to-hip ratio (WHR), body fat mass (kg), and body fat (%),[12–19] but only one study investigated a muscle mass parameter, lean body mass (kg).[17] Studies using high-throughput proteomics to assess associations with muscle and fat mass parameters in combination are lacking. The aim of this study is to identify new protein biomarkers of low muscle, high fat mass, and their combination as well as their changes over a 14 year follow-up period.

Methods

Study population

The analysis is based on data from the population-based Cooperative Health Research in the Region of Augsburg (KORA) study, conducted in Southern Germany. 4261 individuals participated in the KORA S4 baseline examinations,[20] and 2279 additionally participated in the second follow-up study KORA FF4 (2013–2014).

The present analysis was restricted to participants aged 55–74 years at baseline (n = 1653), who were invited to the study centre after an overnight fast of at least 8 h. After exclusions, the cross-sectional analysis included 1478 participants (756 men and 722 women) of which 1315 participants complied with the overnight fasting and 163 participants did not. Out of these 1478 participants, 608 participants (315 men and 293 women) with a median follow-up time of 13.5 years (25th percentile: 13.5 years, 75th percentile: 13.6 years) remained for the longitudinal analysis. Exclusion criteria of the cross-sectional and longitudinal analysis are illustrated in Supporting information, *Figure* S1.

At the S4 and FF4 surveys, all participants were examined by trained medical personnel. In the S4 survey, sociodemographic data, lifestyle, medical history, and medication use were assessed in a standardized face-to-face interview.[20]

Exposure

Plasma samples collected at S4 in 1999–2001 were used to measure CVD- and inflammation-related protein biomarkers. Protein measurements were performed using proximity extension assay (PEA) technology developed by Olink® (Olink Proteomics, Uppsala, Sweden) with the three panels Olink® Multiplex CVDII, CVDIII, and Inflammation, each comprising 92 protein biomarkers. Details regarding measurement protocol are described elsewhere.[21] The Olink® platform provided the protein biomarkers as log2-normalized protein expression (NPX) values. We further divided the values by their respective standard deviation using the total study population with available data before exclusions. After exclusions, 233 protein

biomarkers remained for the present analysis. Exclusion criteria are described in Supporting information, *Figure* S2. Supporting information, *Tables* S1–S3 provide detailed information regarding all 276 measured biomarkers of the three panels before exclusions.

Outcome

The parameters requisite to calculate the continuous outcome variables ASMM in kilogram and body fat mass index (BFMI) in kilograms per square meter were assessed at S4 and FF4 using bioelectrical impedance analysis (BIA) with the BIA 2000-S (DATA-INPUT GmbH, Frankfurt, Germany). The calculations are included in the Supporting information. The binary outcomes included the risk group low ASMM, representing the 25% (n = 370) of participants with the lowest ASMM and its corresponding control group, the remaining 75% (n = 1108). The risk group of the outcome high BFMI included the 25% (n = 370) of participants with the highest BFMI and its corresponding control group, the remaining 75% (n = 1108). Sex-specific cut points were used for this purpose. The risk group for the combined outcome of low ASMM and high BFMI was determined by intersecting the 40% of participants with the lowest ASMM and the 40% of participants with the highest BFMI. This group consists of 7% (n = 110) of the total study population and the corresponding control group of the remaining 93% (n = 1368). Cut points of 40% were chosen for this outcome to ensure a sufficiently large size of the risk group while preserving a relatively extreme value of low ASMM and high BFMI. Currently, no standardized definition for the combination of both outcomes exists for European populations. For the longitudinal analysis, we used the changes of ASMM and BFMI between baseline and follow-up relative to baseline. Therefore, we changed the variables' descriptions from 'low ASMM' to 'strong decrease in ASMM' and 'high BFMI' to 'strong increase in BFMI', while the cut points based on the percentages remained the same. Detailed descriptions of the cross-sectional and longitudinal outcomes are included in Supporting information, *Table* S4, *Figure* S3, and *Table* S5.

Covariates

The covariates (association analysis)/classical risk factors (prediction analysis) included age, high-density lipoprotein, triglycerides, glycated haemoglobin, estimated glomerular filtration rate (eGFR), albumin (all continuous), sex (female/male), physical activity (high/moderate/low/no activity), hypertension (no/yes), smoking status (never/former/current smoker), education (>10 years/≤10 years), alcohol intake [0 g/day, 0.1–39.9 g/day (men)/0.1–19.9 g/day (women), ≥40 g/day [men]/≥20 g/day (women)], and intake of lipid-lowering medication (no/yes). Detailed information describing their measurements are available in the Supporting information.

Statistical analysis

Test results with two-sided P value <0.05 were considered statistically significant. Analysis workflow is depicted in *Figure* 1 and described in the Supporting information. We implemented the same statistical approach in both, cross-sectional and longitudinal analyses.

We separated the analysis into two parts to investigate two different analysis goals, 'association' and 'prediction'. As both terms have various applications, in the following, we specify this paper's meaning of the terms. The goal of the association analysis comprised the accurate selection of biomarkers associated with the outcomes independent of covariates. Therefore, we implemented boosting with stability selection as it allows finite error control of false positives enabling accurate variable selection. The paper validating this method explains that its prediction accuracy can suffer as the true positive rate is due to a tight error control usually lower compared with prediction methods without stability selection. 'Prediction and variable selection are two different goals.'[22] Our goal of the prediction analysis was to identify biomarker models able to predict unknown data using methods with a high predictive accuracy. The sensitivity analysis was employed to compare the highest ranked variables between these methods.

Results

Participant characteristics of the analysed population are listed in Supporting information, *Tables* S6 and S7. Partial correlation analysis between ASMM and BFMI adjusted for age and sex resulted in a Spearman rank correlation coefficient of 0.57. Coefficients to other body composition parameters constituted for BFMI and BMI 0.93, ASMM and BMI 0.68, BFMI and WC 0.84, ASMM and WC 0.68, BFMI and WHR 0.52, and ASMM and WHR 0.30.

Cross-sectional association of appendicular skeletal muscle mass/body fat mass index with protein biomarkers

Table 1 displays the strength of associations of protein biomarkers selected by boosting with stability selection with the outcomes adjusted for Models 1 and 2. *Figure* 2 illustrates a comparison of the selected biomarkers between the outcomes.

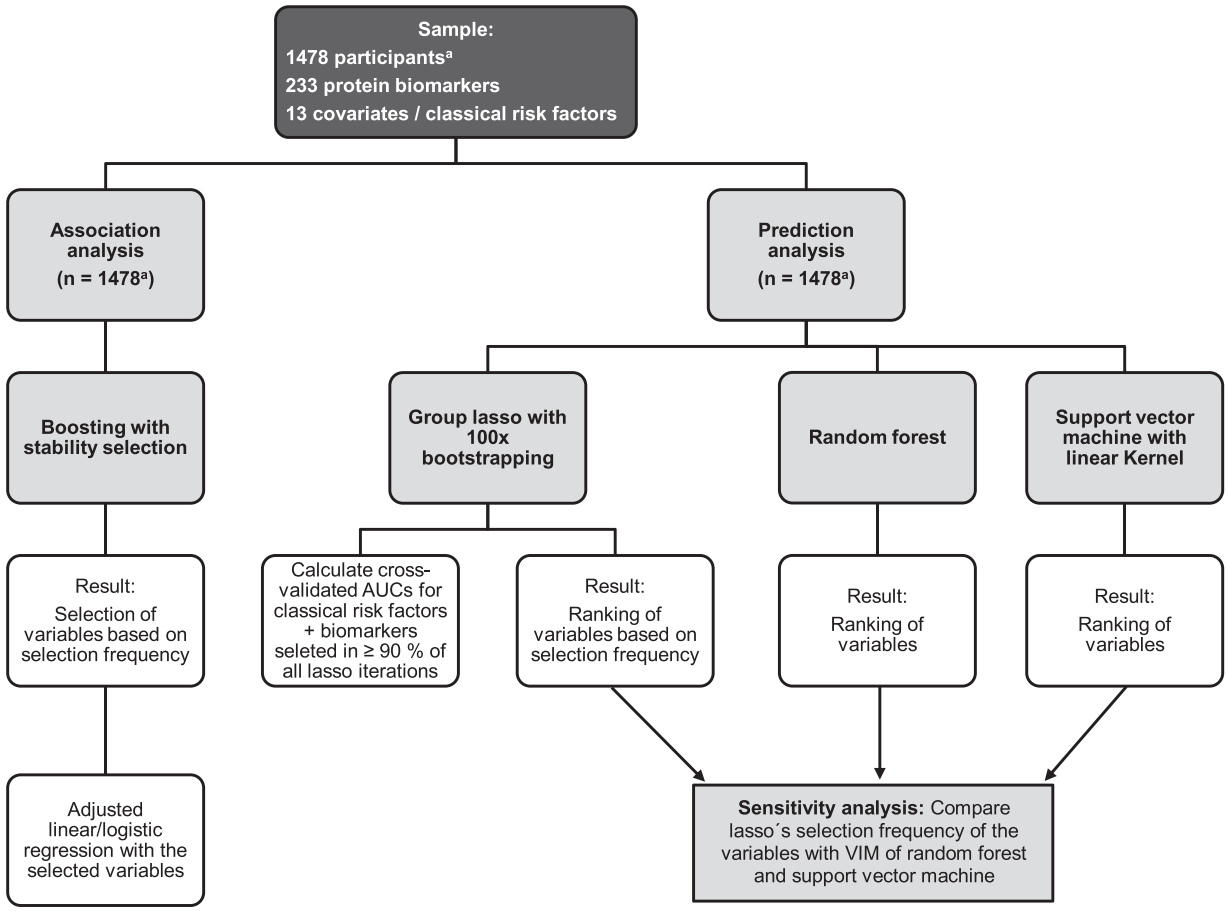

Figure 1 Statistical analysis plan. AUC, area under the curve; lasso, least absolute shrinkage and selection operator; VIM, variable importance measure. [a]1478 participants in the cross-sectional analysis; 608 participants in the longitudinal analysis.

Concerning the association analysis, leptin (LEP) was the only protein biomarker that was selected for all five outcomes. Insulin-like growth factor-binding protein (IGFBP) 1 and 2, C-C motif chemokine (CCL) 28, growth/differentiation factor 2 (GDF2), and growth hormone (GH) were selected for both, muscle and fat mass parameters. Kallikrein-6 (KLK6), myoglobin (MB), and tissue factor pathway inhibitor (TFPI) were only selected for the two muscle mass parameters ASMM and low ASMM. Adrenomedullin (ADM), fatty acid-binding protein 4 (FABP4), serine protease 27 (PRSS27), and paraoxonase (PON3) were only selected for the two fat mass parameters BFMI and high BFMI. LEP, CCL28, and metalloproteinase inhibitor 4 (TIMP4) were selected for the combination of low ASMM and high BFMI (*Table* 1).

Table 1 illustrates that after adjusting for the other outcome in Model 2, the associations of thrombospondin-2 (THBS2) and GDF2 with ASMM, of CCL28 and IGFBP2 with BFMI as well as of GH with low ASMM became non-significant. The association of LEP with ASMM was still significant but became inverse. After further including an interaction term between the above-listed proteins and the other outcome, only the interaction between GDF2 and BFMI for the outcome of ASMM was significant [beta coefficient (β) (95% confidence interval, CI): −0.03 (−0.06, 0.00), P = 0.041].

Cross-sectional analysis: prediction of appendicular skeletal muscle mass/body fat mass index by classical risk factors and protein biomarkers

Table 2 displays the cross-validated area under the curve (AUC) of a logistic regression model including 13 classical risk factors (AUC_{basic}) and a model additionally including protein biomarkers (listed in Supporting information, *Table* S8) that were selected in ≥90% of the 100 group least absolute shrinkage and selection operator (lasso) bootstrap iterations ($AUC_{extended}$) as well as their cross-validated delta AUC ($AUC_{extended} - AUC_{basic}$). The receiver operating characteristic (ROC) curves of the AUC cross-validation are included in Supporting information, *Figure* S4.

Table 1 Association analysis — boosting with stability selection and regression analyses

Boosting with stability selection		Linear regression models			
Selected variables	Selection frequency	β (95% CI)	*P* value	β (95% CI)	*P* value
		ASMM (kg)			
		Model 1		Model 2 (Model 1 + BFMI)	
IGFBP1	100%	**−0.38** (−0.51, −0.26)	**4.74e-09**	**−0.24** (−0.35, −0.12)	**6.05e-05**
LEP	100%	**0.69** (0.54, 0.84)	**<2e-16**	**−0.22** (−0.39, −0.05)	**0.010667**
CCL28	100%	**−0.29**(−0.39, −0.18)	**6.37e-08**	**−0.21** (−0.30, −0.11)	**1.80e-05**
KLK6	98%	**−0.46** (−0.58, −0.33)	**3.41e-12**	**−0.34** (−0.46, −0.23)	**5.95e-09**
IGFBP2	94%	**−0.25** (−0.39, −0.10)	**0.000764**	**−0.22** (−0.35, −0.09)	**0.000843**
THBS2	83%	**0.18** (0.06, 0.29)	**0.003159**	0.08 (−0.03, 0.19)	0.142810
MB	76%	**0.28** (0.16, 0.41)	**1.25e-05**	**0.20** (0.08, 0.31)	**0.000690**
TFPI	75%	**−0.21** (−0.33, −0.10)	**0.000147**	**−0.22** (−0.32, −0.12)	**2.40e-05**
Notch3	73%	**0.52** (0.37, 0.66)	**7.72e-12**	**0.43** (0.30, 0.57)	**1.84e-10**
DNER	66%	**−0.19** (−0.31, −0.08)	**0.000910**	**−0.12** (−0.23, −0.02)	**0.021671**
GDF2	63%	**−0.21** (−0.34, −0.09)	**0.000496**	−0.07 (−0.18, 0.04)	0.212388
		BFMI (kg/m^2)			
		Model 1		Model 2 (Model 1 + ASMM)	
IGFBP1	100%	**−0.36** (−0.47, −0.24)	**1.46e-09**	**−0.23** (−0.33, −0.12)	**1.74e-05**
LEP	100%	**1.32** (1.15, 1.49)	**<2e-16**	**1.09** (0.93, 1.24)	**<2e-16**
GDF2	97%	**−0.26** (−0.37, −0.16)	**1.42e-06**	**−0.20** (−0.30, −0.11)	**2.32e-05**
ADM	97%	**0.30** (0.16, 0.43)	**2.45e-05**	**0.25** (0.13, 0.37)	**7.27e-05**
FABP4	93%	**0.78** (0.62, 0.93)	**<2e-16**	**0.70** (0.56, 0.83)	**<2e-16**
CCL28	79%	**−0.17** (−0.26, −0.07)	**0.000425**	−0.05 (−0.13, 0.03)	0.257357
PRSS27	76%	**−0.17** (−0.27, −0.08)	**0.000399**	**−0.12** (−0.21, −0.03)	**0.005940**
GH	72%	**−0.13** (−0.24, −0.03)	**0.013998**	**−0.12** (−0.22, −0.03)	**0.012302**
HO1	71%	0.08 (−0.02, 0.18)	0.124910	0.03 (−0.05, 0.12)	0.444350
PON3	68%	**−0.27** (−0.39, −0.16)	**4.55e-06**	**−0.20** (−0.30, −0.09)	**0.000206**
IGFBP2	64%	**−0.18** (−0.31, −0.04)	**0.010195**	−0.08 (−0.20, 0.04)	0.181302
		Logistic regression models			
Selected variables	Selection frequency	OR (95% CI)	*P* value	OR (95% CI)	*P* value
		Low ASMM			
		Model 1		Model 2 (Model 1 + high BFMI)	
IGFBP2	100%	1.14 (0.94, 1.39)	0.180094	1.14 (0.93, 1.39)	0.207678
IGFBP1	100%	**1.45** (1.19, 1.75)	**0.000166**	**1.40** (1.15, 1.70)	**0.000844**
LEP	100%	**0.51** (0.42, 0.64)	**6.21e-10**	**0.67** (0.54, 0.84)	**0.000537**
KLK6	99%	**1.63** (1.37, 1.95)	**3.80e-08**	**1.61** (1.35, 1.93)	**1.50e-07**
CCL28	95%	**1.31** (1.14, 1.51)	**0.000177**	**1.29** (1.12, 1.49)	**0.000590**
MB	74%	**0.62** (0.52, 0.75)	**2.88e-07**	**0.64** (0.53, 0.77)	**3.03e-06**
TFPI	65%	**1.24** (1.06, 1.45)	**0.006704**	**1.23** (1.05, 1.45)	**0.008969**
GH	63%	**1.18** (1.00, 1.39)	**0.048414**	1.16 (0.98, 1.37)	0.083713

(*Continues*)

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		High BFMI		
		Model 1		Model 2 (Model 1 + low ASMM)
LEP	100%	**8.08** (5.39, 12.13)	**<2e-16**	**7.55** (4.95, 11.52)	**<2e-16**
PON3	99%	**0.69** (0.57, 0.83)	**0.000123**	**0.71** (0.58, 0.86)	**0.000450**
IGFBP1	98%	**0.74** (0.61, 0.90)	**0.002295**	**0.76** (0.62, 0.93)	**0.006683**
FABP4	96%	**1.91** (1.46, 2.51)	**2.95e-06**	**2.02** (1.52, 2.68)	**9.68e-07**
ADM	90%	**1.50** (1.16, 1.94)	**0.001884**	**1.55** (1.18, 2.03)	**0.001447**
GH	74%	**0.77** (0.63, 0.93)	**0.007026**	**0.81** (0.66, 1.00)	**0.044724**
PRSS27	69%	**0.73** (0.61, 0.86)	**0.000264**	**0.75** (0.63, 0.90)	**0.001481**
RAGE	63%	**0.70** (0.58, 0.85)	**0.000362**	**0.71** (0.58, 0.86)	**0.000668**
		Combination low ASMM and high BFMI		
		Model 1		
LEP	94%	**2.64** (1.84, 3.77)	**1.03e-07**	
TIMP4	80%	**1.28** (1.03, 1.59)	**0.02760**	
CCL28	63%	**1.32** (1.08, 1.61)	**0.00712**	

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; β, beta coefficient; CI, confidence interval; OR, odds ratio.
The cut point for variable selection in the boosting with stability selection was a selection frequency of 63%, which was determined by the algorithm based on the number of variables available for selection, the number of selected variables per iteration, and the maximum number of tolerable false positives.
Effect estimates have been calculated per 1 SD increase in normalized protein expression values on a log2 scale.
Model 1: adjustment for all 13 covariates (age, high-density lipoprotein, triglycerides, glycated haemoglobin, estimated glomerular filtration rate, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication) as well as all other in the boosting with stability selection selected variables of the corresponding outcome.
Bold print indicates significance. Grey shading indicates change of the direction of association or attenuation of the association (i.e. non-significant effect estimates) after adjustment in Model 2 compared with Model 1.

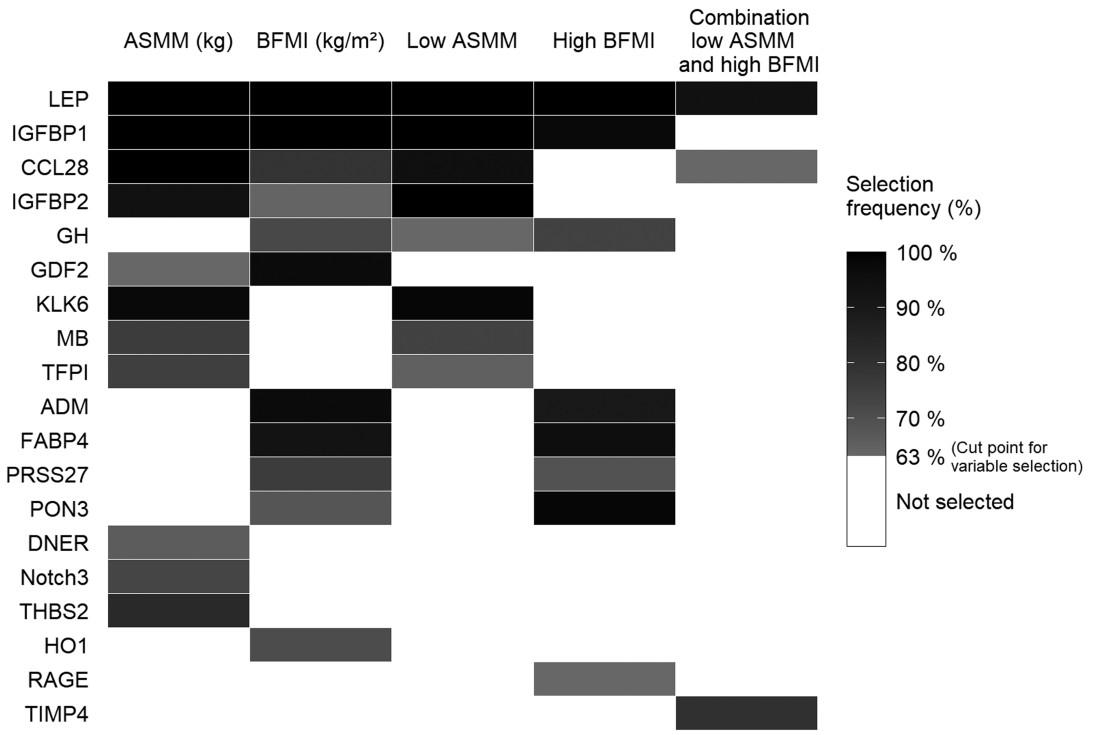

Figure 2 Association analysis — boosting with stability selection — comparison of protein biomarker selection between the outcomes. Protein biomarkers are primarily ordered according to the number of outcomes the biomarkers were selected for and secondary according to their selection for the outcomes in the table from left to right. Only protein biomarkers are included that were selected for at least one outcome. The cut point for variable selection was a selection frequency of 63%, which was determined by the algorithm based on the number of variables available for selection, the number of selected variables per iteration, and the maximum number of tolerable false positives. ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.

Table 2 Prediction analysis — cross-validated AUCs of logistic regression models with classical risk factors (mean AUC_{basic}) and protein biomarkers in addition to classical risk factors (mean $AUC_{extended}$)

Outcome	Mean AUC_{basic} (95% CI)	Mean $AUC_{extended}$ (95% CI)	Mean delta AUC (95% CI)
Low ASMM	0.67 (0.65, 0.71)	0.83 (0.82, 0.87)	0.16 (0.13, 0.20)
High BFMI	0.67 (0.65, 0.72)	0.89 (0.88, 0.92)	0.22 (0.18, 0.25)
Combination low ASMM and high BFMI	0.73 (0.69, 0.80)	0.85 (0.83, 0.90)	0.12 (0.08, 0.17)

ASMM, appendicular skeletal muscle mass; AUC, area under the curve; BFMI, body fat mass index; CI, confidence interval.
AUC_{basic}: AUC of a logistic regression model including 13 classical risk factors (age, high-density lipoprotein, triglycerides, glycated haemoglobin, estimated glomerular filtration rate, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication). $AUC_{extended}$: AUC of the basic model plus all protein biomarkers selected in ≥90% of the group least absolute shrinkage and selection operator bootstrap iterations (variables are listed in Supporting information, *Table* S8). Delta AUC: $AUC_{extended} - AUC_{basic}$. AUCs and delta AUCs are arithmetic means of 10-fold cross-validation. The confidence intervals of AUCs and delta AUCs were calculated via 100-fold percentile bootstrapping.

Figure 3 illustrates the results of the sensitivity analysis including the comparison of variables between the outcomes regarding the number of methods (group lasso with bootstrapping, random forest, and support vector machine) that ranked the variables in the top 10.

In the prediction analysis, the protein biomarkers were ranked equal to or even higher than classical risk factors (Supporting information, *Tables* S8 and S9) and were ranked in the top 10 in all three methods more consistently compared with classical risk factors (*Figure* 3).

Longitudinal analysis

Detailed results regarding the longitudinal analysis are included in Supporting information, *Tables* S10–S13 and *Figures* S5 and S6. Most relevant results of the association analysis include that N-terminal prohormone brain natriuretic peptide (NT-proBNP) was the only protein biomarker selected for a strong decrease in ASMM and the combination of a strong decrease in ASMM and a strong increase in BFMI. In logistic regression analyses, NT-proBNP was positively associated

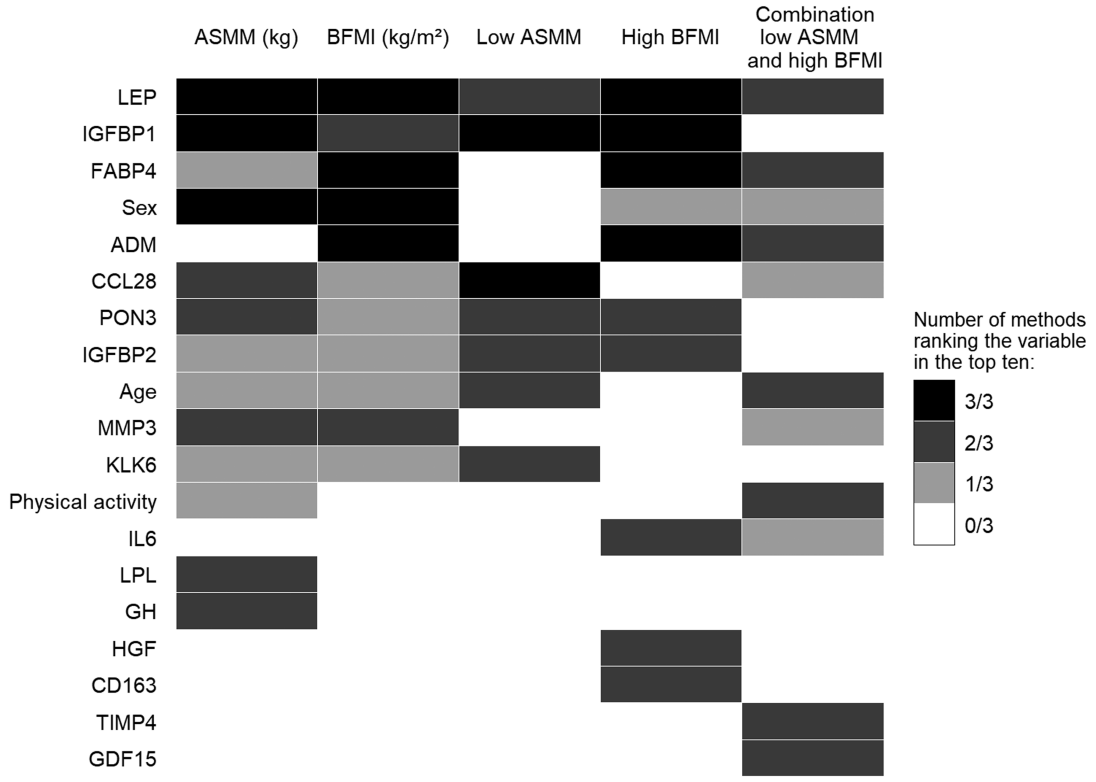

Figure 3 Sensitivity analysis — comparison of variables between the outcomes regarding the number of methods that ranked the variables in the top 10. Only variables are included that were ranked in the top 10 in at least two of the three analysis methods (group least absolute shrinkage and selection operator with 100× bootstrapping, random forest, and support vector machine) in at least one of the five outcomes. Variables are primarily ordered descending according to the total number (sum of all outcomes) of methods that ranked the variable in the top 10, and secondary according to the outcome in the table from left to right based on the number of methods that ranked the variable in the top 10 for the outcome. ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.

with a strong decrease in ASMM [odds ratio (OR) (95% CI): 1.40 (1.10, 1.77) and the combined outcome (OR (95% CI): 1.60 (1.15, 2.24)] after adjustment for all 13 covariates. CCL4, CCL15, and a disintegrin and metalloproteinase with thrombospondin motifs 13 were selected for relative change in BFMI and protein delta homolog 1 for strong increase in BFMI. In the prediction analysis, group lasso with bootstrapping ranked NT-proBNP in first place for both, strong decrease in ASMM and the combined outcome. Sensitivity analysis presents age for relative change in ASMM and CCL4 for relative change in BFMI as the only variables ranked in the top 10 of all three methods for any outcome.

Discussion

This study aimed to identify new protein biomarkers of low muscle mass, high fat mass, and their combination as well as their changes over a 14 year follow-up period. In our cross-sectional analysis, we identified KLK6, CCL28, and TFPI as novel protein biomarkers associated with muscle mass and PRSS27 with fat mass. CCL28 and TIMP4 are newly detected biomarkers associated with the combination of low muscle and high fat mass. In the longitudinal analysis, NT-proBNP was the only biomarker that was selected for a strong decrease in ASMM and the combination of a strong decrease in ASMM and a strong increase in BFMI over 14 years.

To the best of our knowledge, this is the first study to investigate the pathological condition of combined low muscle and high fat mass using proteomics. However, a few previous studies investigated related body composition parameters. Six studies investigated proteomics measured with PEA technology by Olink® using the CVDII panel with BMI-defined obesity,[12] inflammation panel with BMI and WC,[13] CVDI panel with changes in BMI and WHR,[14] inflammation, cardiometabolic, CVDII, and CVDIII panels with BMI-defined obesity,[15] immuno-oncology panel with BMI,[19] and a large-scale mapping of genetics of the proteome investigated causal relationships of CVDI panel with BMI, body fat (%), and WHR.[18] No previous study investigated PEA-measured proteomics and

muscle mass. Other studies implemented the aptamer-based proteomics approach SOMAscan by SomaLogic (Boulder, Colorado, USA), which is as PEA a relative quantification method, but instead of antibodies, aptamers are used, which are randomly generated nucleotide sequences.[23] Aptamer-based proteomics were used to investigate dual-energy X-ray absorptiometry (DXA)-measured body fat mass (kg)[16] as well as DXA-measured body fat (%) and lean body mass (kg).[17]

The comparison of our results with those of other studies has to be viewed with caution as different ethnicities can show varying body composition and numerous different parameters and measurement methods have been used to define muscle and fat mass. As BFMI showed strong correlations to BMI and WC in our data, comparisons of our results with those of studies using these parameters are feasible to some extent.

Relevant protein biomarkers

In the cross-sectional analysis, we identified various protein biomarkers associated with both, muscle and fat mass parameters, among them IGFBP1 and IGFBP2. In line with our results, both proteins are reduced with increasing obesity[24] and an US-American cohort study using single biomarkers measured with radioimmunoassays showed that higher total per cent fat and higher visceral fat were associated with lower IGFBP1 and IGFBP2.[25] In a Swedish PEA-based-proteomics study, IGFBP1 was inversely associated with BMI-defined obesity[15] and by using aptamer methodology, IGFBP1 was inversely associated with fat mass (kg).[16] Concerning muscle mass, IGFBP1 was inversely associated with DXA-measured low relative muscle mass in a Swedish cohort of elderly women[26] and IGFBP2 was inversely associated with DXA-measured total muscle mass in an US-American study.[27] Both IGFBPs have been related to glucose and insulin levels and are known to be suppressed by GH.[24] In our study, GH was selected for some of the same outcomes that the IGFBPs were selected for as well. A longitudinal PEA-based study observed that a decrease in GH was associated with an increase in BMI and WHR over a 10 year period.[14] Payette *et al*. summarized the characteristics of GH among others with a decreased secretion in obesity and in contrast to our results inducing anabolic effects on skeletal muscle. GH therapy can increase muscle mass, however with deleterious side effects.[28] Even though the literature is clear regarding a positive association of GH and muscle mass, we observed an opposite association. This may be explained as follows: first, GH secretion is pulsatile and therefore difficult to interpret as an individual value measured at one timepoint.[29] Second, under conditions of cachexia, that is, body wasting including a decrease in muscle and fat mass, observed usually in patients with chronic diseases such as heart failure (HF), GH resistance can develop.[30] This is characterized by increased secretion of GH and reduced insulin-like growth factor 1 (IGF-1) as GH is ineffective in stimulating IGF-1 production,[30,31] diminishing the highly relevant effect of IGF-1 on muscle regeneration and decelerating muscle wasting under conditions of high GH concentrations.[30] Even though we cannot prove this malfunction in our participants with low ASMM, this process might give an insight into the inverse relationship of GH with ASMM in our study.

KLK6, MB, and TFPI were only associated with the continuous and categorical parameters of ASMM. MB is an already known biomarker for increased muscle mass. MB further increases as a result of exercise induced through the degradation of protein structures within the muscle. In addition to its role in oxygen storage and transport, MB is thought to influence nitric oxide at the microvascular and tissue level.[32] KLK6 and TFPI are new biomarkers associated with muscle mass. Due to a lack of previous studies related to body composition, we described the main hallmarks of the new biomarkers. The over-expression of KLK6 transcript and protein has been recognized in numerous cancer types, such as breast, renal, pancreatic, ovarian, colorectal, and lung cancer.[33] In our study, KLK6 was a risk factor for low muscle mass, notably, in participants without cancer. Moreover, KLK6 is linked to inflammatory pathways due to its ability to activate protease-activated receptors, which are relevant in driving inflammatory processes. KLK6 is further attributed to participate in angiogenesis and apoptosis pathways.[33] Regarding TFPI, recent articles investigated the biomarker as a potential treatment against haemophilia, due to its role in thrombin generation and coagulation processes.[34] To our knowledge, the relations of KLK6 and TFPI to muscle mass have not been observed before.

FABP4, ADM, PRSS27, and PON3 were only associated with the continuous and categorical parameters of BFMI. In a previous PEA-proteomics study, ADM and FABP4 were positively associated with BMI-defined obesity.[15] An increase in FABP4 was also associated with an increase in BMI and WHR over 10 years.[14] Large-scale mapping of genetics of the proteome identified that BMI and body fat (%) causally affected PEA-measured ADM and FABP4 positively and WHR affected these biomarkers inversely.[18] PRSS27 was the only new biomarker associated with fat mass in our study. The protease is largely unknown, and there are only a few articles mentioning PRSS27, for example, as a possible prognostic marker of oesophageal squamous cell carcinoma in patients with preoperative treatment.[35]

LEP, CCL28, and TIMP4 were associated with the combination of low ASMM and high BFMI. Associations of protein biomarkers to the combined outcome can only be expected if the associations to ASMM and BFMI are aligned in opposite directions or if the strengths of the associations differ to a high extent. If the associations of a biomarker to ASMM and BFMI are similar, a significant association to the

combined outcome cannot be assumed as the combined outcome consists of opposite extremes (low ASMM and high BFMI). LEP is known to increase muscle mass and is inextricable from fat mass as it regulates energy expenditure and LEP sensitivity decreases in obesity.[36] In relation to our results, previous European studies using PEA technology displayed that LEP was positively associated with BMI-defined obesity[12,15] and changes in BMI and WHR.[14] LEP measured with aptamer-based proteomics was positively associated with fat mass (kg) in a European cohort[16] and was selected as one of the top three proteins for body fat (%) but not lean mass (kg) in a Finnish cohort.[17] In large-scale mapping of genetics of the proteome, BMI and body fat (%) causally affected LEP positively and WHR affected LEP inversely.[18] Underlying mechanisms connecting LEP to muscle and fat mass might constitute that under physiological conditions, LEP binds to its receptors in skeletal muscle and fat cells, which can initiate energy dissipation and reduce fatty acid accumulation as well as lipotoxicity in the muscle and fat cells. In obesity, LEP is increased but cannot bind to its receptors; thus, processes of fatty acid oxidation might be impaired, which can lead to intracellular accumulation of lipid intermediates.[36] CCL28 is a new marker for muscle mass and as well as TIMP4 a new marker for the combination of low muscle and high fat mass. CCL28 has only recently and for the first time been inversely associated with the metabolic syndrome in Japanese adults also using PEA proteomics.[37] Generally in line with this finding, we observed an inverse relationship of CCL28 with BFMI. TIMP4 is highly expressed in adipose tissue and was reported to promote high fat-induced obesity, fatty liver, and dyslipidaemia in a study using TIMP4-deficient mice exposed to high-fat diet. The underlying mechanism may be the promotion of intestinal lipid absorption by TIMP4 through the reduction of the proteolytic processing of CD36, a fatty acid transporter in the small intestine. In addition, mice with deficient TIMP4 were protected against skeletal muscle triglyceride accumulation in the quadriceps.[38] Our observations are in line with these reports, as higher levels of TIMP4 were associated with the combination of lower muscle mass and higher fat mass.

In the longitudinal analysis, NT-proBNP was the only protein biomarker selected for a strong decrease in ASMM and the combination of a strong decrease in ASMM and a strong increase in BFMI. In a longitudinal cohort study, a decrease in NT-proBNP was associated with an increase in BMI and WHR over 10 years.[14] NT-proBNP levels are increased in severe muscle wasting and the components of NT-proBNP might be involved in lipolysis in adipose tissue.[39] Furthermore, NT-proBNP is already established in clinical application as a marker of HF. Muscle mass reduction as a part of body wasting is described as a complication of HF by the term cardiac cachexia.[30] This could represent the linkage between higher baseline NT-proBNP values and a stronger decrease in muscle mass over time. We are not able to directly verify this as PEA values are relative and not absolute protein concentrations necessary for HF classification. Additionally, compared with the association solely with a strong decrease in muscle mass, we observed a stronger association of NT-proBNP with a strong decrease in muscle mass combined with a strong increase in fat mass, whereas cardiac cachexia is usually accompanied by reduced muscle and reduced fat mass. However, because not all HF patients show a decrease in fat mass as for instance over 80% of HF patients with preserved ejection fraction are overweight or obese,[30] a decreased heart function reflected by NT-proBNP could still be involved in this association.

Cross-sectional prediction analysis

Protein biomarkers were ranked equally high or even higher than most classical risk factors. In sensitivity analysis, protein biomarkers were ranked in the top 10 by all three methods more consistently compared with classical risk factors. The prediction performance reflected by the AUC for all three binary outcomes distinctly increased when protein biomarkers selected in ≥90% of group lasso bootstraps were added to the classical risk factors. This highlights the importance of protein biomarkers in addition to classical risk factors for optimal prediction of low muscle, high fat mass, and their combination.

Comparison: cross-sectional and longitudinal analysis

Prediction analysis in the longitudinal data yielded distinctly lower AUCs concerning all three, AUC_{basic} (only classical risk factors), $AUC_{extended}$ (classical risk factors plus protein biomarkers), and delta AUC ($AUC_{extended} - AUC_{basic}$), compared with the cross-sectional data. Moreover, the overlap of biomarkers that were selected in both, longitudinal and cross-sectional analyses, was lacking. The main reason likely is that our prospective data force the relation into the direction of baseline protein biomarkers leading to changes in body composition as proteomics data were only available at baseline, and we were therefore unable to investigate the changes in proteomics with the changes in body composition. If, in turn, body composition would affect the biomarkers, only cross-sectional analyses without pre-specified direction would be able to identify the association. A recently published large-scale mapping of genetics of the proteome supports this concept as it demonstrated that body fat (%) causally affected LEP, ADM, and FABP4, but there was only weak evidence of FABP4 and LEP and no evidence of ADM causally affecting body fat (%).[18] In our analysis, these biomarkers were selected in cross-sectional but not in

longitudinal analysis, possibly due to the lacking causal effect of the biomarkers on fat mass.

Strengths and limitations

A major strength of this project presents the usage of proteomics in addition to classical risk factors enabling us to simultaneously analyse 233 protein biomarkers. Furthermore, we employed multiple machine learning approaches to analyse the data based on different aspects. Another strength encompasses the usage of stability selection, which strongly minimizes false positives in the association analysis. Only protein biomarkers with good measurement quality were included in the analysis. Concerning the comparison of the outcomes, bias was minimized as muscle and fat mass were calculated based on the same BIA measurements. Another strength constitutes the implementation of both cross-sectional and longitudinal approaches.

A few limitations of the present study also require acknowledgement. First, generalizability of the results is limited for younger adults and other ethnicities, because the study included primarily white Europeans aged 55–74 years. Second, as the number of participants in the combined outcome was relatively low, we had to implement different cut points for the combined outcome compared with the single outcomes. Third, the PEA technique used for proteomics measurements provides only relative and not absolute protein concentration. Fourth, as we used a targeted proteomics approach with proteins selected for inflammation and CVDs, other non-targeted proteins could also be relevant for muscle and fat mass.

Conclusion

To the best of our knowledge, we identified KLK6, CCL28, and TFPI as novel protein biomarkers associated with muscle mass and PRSS27 with fat mass. CCL28 and TIMP4 are new biomarkers associated with the combination of a low muscle and a high fat mass. NT-proBNP was the only biomarker selected for a strong decrease in muscle mass and the combination of a strong decrease in muscle mass and a strong increase in fat mass over 14 years. In the cross-sectional analysis, proteomics substantially improved the prediction of low muscle, high fat mass, and their combination on top of classical risk factors.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S6. Sensitivity Analysis – Comparison of variables between the outcomes regarding the number of methods that ranked the variables in the top ten – Longitudinal analysis

Conflict of interest

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

Ethical guidelines statement

The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.[40] All study methods were approved by the ethics committee of the Bavarian Chamber of Physicians, Munich (S4: EC No. 99186, FF4: EC No. 06068), and were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All participants gave their written informed consent.

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Supporting information

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Proteomic profiling of low muscle and high fat mass: a machine learning approach in the KORA S4/FF4 study

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Supporting information

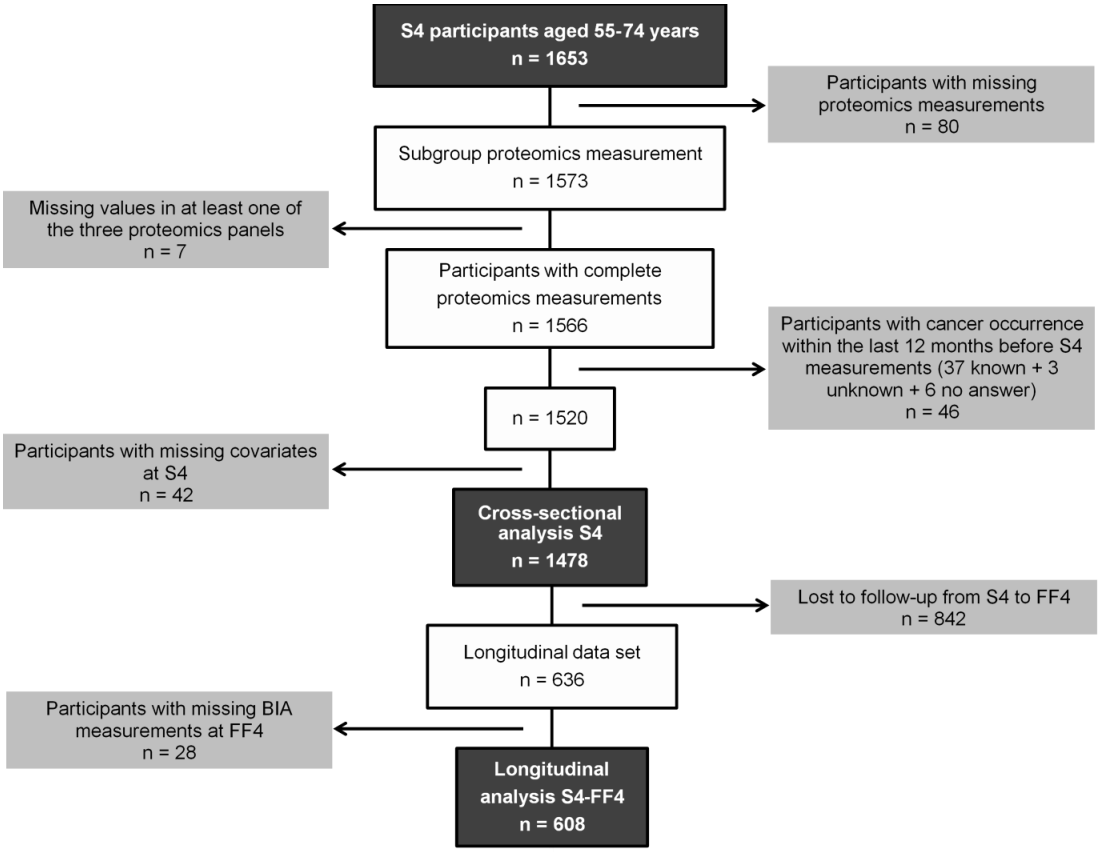

Figure S1: Flow chart of participant exclusions of cross-sectional and longitudinal analysis

BIA, bioelectrical impedance analysis; n, number of participants.

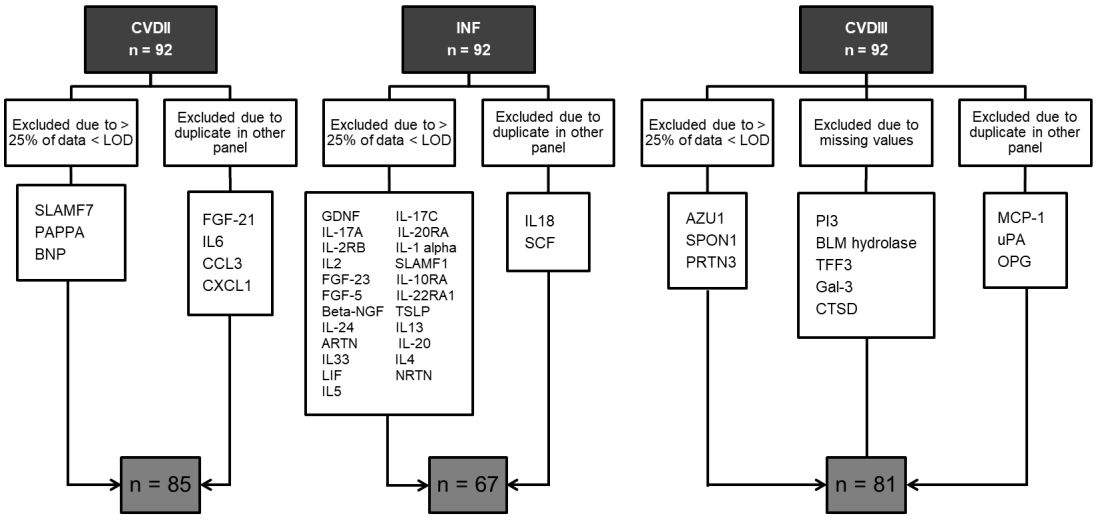

Figure S2: Biomarker exclusions in the three proteomics panels

CVD, cardiovascular disease; INF, inflammation; LOD, limit of detection; n, number of protein biomarkers.

Details regarding exclusions of the protein biomarkers:

We excluded three biomarkers of panel CVDII, three biomarkers of the panel CVDIII, and 23 biomarkers of the panel Inflammation due to values below the limit of detection (LOD) in > 25 % of all data before participant exclusions. From the remaining data, nine biomarkers were measured in duplicate for all participants in two different panels: Six biomarkers were enclosed in both CVDII and Inflammation panels and three biomarkers were included in both CVDIII and Inflammation panels. We decided to exclude the values of the panel in which the data entailed more values below the LOD and if not applicable, a higher inter-assay coefficient of variation. Resulting from this, four biomarkers of CVDII, three biomarkers of CVDIII, and two biomarkers of Inflammation were excluded. Additionally, five biomarkers were excluded in CVDIII, because of missing values not resulting from values below LOD. This concludes to a total number of 233 different protein biomarkers incorporated into the analysis (Figure S2). For all biomarkers that were not excluded and contained values < LOD, the values < LOD remained in the data and were not substituted.

Table S1: Biomarker information CVDII panel

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
ACE2	Angiotensin-converting enzyme 2	Q9BYF1	1.18	0	0	11	13.73
ADAM-TS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	Q76LX8	1.88	0	0	5.58	6.92
ADM	Adrenomedullin	P35318	1.06	0	0	10.15	12.5
AGRP	Agouti-related protein	O00253	0.47	0	0	5.16	12.19
AMBP	Protein AMBP	P02760	0.83	0	0	3.42	4.93
ANGPT1	Angiopoietin-1	Q15389	0.86	0	0	6.82	23.38
BMP-6	Bone morphogenetic protein 6	P22004	2.08	0	0	5.98	17.1
BNP	Natriuretic peptides B	P16860	1.55	1092	69.55	[a]	[a]
BOC	Brother of CDO	Q9BWV1	0.61	0	0	6.77	10.52
CA5A	Carbonic anhydrase 5A, mitochondrial	P35218	1.51	317	20.19	11.19	14.66

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
CCL17	C-C motif chemokine 17	Q92583	0.83	0	0	6.11	30.36
CCL3	C-C motif chemokine 3	P10147	0.58	0	0	5.58	13.28
CD4	T-cell surface glycoprotein CD4	P01730	1.26	0	0	5.65	15.22
CD40-L	CD40 ligand	P29965	2.46	4	0.25	6.73	63.53
CD84	SLAM family member 5	Q9UIB8	2.08	0	0	4.64	18.96
CEACAM8	Carcinoembryonic antigenrelated cell adhesion molecule 8	P31997	1.91	0	0	5.55	16.21
CTRC	Chymotrypsin C	Q99895	1.42	0	0	5.15	10.92
CTSL1	Cathepsin L1	P07711	0.5	0	0	4.06	10.3
CXCL1	C-X-C motif chemokine 1	P09341	3.84	0	0	4.09	43.33
DCN	Decorin	P07585	1.13	0	0	3.86	9.15
DECR1	2,4-dienoyl-CoA reductase, mitochondrial	Q16698	4.95	25	1.59	12.07	39.82
Dkk-1	Dickkopf-related protein 1	O94907	0.93	0	0	5.11	16.65
FABP2	Fatty acid-binding protein, intestinal	P12104	1.66	0	0	5.38	15.76
FGF-21	Fibroblast growth factor 21	Q9NSA1	1.84	0	0	7.12	13.39
FGF-23	Fibroblast growth factor 23	Q9GZV9	2.96	28	1.78	4.22	10.24
FS	Follistatin	P19883	2.08	0	0	4.26	9.51
Gal-9	Galectin-9	O00182	0.46	0	0	3.57	8.49
GDF-2	Growth/differentiation factor 2	Q9UK05	2.81	0	0	7.02	12.5
GH	Growth hormone	P01241	1.1	0	0	3.7	28.29
GIF	Gastric intrinsic factor	P27352	1.7	3	0.19	10.09	11.65
GLO1	Lactoylglutathione lyase	Q04760	1.86	0	0	5.67	66.17
GT	Gastrotropin	P51161	0.75	34	2.17	6.18	11.21
HAOX1	Hydroxyacid oxidase 1	Q9UJM8	1.1	0	0	8.89	17.66
HB-EGF	Proheparin-binding EGF-like growth factor	Q99075	0.61	0	0	6.36	17.6
HO-1	Heme oxygenase 1	P09601	0.97	0	0	5.84	9.94
hOSCAR	Osteoclast-associated immunoglobulin-like receptor	Q8IYS5	2.29	0	0	6.19	6.87
HSP 27	Heat shock 27 kDa protein	P04792	4.29	0	0	5.26	8.73
IDUA	Alpha-L-iduronidase	P35475	1.73	0	0	5.5	15.53

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
IgG Fc receptor II-b	Low affinity immunoglobulin gamma Fc region receptor II-b	P31994	2.2	122	7.77	7.24	13.47
IL-17D	Interleukin-17D	Q8TAD2	1.95	52	3.31	9.15	12.73
IL-1RA	Interleukin-1 receptor antagonist protein	P18510	1.79	0	0	4.49	18.48
IL-27	Interleukin-27	Q8NEV9 ,Q14213	0.99	0	0	4.56	6.7
IL-4RA	Interleukin-4 receptor subunit alpha	P24394	0.88	0	0	4.91	16.73
IL16	Pro-interleukin-16	Q14005	2.47	0	0	7.43	71.9
IL18	Interleukin-18	Q14116	0.45	0	0	5.68	10.41
IL1RL2	Interleukin-1 receptor-like 2	Q9HB29	2.14	0	0	7.33	12.14
IL6	Interleukin-6	P05231	1.57	50	3.18	3.98	11.3
ITGB1BP2	Melusin	Q9UKP3	4.89	123	7.83	[a]	9.72
KIM1	Kidney Injury Molecule	Q96D42	2.01	0	0	5.14	10.28
LEP	Leptin	P41159	1.59	0	0	5.82	14.32
LOX-1	Lectin-like oxidized LDL receptor 1	P78380	1.08	0	0	4.87	12.58
LPL	Lipoprotein lipase	P06858	2.36	0	0	3.13	7.99
MARCO	Macrophage receptor MARCO	Q9UEW 3	1.42	0	0	4.16	11.34
MERTK	Tyrosine-protein kinase Mer	Q12866	2.08	0	0	4.84	12.96
MMP12	Matrix metalloproteinase-12	P39900	0.76	0	0	4.62	10.53
MMP7	Matrix metalloproteinase-7	P09237	1.36	0	0	3.31	22.69
NEMO	NF-kappa-B essential modulator	Q9Y6K9	3.74	2	0.13	7.78	68.32
PAPPA	Pappalysin-1	Q13219	2.99	1229	78.28	8.53	8.92
PAR-1	Proteinase-activated receptor 1	P25116	1.38	0	0	5.33	23.68
PARP-1	Poly [ADP-ribose] polymerase 1	P09874	3.96	315	20.06	24.09	19.74
PD-L2	Programmed cell death 1 ligand 2	Q9BQ51	1.55	2	0.13	5.13	10.54
PDGF subunit B	Platelet-derived growth factor subunit B	P01127	2.26	0	0	7.82	25.22
PGF	Placenta growth factor	P49763	1.08	0	0	8.32	11.87
PIgR	Polymeric immunoglobulin receptor	P01833	2.15	0	0	4.19	6.07
PRELP	Prolargin	P51888	0.39	0	0	3.89	6.32
PRSS27	Serine protease 27	Q9BQR3	0.62	0	0	4.02	6.18

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
PRSS8	Prostasin	Q16651	2.1	0	0	3.75	7.54
PSGL-1	P-selectin glycoprotein ligand 1	Q14242	1.12	0	0	6.16	7.38
PTX3	Pentraxin-related protein PTX3	P26022	1.6	0	0	6.27	9.3
RAGE	Receptor for advanced glycosylation end products	Q15109	1.15	0	0	4.97	10.14
REN	Renin	P00797	0.76	0	0	7.56	10.6
SCF	Stem cell factor	P21583	0.89	0	0	3.26	7
SERPINA12	Serpin A12	Q8IW75	-0.16	2	0.13	5.18	13.01
SLAMF7	SLAM family member 7	Q9NQ25	3.51	498	31.72	6.97	13.61
SOD2	Superoxide dismutase [Mn], mitochondrial	P04179	0.66	0	0	3.86	4.91
SORT1	Sortilin	Q99523	1.45	0	0	3.47	8.43
SPON2	Spondin-2	Q9BUD6	0.62	0	0	4.14	7.96
SRC	Proto-oncogene tyrosine-protein kinase Src	P12931	1.29	0	0	5.37	29.18
STK4	Serine/threonine-protein kinase 4	Q13043	2.64	15	0.96	7.99	26.11
TF	Tissue factor	P13726	0.06	0	0	4.2	8.53
TGM2	Protein-glutamine gamma-glutamyltransferase 2	P21980	2.44	0	0	2.43	12.44
THBS2	Thrombospondin-2	P35442	0.29	0	0	3.67	4.81
THPO	Thrombopoietin	P40225	0.14	0	0	5.72	11.97
TIE2	Angiopoietin-1 receptor	Q02763	1.43	0	0	5.24	7.67
TM	Thrombomodulin	P07204	3.73	0	0	4.74	8.32
TNFRSF10A	Tumor necrosis factor receptor superfamily member 10A	O00220	2.01	0	0	5.44	9.71
TNFRSF11A	Tumor necrosis factor receptor superfamily member 11A	Q9Y6Q6	1.24	0	0	4.9	10.96
TNFRSF13B	Tumor necrosis factor receptor superfamily member 13B	O14836	1.84	0	0	4.75	8.33
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2	O14763	1.68	0	0	6.33	9.24
VEGFD	Vascular endothelial growth factor D	O43915	0.32	0	0	5.61	6.81
VSIG2	V-set and immunoglobulin domain-containing protein 2	Q96IQ7	2.16	0	0	7.83	11.65

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
XCL1	Lymphotactin	P47992	0.5	0	0	4.61	10.06

CV, coefficient of variation; LOD, limit of detection; UniProt ID, universal protein database identification.

[a] All or nearly all values of the control samples, which are requisite to calculate the CVs, were < LOD. If the values of the control samples are < LOD, they are not included in the calculation of the CVs. Therefore, the number of available values was too low to estimate the CV.

Table S2: Biomarker information CVDIII panel

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
ALCAM	CD166 antigen	Q13740	0.71	0	0	6.04	16.21
AP-N	Aminopeptidase N	P15144	0.63	0	0	5.42	13.16
AXL	Tyrosine-protein kinase receptor UFO	P30530	2.95	0	0	6.62	18.45
AZU1	Azurocidin	P20160	3.06	841	53.46	[a]	[a]
BLM hydrolase	Bleomycin hydrolase	Q13867	1.45	52	3.31	5.41	18.12
CASP-3	Caspase-3	P42574	3.14	4	0.25	6.18	69.48
CCL15	C-C motif chemokine 15	Q16663	0.76	0	0	6.81	17.15
CCL16	C-C motif chemokine 16	O15467	0.58	0	0	8.22	19.65
CCL24	C-C motif chemokine 24	O00175	0.61	0	0	6.45	17.3
CD163	Scavenger receptor cysteine-rich type 1 protein M130	Q86VB7	0.88	0	0	6.62	16.62
CD93	Complement component C1q receptor	Q9NPY3	1.21	0	0	5.14	15.99
CDH5	Cadherin-5	P33151	1.24	0	0	7.06	18.09
CHI3L1	Chitinase-3-like protein 1	P36222	2.86	164	10.43	4.95	13.02
CHIT1	Chitotriosidase-1	Q13231	1.58	87	5.53	5.22	17.57
CNTN1	Contactin-1	Q12860	0.69	0	0	7.06	17.89
COL1A1	Collagen alpha-1(I) chain	P02452	0.35	0	0	5.16	14.62
CPA1	Carboxypeptidase A1	P15085	0.40	0	0	5.61	13.75
CPB1	Carboxypeptidase B	P15086	0.20	0	0	5.64	13.64
CSTB	Cystatin-B	P04080	3.26	47	2.99	6.83	22.64
CTSD	Cathepsin D	P07339	1.26	0	0	4.51	14.86

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
CTSZ	Cathepsin Z	Q9UBR2	0.64	0	0	5.51	15.3
CXCL16	C-X-C motif chemokine 16	Q9H2A7	0.69	0	0	5.42	15.85
DLK-1	Protein delta homolog 1	P80370	0.62	0	0	6.24	17.52
EGFR	Epidermal growth factor receptor	P00533	0.90	0	0	5.66	13.34
Ep-CAM	Epithelial cell adhesion molecule	P16422	2.29	0	0	5.69	21.07
EPHB4	Ephrin type-B receptor 4	P54760	1.40	0	0	5.17	15.22
FABP4	Fatty acid-binding protein 4	P15090	1.61	0	0	5.85	21.23
FAS	Tumor necrosis factor receptor superfamily member 6	P25445	-0.16	0	0	5.81	16.09
Gal-3	Galectin-3	P17931	0.61	0	0	5.64	14.55
Gal-4	Galectin-4	P56470	0.61	0	0	7.03	14.98
GDF-15	Growth/differentiation factor 15	Q99988	0.51	0	0	5.07	14.89
GP6	Platelet glycoprotein VI	Q9HCN6	1.00	37	2.35	5.82	34.23
GRN	Granulins	P28799	1.97	0	0	5.44	13.83
ICAM-2	Intercellular adhesion molecule 2	P13598	1.10	0	0	6.59	15.94
IGFBP-1	Insulin-like growth factor-binding protein 1	P08833	1.33	0	0	5.44	17.38
IGFBP-2	Insulin-like growth factor-binding protein 2	P18065	1.54	0	0	5.52	17.79
IGFBP-7	Insulin-like growth factor-binding protein 7	Q16270	1.19	0	0	6.37	16.87
IL-17RA	Interleukin-17 receptor A	Q96F46	1.36	0	0	6.3	24.57
IL-18BP	Interleukin-18-binding protein	O95998	0.90	0	0	6.13	15.4
IL-1RT1	Interleukin-1 receptor type 1	P14778	2.17	0	0	5.74	15.31
IL-1RT2	Interleukin-1 receptor type 2	P27930	2.85	0	0	5.66	14.4
IL-6RA	Interleukin-6 receptor subunit alpha	P08887	2.39	0	0	5.22	15.24
IL2-RA	Interleukin-2 receptor subunit alpha	P01589	0.56	0	0	5.13	13.17
ITGB2	Integrin beta-2	P05107	3.61	0	0	5.04	31.13
JAM-A	Junctional adhesion molecule A	Q9Y624	2.45	1	0.06	7.28	64.49
KLK6	Kallikrein-6	Q92876	1.59	75	4.77	6.87	14.04
LDL receptor	Low-density lipoprotein receptor	P01130	0.73	0	0	5.71	16.18
LTBR	Lymphotoxin-beta receptor	P36941	0.42	0	0	4.95	15.07

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
MB	Myoglobin	P02144	2.16	0	0	6.48	15.06
MCP-1	Monocyte chemotactic protein 1	P13500	0.49	0	0	5.65	19.24
MEPE	Matrix extracellular phosphoglycoprotein	Q9NQ76	1.11	0	0	8.96	19.91
MMP-2	Matrix metalloproteinase-2	P08253	0.40	0	0	6.31	18.11
MMP-3	Matrix metalloproteinase-3	P08254	1.30	0	0	6.64	17.82
MMP-9	Matrix metalloproteinase-9	P14780	2.06	0	0	6.13	23.75
MPO	Myeloperoxidase	P05164	3.22	347	22.06	3.73	12.41
Notch 3	Neurogenic locus notch homolog protein 3	Q9UM47	0.87	0	0	7.24	18.32
NT-proBNP	N-terminal prohormone brain natriuretic peptide	NA	2.34	236	15	6.62	17.19
OPG	Osteoprotegerin	O00300	0.73	0	0	5.78	15.64
OPN	Osteopontin	P10451	1.04	0	0	6.22	19.29
PAI	Plasminogen activator inhibitor 1	P05121	1.35	0	0	5.89	22.87
PCSK9	Proprotein convertase subtilisin/kexin type 9	Q8NBP7	0.82	0	0	8.13	18.11
PDGF subunit A	Platelet-derived growth factor subunit A	P04085	2.08	57	3.62	5.5	36.9
PECAM-1	Platelet endothelial cell adhesion molecule	P16284	0.99	0	0	5.32	37.48
PGLYRP1	Peptidoglycan recognition protein 1	O75594	1.63	0	0	5.36	16.09
PI3	Elafin	P19957	1.13	66	4.2	10.15	38.14
PLC	Perlecan	P98160	3.40	0	0	4.73	14.56
PON3	Paraoxonase	Q15166	0.68	0	0	6.92	18.11
PRTN3	Myeloblastin	P24158	3.66	429	27.27	4.07	21.1
PSP-D	Pulmonary surfactant-associated protein D	P35247	1.40	16	1.02	8.65	12.08
RARRES2	Retinoic acid receptor responder protein 2	Q99969	1.39	0	0	7.64	14.89
RETN	Resistin	Q9HD89	2.85	0	0	5.26	15.52
SCGB3A2	Secretoglobin family 3A member 2	Q96PL1	0.20	0	0	6.24	17.44
SELE	E-selectin	P16581	3.23	0	0	4.84	13.58
SELP	P-selectin	P16109	1.92	0	0	6.47	40.36
SHPS-1	Tyrosine-protein phosphatase non-receptor type substrate 1	P78324	1.04	0	0	6.23	17.31
SPON1	Spondin-1	Q9HCB6	1.51	746	47.43	5.6	10.9

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
ST2	ST2 protein	Q01638	1.62	1	0.06	8.81	14.62
t-PA	Tissue-type plasminogen activator	P00750	2.44	0	0	5.6	25.63
TFF3	Trefoil factor 3	Q07654	2.68	0	0	6.49	15.5
TFPI	Tissue factor pathway inhibitor	P10646	0.53	0	0	6.2	15.91
TIMP4	Metalloproteinase inhibitor 4	Q99727	0.58	0	0	5.78	14.53
TLT-2	Trem-like transcript 2 protein	Q5T2D2	2.47	0	0	7.23	22
TNF-R1	Tumor necrosis factor receptor 1	P19438	1.36	0	0	6.46	15.15
TNF-R2	Tumor necrosis factor receptor 2	P20333	2.17	0	0	6.08	15.06
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10C	O14798	1.77	0	0	5.94	14.54
TNFRSF14	Tumor necrosis factor receptor superfamily member 14	Q92956	1.85	0	0	5.39	18.65
TNFSF13B	Tumor necrosis factor ligand superfamily member 13B	Q9Y275	1.28	0	0	6.02	16.91
TR	Transferrin receptor protein 1	P02786	0.57	0	0	4.34	12.57
TR-AP	Tartrate-resistant acid phosphatase type 5	P13686	1.97	0	0	5.58	14.63
U-PAR	Urokinase plasminogen activator surface receptor	Q03405	1.86	0	0	6.13	18.69
uPA	Urokinase-type plasminogen activator	P00749	1.09	0	0	5.65	15.43
vWF	von Willebrand factor	P04275	1.09	0	0	11.49	38.74

CV, coefficient of variation; LOD, limit of detection; UniProt ID, universal protein database identification.

[a] All or nearly all values of the control samples, which are requisite to calculate the CVs, were < LOD. If the values of the control samples are < LOD, they are not included in the calculation of the CVs. Therefore, the number of available values was too low to estimate the CV.

Table S3: Biomarker information Inflammation panel

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1	Q13541	2.19	0	0	5.78	64.17
ADA	Adenosine Deaminase	P00813	1.06	0	0	6.88	29.35

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
ARTN	Artemin	Q5T4W7	0.97	1476	93.95	[a]	[a]
AXIN1	Axin-1	O15169	2.24	26	1.65	5.08	48.36
Beta-NGF	Beta-nerve growth factor	P01138	1.68	1549	98.6	[a]	[a]
CASP-8	Caspase-8	Q14790	1.64	215	13.69	6.17	48
CCL11	Eotaxin	P51671	0.75	0	0	5.83	14.16
CCL19	C-C motif chemokine 19	Q99731	1.82	0	0	5.45	15.73
CCL20	C-C motif chemokine 20	P78556	1.64	0	0	7.31	21.3
CCL23	C-C motif chemokine 23	P55773	1.63	0	0	5.38	12.34
CCL25	C-C motif chemokine 25	O15444	0.55	0	0	5.97	10.84
CCL28	C-C motif chemokine 28	Q9NRJ3	0.71	0	0	7.38	12.03
CCL3	C-C motif chemokine 3	P10147	0.14	0	0	6.48	12.74
CCL4	C-C motif chemokine 4	P13236	0.45	0	0	6.06	14.57
CD244	Natural killer cell receptor 2B4	Q9BZW8	2.03	0	0	7.16	21.25
CD40	CD40L receptor	P25942	2.21	0	0	5.33	25.34
CD5	T-cell surface glycoprotein CD5	P06127	0.7	0	0	6.09	20.84
CD6	T cell surface glycoprotein CD6 isoform	P30203	1.5	0	0	11.63	28.56
CD8A	T-cell surface glycoprotein CD8 alpha chain	P01732	1.23	0	0	8.41	14.18
CDCP1	CUB domain-containing protein 1	Q9H5V8	-0.06	0	0	10.33	12.52
CSF-1	Macrophage colony-stimulating factor 1	P09603	1.24	0	0	5.66	8.18
CST5	Cystatin D	P28325	0.57	0	0	4.77	11.88
CX3CL1	Fractalkine	P78423	1.11	0	0	8.39	13.04
CXCL1	C-X-C motif chemokine 1	P09341	2.49	0	0	5.49	42.98
CXCL10	C-X-C motif chemokine 10	P02778	2.98	0	0	5.96	15.71
CXCL11	C-X-C motif chemokine 11	O14625	1.89	0	0	5.59	37.97
CXCL5	C-X-C motif chemokine 5	P42830	3.17	0	0	4.49	40.11
CXCL6	C-X-C motif chemokine 6	P80162	1.28	0	0	5.63	30.23
CXCL9	C-X-C motif chemokine 9	Q07325	1.38	0	0	5.45	12.76

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
DNER	Delta and Notch-like epidermal growth factor-related receptor	Q8NFT8	1.31	0	0	4.22	9.55
EN-RAGE	Protein S100-A12	P80511	0.13	0	0	7.89	28.18
FGF-19	Fibroblast growth factor 19	O95750	0.89	0	0	5.69	13.34
FGF-21	Fibroblast growth factor 21	Q9NSA1	1.6	0	0	6.26	11.12
FGF-23	Fibroblast growth factor 23	Q9GZV9	2.38	1279	81.41	8.34	9.23
FGF-5	Fibroblast growth factor 5	P12034	0.68	1203	76.58	8.94	8.53
Flt3L	Fms-related tyrosine kinase 3 ligand	P49771	1.89	0	0	6.28	11.58
GDNF	Glial cell line-derived neurotrophic factor	P39905	2.07	721	45.89	8.89	12.11
HGF	Hepatocyte growth factor	P14210	0.93	0	0	5.3	14.82
IFN-gamma	Interferon gamma	P01579	3.08	0	0	6.9	14.08
IL-1 alpha	Interleukin-1 alpha	P01583	-0.62	1493	95.04	[a]	[a]
IL-10RA	Interleukin-10 receptor subunit alpha	Q13651	0.34	512	32.59	8.22	9.7
IL-10RB	Interleukin-10 receptor subunit beta	Q08334	0.73	0	0	6.45	9.64
IL-12B	Interleukin-12 subunit beta	P29460	0.19	0	0	6.62	13.25
IL-15RA	Interleukin-15 receptor subunit alpha	Q13261	0.22	0	0	8.66	9.87
IL-17A	Interleukin-17A	Q16552	1.1	725	46.15	9.99	13.14
IL-17C	Interleukin-17C	Q9P0M4	1.48	878	55.89	6.67	5.76
IL-18R1	Interleukin-18 receptor 1	Q13478	1.36	0	0	5.29	11.1
IL-20	Interleukin-20	Q9NYY1	0.73	1515	96.44	[a]	[a]
IL-20RA	Interleukin-20 receptor subunit alpha	Q9UHF4	1.03	1394	88.73	7.64	9.21
IL-22 RA1	Interleukin-22 receptor subunit alpha-1	Q8N6P7	2.63	1369	87.14	9.04	14.66
IL-24	Interleukin-24	Q13007	1.99	1501	95.54	[a]	[a]
IL-2RB	Interleukin-2 receptor subunit beta	P14784	1.6	1443	91.85	[a]	1.77
IL10	Interleukin-10	P22301	1.99	5	0.32	9.48	16.18
IL13	Interleukin-13	P35225	1.14	1423	90.58	6.96	11.88
IL18	Interleukin-18	Q14116	0.35	0	0	5.62	14.9
IL2	Interleukin-2	P60568	1.48	1568	99.81	[a]	[a]
IL33	Interleukin-33	O95760	1.41	1542	98.15	[a]	[a]

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
IL4	Interleukin-4	P05112	1.07	1409	89.69	[a]	2.41
IL5	Interleukin-5	P05113	1.34	1415	90.07	8.35	15.58
IL6	Interleukin-6	P05231	1.48	23	1.46	5.27	12.44
IL7	Interleukin-7	P13232	0.96	0	0	7.71	20.38
IL8	Interleukin-8	P10145	0.85	0	0	5.72	13.86
LAP TGF-beta-1	Latency-associated peptide transforming growth factor beta-1	P01137	1.08	0	0	5.26	17.29
LIF	Leukemia inhibitory factor	P15018	0.88	1504	95.74	5.44	7.43
LIF-R	Leukemia inhibitory factor receptor	P42702	0.81	0	0	7.19	11.11
MCP-1	Monocyte chemotactic protein 1	P13500	1.77	0	0	4.94	11.15
MCP-2	Monocyte chemotactic protein 2	P80075	1.93	0	0	8.85	16.51
MCP-3	Monocyte chemotactic protein 3	P80098	1.24	165	10.5	7.67	9.12
MCP-4	Monocyte chemotactic protein 4	Q99616	3.47	0	0	4.77	32.72
MMP-1	Matrix metalloproteinase-1	P03956	1.73	0	0	4.7	28.16
MMP-10	Matrix metalloproteinase-10	P09238	2.43	0	0	4.92	11.4
NRTN	Neurturin	Q99748	1.02	1502	95.61	[a]	[a]
NT-3	Neurotrophin-3	P20783	1.25	6	0.38	7.69	12.76
OPG	Osteoprotegerin	O00300	1.68	0	0	4.57	12.03
OSM	Oncostatin-M	P13725	0.7	0	0	6.52	16.86
PD-L1	Programmed cell death 1 ligand 1	Q9NZQ7	2.82	0	0	5.95	15.45
SCF	Stem cell factor	P21583	1.06	0	0	4.17	8.85
SIRT2	SIR2-like protein 2	Q8IXJ6	4.28	205	13.05	7.04	100.19
SLAMF1	Signaling lymphocytic activation molecule	Q13291	1.49	1007	64.1	[a]	4.39
ST1A1	Sulfotransferase 1A1	P50225	2.67	204	12.99	[a]	[a]
STAMBP	STAM-binding protein	O95630	2.29	0	0	6.65	86.4
TGF-alpha	Transforming growth factor alpha	P01135	0.49	0	0	8.98	10.53
TNF	Tumor necrosis factor	P01375	0.21	0	0	6.08	9.45
TNFB	TNF-beta	P01374	1.21	0	0	7.71	10.28
TNFRSF9	Tumor necrosis factor receptor superfamily member 9	Q07011	1.59	0	0	6.27	10.92

Biomarker ID	Full name	UniProt ID	LOD	Values <LOD (n)	Values <LOD (%)	Intra-Assay CV (%)	Inter-Assay CV (%)
TNFSF14	Tumor necrosis factor ligand superfamily member 14	O43557	1.47	0	0	6.56	35.24
TRAIL	TNF-related apoptosis-inducing ligand	P50591	1.16	0	0	4.94	9.24
TRANCE	TNF-related activation-induced cytokine	O14788	1.02	0	0	8.16	13.39
TSLP	Thymic stromal lymphopoietin	Q969D9	0.64	1476	93.95	[a]	[a]
TWEAK	Tumor necrosis factor (Ligand) superfamily, member 12	O43508	1.67	0	0	6.5	13.86
uPA	Urokinase-type plasminogen activator	P00749	1.69	0	0	4.39	11.53
VEGFA	Vascular endothelial growth factor A	P15692	1.95	0	0	6.67	13.17

CV, coefficient of variation; LOD, limit of detection; UniProt ID, universal protein database identification.

[a] All or nearly all values of the control samples, which are requisite to calculate the CVs, were < LOD. If the values of the control samples are < LOD, they are not included in the calculation of the CVs. Therefore, the number of available values was too low to estimate the CV.

Detailed description concerning the calculations of the outcomes:

Based on the impedance, the BIA generates the parameters resistance and reactance, which were used for the calculations of the variables appendicular skeletal muscle mass (ASMM) and body fat mass index (BFMI). ASMM was calculated using the Sergi equation: ASMM(kg) = -3.964 + 0.227 * resistive index + 0.095 * weight + 1.384 * sex + 0.064 * reactance [1], recommended by the European Working Group on Sarcopenia in Older People in 2019 [2]. Concerning the Sergi equation, the resistive index is the resistance normalized by stature (height^2 / resistance). Sex was coded as female = 0 and male = 1. BFMI was calculated using the equation of Kyle et al. [3]. This included first the calculation of fat free mass (FFM) in kg using the formula: FFM = -4.104 + 0.518 * (height^2 / resistance) + 0.231 * weight + 0.130 * reactance + 4.229 * sex [4], followed by the calculation of body fat in kg (body fat = weight - FFM) and subsequently the calculation of BFMI (BFMI = body fat / height^2).

In the following, we describe the choice of using BIA measurements for our study. Apart from the lower costs, BIA does not expose the participants to radiation as opposed to dual X-ray absorptiometry (DXA) and computed tomography (CT) [5]. This could increase the compliance of the participants and therefore reduce selection bias. Moreover, we specifically used equations to calculate muscle [1] and fat mass [4] for which DXA was used as the reference method. The consensus of the European Working Group on Sarcopenia in Older People from 2019 on which we based our choice to use the Sergi equation for ASMM of BIA measurements, advised the BIA as well as DXA, CT or magnetic resonance imaging (MRI) in research studies to confirm sarcopenia through measuring muscle quantity or quality [2].

Table S4: Definition of the outcomes in the cross-sectional analysis

Outcome variable	**Type**	**Coding**	**N**
ASMM	Continuous (kg)	-	1478
BFMI	Continuous (kg/m^2)	-	1478
Low ASMM[a]	Binary	1: ASMM < 25^{th} sex-specific percentile 0: ASMM ≥ 25^{th} sex-specific percentile	1: 370 0: 1108
High BFMI[b]	Binary	1: BFMI > 75^{th} sex-specific percentile 0: BFMI ≤ 75^{th} sex-specific percentile	1: 370 0: 1108
Combination low ASMM[c] & high BFMI[d]	Binary	1: ASMM < 40^{th} sex-specific percentile & BFMI > 60^{th} sex-specific percentile 0: Remaining participants	1: 110 0: 1368

[a] Cut point for women: 15.26 kg, cut point for men: 21.18 kg

[b] Cut point for women: 13.42 kg/m^2, cut point for men: 9.78 kg/m^2

[c] Cut point for women: 16.08 kg, cut point for men: 22.27 kg

[d] Cut point for women: 12.03 kg/m^2, cut point for men: 8.79 kg/m^2

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; N, number of participants

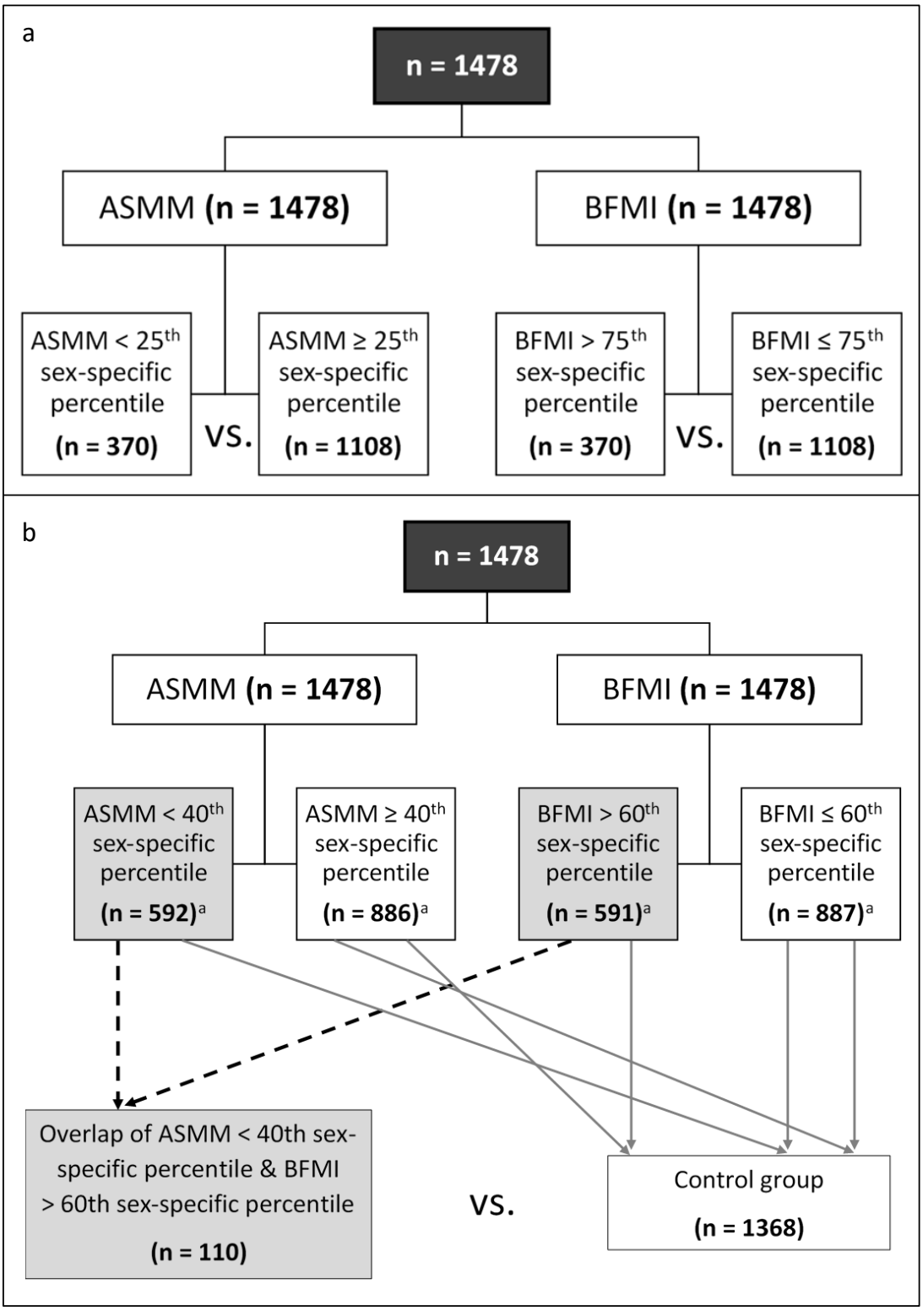

Figure S3: Definition of the outcomes in the cross-sectional analysis

(a) The binary outcome low ASMM consists of the risk group including participants representing the 25 % (n = 370) of participants with the lowest ASMM and its corresponding control group, the remaining 75

% (n = 1108). The binary outcome high BFMI included the 25 % (n = 370) of participants with the highest BFMI and its corresponding control group, the remaining 75 % (n = 1108). (b) The risk group for the combined outcome of low ASMM and high BFMI was determined by intersecting the 40 % of participants with the lowest ASMM and the 40 % of participants with the highest BFMI, illustrated in light grey. This group consists of 7 % (n = 110) of the total study population and the corresponding control group of the remaining participants (n = 1368).

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; n, number of participants.

[a] For the group of male participants, one participant had the same value as the cutoff for BFMI. Therefore, the one participant did count into the group of ≤ 60 %. As this was not the case for ASMM, there is one participant less in the group of ASMM ≥ 40 % compared to the group of BFMI ≤ 60 %.

Table S5: Definition of the outcomes in the longitudinal analysis

Outcome variable	**Type**	**Coding**	**N**
Relative change in ASMM	Continuous (%)	(follow-up – baseline) / baseline) * 100	608
Relative change in BFMI	Continuous (%)	(follow-up – baseline) / baseline) * 100	608
Strong decrease in ASMM[a]	Binary	1: ASMM relative change < 25th sex-specific percentile 0: ASMM relative change ≥ 25th sex-specific percentile	1: 152 0: 456
Strong increase in BFMI[b]	Binary	1: BFMI relative change > 75th sex-specific percentile 0: BFMI relative change ≤ 75th sex-specific percentile	1: 152 0: 456
Combination strong decrease in ASMM[c] & strong increase in BFMI[d]	Binary	1: ASMM relative change < 40th sex-specific percentile & BFMI relative change > 60th sex-specific percentile 0: Remaining participants	1: 57 0: 551

[a] Cut point for women: -6.81 %, cut point for men: -5.28 %

[b] Cut point for women: 13.19 %, cut point for men: 14.21 %

[c] Cut point for women: -4.63 %, cut point for men: -2.75 %

[d] Cut point for women: 7.78 %, cut point for men: 5.08 %

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; N, number of participants.

Detailed description of the covariates:

Albumin was measured in EDTA-plasma with nephelometry using a BN 2 analyzer. Glycated hemoglobin (HbA1c) was analyzed in whole blood with a turbidimetric inhibition immunoassay (TINIA) using a Hitachi 717 (Roche Diagnostics, Mannheim, Germany) [6]. The measurements of high-density lipoprotein (HDL) and triglycerides were described elsewhere [7]. For this analysis, the covariate triglycerides was transformed with natural logarithmic transformation. Estimated glomerular filtration rate (eGFR) was calculated based on measurements of creatinine. Creatinine was measured in serum using enzymatic color test on a Hitachi 917 (Boehringer Mannheim, Mannheim, Germany). The calculations of eGFR with creatinine were based on the publication of Inker et al. in 2012 [8].

The categories of smoking status included never, former or current (at least one cigarette per day) smoker. The definition of the variable physical activity was described elsewhere [9]. The variable education was classified as either > 10 years or ≤ 10 years of education. For the variable alcohol intake, the participants were asked about their consumption of alcoholic beverages on the previous workday and during the previous weekend to estimate the alcohol intake as grams per day. Based on the continuous variable of grams per day, alcohol intake was classified into three categories: men: 0 g/day, 0.1-39.9 g/day, and ≥ 40 g/day; women: 0 g/day, 0.1-19.9 g/day, and ≥ 20 g/day [10]. Blood pressure measurements were described elsewhere [7]. Hypertension was identified if participants had a blood pressure of > 140/90 mmHg or if the participant claimed the intake of antihypertensive medication and was aware of having hypertension [6]. Intake of lipid-lowering medication was defined as intake of at least one medication including Simvastatin, Lovastatin, Pravastatin, Fluvastatin, Atorvastatin, Cerivastatin, Bezafibrat, Gemfirolzil, Fenofibrat, and Etofibrat. Plant-based medication was not included.

Detailed description of the statistical analysis:

All statistical analyses were performed using R, V.3.6.2 [11]. We performed association analysis using the combined method boosting with stability selection [12]. Thereby,

component-wise functional gradient descent boosting of a linear / logistic regression model is combined with the method stability selection, which enables strong control of false positives. We used the R package mboost [13] for boosting and the R package stabs [14] for stability selection. We performed the boosting with an offset encompassing a model including the 13 covariates age, HDL, triglycerides, HbA1c, eGFR, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication. As a result, only protein biomarkers that were associated with the outcome independent of the covariates were selected. In a second step, we calculated logistic / linear regression models with the single selected biomarkers adjusted for all 13 covariates and other selected protein biomarkers of the corresponding outcome (model 1). In model 2, we included in addition to model 1 the opponent outcome as a further covariate, i.e. for the outcome ASMM we adjusted for BFMI and vice versa. For all protein biomarkers of which the coefficients became non-significant or changed directions in model 2 compared to model 1, we further included an interaction term of the concerned protein biomarker and the opponent outcome.

The prediction analysis encompassed the calculation of group least absolute shrinkage and selection operator (lasso) using R package grpreg [15] with 100 bootstrap iterations. Based on the 100 lasso calculations in all training samples of the bootstrapping and therefore 100 results concerning the selected variables, we determined the selection frequency of the variables and based on this the final ranking. All variables with the same selection frequency calculated from lasso with bootstrapping have the same rank; e.g. all variables with a selection frequency of 100% have rank 1. Therefore, more than one variable can be assigned to rank 1. We calculated the area under the curve (AUC) of a logistic regression model including 13 classical risk factors (AUC_{basic}) and a model additionally including protein biomarkers (variables of the cross-sectional analysis are listed in Table S8, variables of the longitudinal analysis in Table S11) that were selected in ≥ 90 % of the group lasso bootstrap iterations ($AUC_{extended}$). We additionally calculated their delta AUC ($AUC_{extended}$-AUC_{basic}) to identify the added prediction performance of the most important protein biomarkers on top of the classical risk factors. Therefore, AUCs and delta AUCs were calculated using the R package fbroc [16]. Cross-

validation was used to calculate the arithmetic means of AUCs and delta AUCs over 10 folds. The confidence intervals (CI) of mean AUCs and mean delta AUCs were calculated via 100-fold percentile bootstrapping using the R package boot [17, 18]. Smoothing the ROC curves enabled us to calculate and plot a mean ROC curve illustrated in Figure S4. We smoothed the ROC curve of each of the 10 folds using the function “smooth” from the R package pROC [19] and created the plots of Figure S4 using the R package ggplot2 [20].

As a sensitivity analysis for the prediction analysis, we further compared the results of lasso with bootstrapping with the results of random forest (RF) and support vector machine (SVM). We performed RF using the R package randomForest [21]. R packages caret [22] and e1071 [23] with the “svmlinear2” method were used for SVM with linear Kernel. The ranking of the variables in RF and SVM was according to variable importance measures (VIM), based on the mean decrease in accuracy for categorical outcomes in RF, percentage increase in mean squared error for continuous outcomes in RF, coefficient of determination R^2 for continuous outcomes in SVM and AUC for categorical outcomes in SVM. The top 10 rankings of the most important variables of the lasso with bootstrapping, RF, and SVM were compared in the sensitivity analysis. In all prediction analyses, the classical risk factors and the protein biomarkers were processed equally as possible predictors. Therefore, all variables (13 classical risk factors and 233 protein biomarkers) were available for the ranking.

In the longitudinal analysis, we used the same statistical approach as in the cross-sectional analysis.

Table S6: Baseline (S4) characteristics of the study population

Characteristic	Cross-sectional (n = 1478)	Longitudinal (n = 608)
Age (years) [a]	63.9±5.4	61.9±4.9
Sex male, n (%)	756 (51.2)	315 (51.8)
Triglycerides (mmol/L) [b]	1.41 (1.01)	1.34 (1.02)
HDL cholesterol (mmol/L) [a]	1.49±0.43	1.51±0.43
HbA1c (mmol/mol) [a]	39.5±7.9	38.8±6.8
HbA1c (%)[a]	5.8±0.7	5.7±0.6
Hypertension, n (%)	831 (56.2)	304 (50.0)
eGFR (ml/min/1.73 m^2) [a]	82.4±13.3	84.2±11.7
Albumin (g/L) [a]	38.2±3.9	38.6±4.1
Intake of lipid-lowering medication, n (%)	172 (11.6)	64 (10.5)
Smoking status, n (%)		
Never	710 (48.0)	309 (50.8)
Former	561 (38.0)	231 (38.0)
Current	207 (14.0)	68 (11.2)
Alcohol intake, n (%)		
0 g/day	415 (28.1)	140 (23.0)
Men 0.1–39.9 g/day Women 0.1–19.9 g/day	765 (51.8)	347 (57.1)
Men ≥40 g/day Women ≥20 g/day	298 (20.2)	121 (19.9)
Physical activity, n (%)		
High activity	256 (17.3)	124 (20.4)
Moderate activity	365 (24.7)	170 (28.0)
Low activity	227 (15.4)	96 (15.8)
No activity	630 (42.6)	218 (35.9)
Education ≤ 10 years, n (%)	927 (62.7)	334 (54.9)
ASMM (kg) [a]	19.9±3.9	20.1±4.0
BFMI (kg/m^2) [a]	10.0±3.1	9.5±2.9
Low ASMM, n (%)[c]	370 (25.0)	152 (25.0)
High BFMI, n (%)[d]	370 (25.0)	152 (25.0)
Combination low ASMM and high BFMI, n (%)[e]	110 (7.4)	39 (6.4)

[a] Continuous variables are presented as arithmetic mean±SD.

[b] Natural logarithmic transformed variables are presented as geometric mean (antilog of SE).

[c] 25 % of participants with the lowest ASMM. Cut points were applied for men and women separately.

[d] 25 % of participants with the highest BFMI. Cut points were applied for men and women separately.

[e] Combination of participants, who were categorized in the group of the 40 % of participants with the lowest ASMM and the group of the 40 % of participants with the highest BFMI. Cut points were applied for men and women separately.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein.

Table S7: Characteristics of the study population in the longitudinal sample

Characteristic	n = 608
Variables measured at baseline (S4)	
ASMM (kg) [a]	20.1±4.0
BFMI (kg/m^2) [a]	9.5±2.88
Low ASMM, n (%)[b]	152 (25.0)
High BFMI, n (%)[c]	152 (25.0)
Combination low ASMM and high BFMI, n (%)[d]	39 (6.4)
Variables measured at follow-up (FF4)	
ASMM (kg) [a]	19.7±4.2
BFMI (kg/m^2) [a]	9.7±3.1
Low ASMM, n (%)[b]	152 (25.0)
High BFMI, n (%)[c]	152 (25.0)
Combination low ASMM and high BFMI, n (%)[d]	44 (7.2)
Variables measured at S4 and FF4	
Relative change in ASMM (%) [a]	-2.2±6.6
Relative change in BFMI (%) [a]	3.8±17.1
Strong decrease in ASMM, n (%)[e]	152 (25.0)
Strong increase in BFMI, n (%)[f]	152 (25.0)
Combination of strong decrease in ASMM and strong increase in BFMI, n (%)[g]	57 (9.4)
Strong decrease in ASMM	
Yes (n = 152), relative change in ASMM (%) [a]	-10.1±4.1
No (n = 456), relative change in ASMM (%) [a]	0.4±5.1
Strong increase in BFMI	
Yes (n = 152), relative change in BFMI (%) [a]	25.7±12.2
No (n = 456), relative change in BFMI (%) [a]	-3.5±11.2

[a] Continuous variables are presented as arithmetic mean±SD.

[b] 25 % of participants with the lowest ASMM. Cut points were applied for men and women separately.

[c] 25 % of participants with the highest BFMI. Cut points were applied for men and women separately.

[d] Combination of participants, who were categorized in the group of the 40 % of participants with the lowest ASMM and the group of the 40 % of participants with the highest BFMI. Cut points were applied for men and women separately.

[e] 25 % of participants with the highest decrease in ASMM. Cut points were applied for men and women separately.

[f] 25 % of participants with the highest increase in BFMI. Cut points were applied for men and women separately.

[g] Combination of participants, who were categorized in the group of the 40 % of participants with the highest decrease in ASMM and the group of the 40 % of participants with the highest increase in BFMI. Cut points were applied for men and women separately.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.

Table S8: Cross-sectional analysis – Prediction analysis – Group lasso with 100x bootstrapping

ASMM (kg)		BFMI (kg/m^2)		Low ASMM		High BFMI		Combination low ASMM and high BFMI	
Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency
Age Sex Physical Activity LEP IGFBP1 KLK6 MMP2 Notch3 CXCL9 CCL28	100 %	Age Sex eGFR Smoking Education ADM LEP FABP4 IGFBP1 KLK6 CCL4 FGF21 CCL28	100 %	Age Alcohol IL1RL2 PRSS8 CCL15 Gal4 IGFBP1 KLK6 MB CST5 CCL28	100 %	Sex eGFR ADM LEP FABP4 IGFBP1	100 %	Age Physical Activity LEP SCGB3A2	100 %
Smoking PRSS27 VSIG2 DCN IGFBP2 TFPI TRAP CST5 DNER	99 %	Triglycerides Alcohol PRSS27 PSGL1 CD8A TWEAK	99 %	SCF LEP SELE TFPI	99 %	Smoking	98 %	Sex MMP2 MMP3	99 %
HbA1c eGFR PRSS8 CPB1 MB	98 %	FGF23 XCL1 IGFBP7 PON3 TNFR1 MCP1 TRAIL	98 %	Physical Activity Intake lipid-lowering medication CD40L VSIG2 CXCL10	98 %	Alcohol VEGFA CCL4	97 %	CCL17	98 %
GDF2 ALCAM EpCAM CCL4	97 %	SOD2	97 %	CD84 SERPINA12 GDF15 OPN vWF MCP3	97 %	TWEAK	96 %	CCL28	97 %
HGF	96 %	Intake lipid-lowering medication IL4RA IL1RL2 GDF2 Notch3 IFNG MCP2	96 %	eGFR CPB1 CTSZ	96 %	Notch3	95 %	MB FGF21	96 %

Alcohol IL7 FGF21	95 %	Hyperten- sion TNFRSF- 13B CCL16 CPB1	95 %	MARCO Notch3	95 %	Age Physical Activity Education PCSK9	94 %	TWEAK	94 %
IL1RL2 THBS2 XCL1 OPN vWF TNFB	94 %	RAGE THBS2	94 %	FABP4	94 %	MMP12 VEGFD	93 %	Education	93 %
TRAILR2 NT-proBNP	93 %	IL17RA TNFRSF- 10C HGF	93 %	RAGE DCN MEPE MMP2 PAI IFNG	92 %	Triglyceride- es PRSS27 GH	91 %	ENRAGE	92 %
ADM	92 %	CCL15 NT-proBNP	92 %	ALCAM LIFR	91 %	TF CD8A PDL1	90 %	MPO	90 %
Intake lipid- lowering medication	91 %	HDL VEGFD PSPD TIMP4 TR	91 %	Smoking CD4	90 %				
HDL TNFRSF- 11A RAGE CD93 CTSZ	90 %	PDL2 LDL-RC	90 %						

In the table, only variables are listed that were selected in ≥ 90 times out of 100 group least absolute shrinkage and selection operator bootstrap iterations.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; lasso, least absolute shrinkage and selection operator.

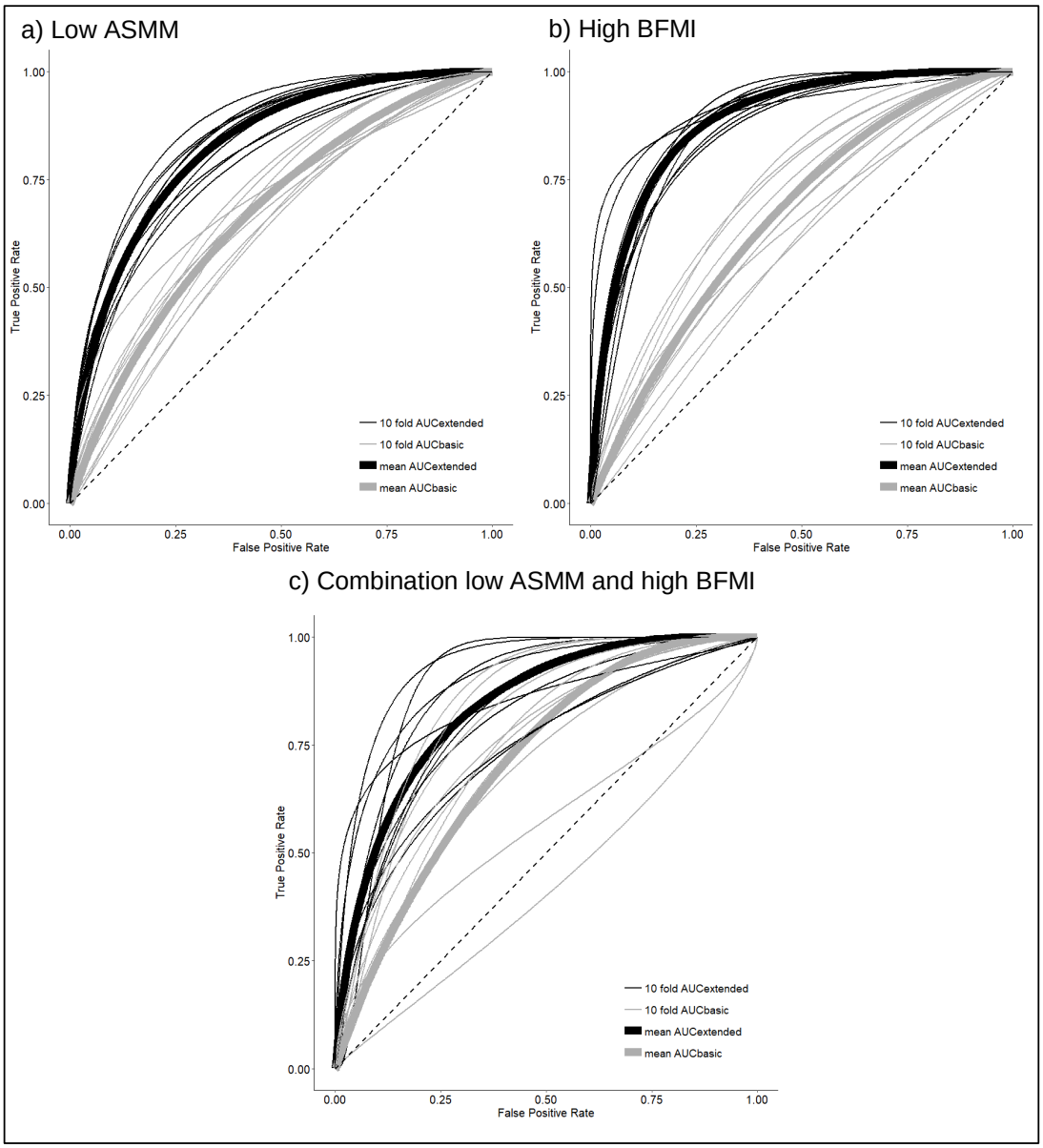

Figure S4: Smoothed ROC curves of 10-fold cross-validation of logistic regression models with classical risk factors (AUC_{basic}) and protein biomarkers in addition to classical risk factors ($AUC_{extended}$)

Smoothed ROC curves of all 10 folds and their mean of the cross-validation are illustrated for the AUCs calculated for a model only including classical risk factors, AUC_{basic} (illustrated in grey), and the AUCs calculated for a model additionally including all protein biomarkers that were selected in ≥ 90 % of the group least absolute shrinkage and selection operator bootstrap iterations, $AUC_{extended}$ (illustrated in black). Bold lines indicate the mean ROC curve of all 10 smoothed ROC curves of the folds, which are

illustrated as thin lines. ROC curves are shown for the outcomes (a) low ASMM, (b) high BFMI, and (c) combination of low ASMM and high BFMI.

AUC_{basic}: AUC of a logistic regression model including 13 classical risk factors (age, high-density lipoprotein, triglycerides, glycated hemoglobin, estimated glomerular filtration rate, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication).

$AUC_{extended}$: AUC of the basic model plus all protein biomarkers selected in ≥ 90 % of the group least absolute shrinkage and selection operator bootstrap iterations (variables are listed in Supporting Information, Table S8).

ASMM, appendicular skeletal muscle mass; AUC, area under the curve; BFMI, body fat mass index; ROC, receiver operating characteristic.

Table S9: Cross-sectional analysis – Sensitivity analysis – Comparison of the top 10 most important variables of lasso, random forest, and support vector machine

Rank	Lasso	Random forest	Support vector machine
		ASMM (kg)	
1	Age / Sex / Physical activity / LEP / IGFBP1 / KLK6 / MMP2 / Notch3 / CXCL9 / **CCL28**	Sex	Sex
2		LEP	**LPL**
3		IGFBP1	**GH**
4		**LPL**	HDL
5		**MMP3**	**MMP3**
6		**GH**	GDF2
7		IGFBP2	ACE2
8		**CCL28**	IGFBP1
9		FABP4	**PON3**
10		**PON3**	LEP
		BFMI (kg/m^2)	
1	Age / Sex / eGFR / Education / Smoking / ADM / LEP / FABP4 / **IGFBP1** / KLK6 / CCL4 / FGF21 / CCL28	LEP	LEP
2		FABP4	FABP4
3		Sex	Sex
4		**IGFBP1**	ADM
5		IGFBP2	RARRES2
6		PON3	THBS2
7		ADM	IL1RL2
8		RAGE	**MMP3**

9		GAL9	TNFRSF11A
10		**MMP3**	IL12B
	Low ASMM		
1	**Age** / Alcohol / IL1RL2 / PRSS8 / CCL15 / Gal4 / IGFBP1 / **KLK6** / MB / CST5 / CCL28	**IGFBP2**	**IGFBP2**
2		IGFBP1	IGFBP1
3		**LEP**	**LEP**
4		**PON3**	**PON3**
5		IL1RA	**KLK6**
6		CCL28	**Age**
7		IL27	CCL28
8		CX3CL1	OPN
9		THPO	SCGB3A2
10		CA5A	TFPI
	High BFMI		
1	Sex / eGFR / ADM / LEP / FABP4 / IGFBP1	LEP	LEP
2	Smoking	FABP4	FABP4
3	Alcohol / VEGFA / CCL4	**PON3**	**PON3**
4		ADM	ADM
5		IGFBP1	IGFBP1
6		**IGFBP2**	**IL6**
7		**HGF**	**HGF**
8		**CD163**	THBS2
9		**IL6**	**IGFBP2**
10		TNFRSF11A	**CD163**
	Combination low ASMM and high BFMI		
1	**Age / Physical Activity / LEP /** SCGB3A2	**ADM**	**Age**
2	Sex / MMP2 / MMP3	TFPI	**TIMP4**
3	CCL17	SCF	**Physical activity**
4	CCL28	**GDF15**	**ADM**
5	MB / FGF21	**FABP4**	**GDF15**
6		**TIMP4**	CXCL9
7		PRSS8	IL6
8		CD93	**LEP**
9		AMBP	UPAR
10		KIM1	**FABP4**

Grey shading indicates that the variable was ranked in the top 10 in all three methods (lasso, random forest, and support vector machine); bold print indicates that the variable was ranked in the top 10 in two of the three methods.

All variables with the same selection frequency calculated from lasso with bootstrapping have the same rank; e.g. all variables with a selection frequency of 100% have rank 1. Therefore, more than one variable can be assigned to rank 1.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; eGFR, estimated glomerular filtration rate; lasso, least absolute shrinkage and selection operator.

Results of the longitudinal analysis

Table S10: Association analysis – Boosting with stability selection – Longitudinal analysis

Boosting with stability selection		Linear regression models	
Selected variables	Selection frequency	β (95% CI) (Model 1)	p value
Relative change in ASMM (%)			
-			
Relative change in BFMI (%)			
CCL4	76%	**-2.29** (-3.72, -0.87)	**0.001700**
ADAMTS13	76%	**-2.22** (-3.55, -0.89)	**0.001123**
CCL15	66 %	**1.92** (0.49, 3.35)	**0.008596**
		Logistic regression models	
Selected variables	Selection frequency	OR (95% CI) (Model 1)	p value
Strong decrease in ASMM			
NT-proBNP	69 %	**1.40** (1.10, 1.77)	**0.00582**
Strong increase in BFMI			
DLK1	65 %	**0.75** (0.60, 0.92)	**0.00681**
Combination strong decrease in ASMM and strong increase in BFMI			
NT-proBNP	72 %	**1.60** (1.15, 2.24)	**0.00524**

The cut point for variable selection in the boosting with stability selection was a selection frequency of 63 %, which was determined by the algorithm based on the number of variables available for selection, the number of selected variables per iteration, and the maximum number of tolerable false positives.

Effect estimates have been calculated per 1 SD increase in normalized protein expression values on a log2 scale.

Model 1: Adjustment for all 13 covariates (age, high-density lipoprotein, triglycerides, glycated hemoglobin, estimated glomerular filtration rate, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication) as well as all other in the boosting with stability selection selected variables of the corresponding outcome.

Bold print indicates significance.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; β, beta coefficient; CI, confidence interval; OR, odds ratio.

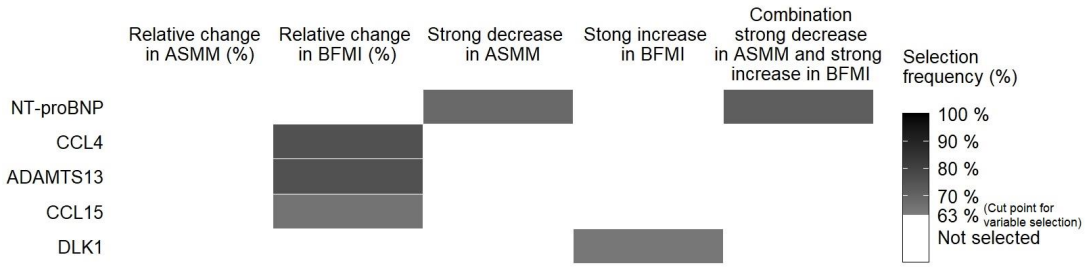

Figure S5: Association analysis – Boosting with stability selection – Comparison of protein biomarker selection between the outcomes – Longitudinal analysis

Protein biomarkers are primarily ordered according to the number of outcomes the biomarkers were selected for and secondary according to their selection for the outcomes in the table from left to right. Only protein biomarkers are included that were selected for at least one outcome. The cut point for variable selection was a selection frequency of 63 %, which was determined by the algorithm based on the number of variables available for selection, the number of selected variables per iteration, and the maximum number of tolerable false positives.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.

Table S11: Prediction analysis – Group lasso with 100x bootstrapping – Longitudinal analysis

Relative change in ASMM (%)		Relative change in BFMI (%)		Strong decrease in ASMM		Strong increase in BFMI		Combination strong decrease in ASMM and strong increase in BFMI	
Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency	Selected variables	Selection frequency
Age	98 %	CCL4	98 %	NT-proBNP	99 %	Age ICAM2	100 %	NT-proBNP	94 %
FAS	95 %	Education Alcohol	96 %	HDL	97 %	PLGR	99 %	PLGR	92 %
FLT3L	90 %	DLK1	95 %			CCL15	96 %		
		ADAMTS-13	93 %			Physical activity	95 %		
		CCL15	92 %			IL6RA	93 %		
		TGM2	90 %			CCL4	92 %		
						CCL16 DLK1	90 %		

In the table, only variables are listed that were selected in ≥ 90 times out of 100 group least absolute shrinkage and selection operator bootstrap iterations.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; HDL, high-density lipoprotein.

Table S12: Prediction analysis – Cross-validated AUCs of logistic regression models with classical risk factors (mean AUC_{basic}) and protein biomarkers in addition to classical risk factors (mean $AUC_{extended}$) – Longitudinal analysis

Outcome	Mean AUC_{basic} (95 % CI)	Mean $AUC_{extended}$ (95 % CI)	Mean delta AUC (95 % CI)
Strong decrease in ASMM	0.54 (0.51, 0.67)	0.57 (0.54, 0.68)	0.03 (0.00, 0.07)
Strong increase in BFMI	0.56 (0.54, 0.68)	0.63 (0.63, 0.75)	0.07 (0.01, 0.11)
Combination strong decrease in ASMM and strong increase in BFMI	0.50 (0.48, 0.70)	0.55 (0.52, 0.72)	0.05 (-0.01, 0.11)

AUC_{basic}: AUC of a logistic regression model including 13 classical risk factors (age, high-density lipoprotein, triglycerides, glycated hemoglobin, estimated glomerular filtration rate, albumin, sex, physical activity, hypertension, smoking status, education, alcohol intake, and intake lipid-lowering medication).

$AUC_{extended}$: AUC of the basic model plus all protein biomarkers selected in ≥ 90 % of the group least absolute shrinkage and selection operator bootstrap iterations (variables are listed in Supporting Information, Table S11).

Delta AUC: $AUC_{extended}$ - AUC_{basic}

AUCs and delta AUCs are arithmetic means of 10-fold cross-validation. The confidence intervals of AUCs and delta AUCs were calculated via 100-fold percentile bootstrapping.

ASMM, appendicular skeletal muscle mass; AUC, area under the curve; BFMI, body fat mass index; CI, confidence interval.

Table S13: Sensitivity Analysis – Comparison of the top 10 most important variables of lasso, random forest, and support vector machine – Longitudinal analysis

Rank	Lasso	Random forest	Support vector machine
	Relative change in ASMM (%)		
1	Age	Age	CA5A
2	FAS	TFPI	Age
3	**FLT3L**	**FLT3L**	CASP8
4	**OPG**	IGFBP2	ALCAM
5	IL6RA	LIFR	IGFBP7
6	CDCP1 / CCL19	CCL23	**OPG**
7	Alcohol / FGF21	IL10RB	HSP27
8	IL12B	GRN	LTBR
9		CSTB	TIE2
10		**IL18R1**	**IL18R1**
	Relative change in BFMI (%)		
1	CCL4	**TPA**	**TPA**
2	Education / Alcohol	CCL4	CCL4
3	**DLK1**	GRN	**LDL-RC**
4	ADAMTS13	**IGFBP2**	IGFBP7
5	**CCL15**	Notch3	Triglycerides
6	TGM2	**CCL15**	**IGFBP2**
7	Smoking	CPB1	**DLK1**
8	CHIT1 / CCL19	MMP9 / HAOX1	FGF21
9		**LDL-RC**	REN
10			AXIN1
	Strong decrease in ASMM		
1	**NT-proBNP**	IGFBP2	**NT-proBNP**
2	HDL	CD40L	HSP27
3	**IL27** / FGF21	**Age**	**vWF**
4	**EGFR**	PCSK9	TNFRSF11A
5	Physical activity	LTBR	**EGFR**
6	RAGE	**TNFR2**	**Age**
7	AGRP / **vWF** / IL12B	TNFRSF10C	RETN
8		ADAMTS13	**IL27**
9		IL17RA / DCN	**TNFR2**
10			GDF15
	Strong increase in BFMI		
1	**Age** / ICAM2	AXIN1	**DLK1**
2	PLGR	NEMO	IGFBP2
3	CCL15	4EBP1	FLT3L
4	Physical activity	PDGFA / MERTK	**IL6RA**
5	**IL6RA**	SIRT2	**Age**
6	**CCL4**	CPB1	LDL-RC
7	CCL16 / **DLK1**	**Triglycerides**	CD163
8	CHIT1	JAMA	CXCL10
9		CXCL16	**CCL4**
10			**Triglycerides** / DCN
	Combination strong decrease in ASMM and strong increase in BFMI		
1	**NT-proBNP**	TIE2	FABP4
2	PLGR	**LEP**	**NT3**
3	**RARRES2**	CXCL9	**LEP**

4	**ADAMTS13**	PCSK9	SCGB3A2
5	**MEPE**	HOSCAR	**NT-proBNP**
6	IL17D	MCP3	**HSP27**
7	**NT3**	CTSZ	**RARRES2**
8	**HSP27**	OPN	**MEPE**
9	IFNG	TNF	IGFBP2
10	MB	IL7	**ADAMTS13**

Grey shading indicates that the variable was ranked in the top 10 in all three methods (lasso, random forest, and support vector machine); bold print indicates that the variable was ranked in the top 10 in two of the three methods.

All variables with the same selection frequency calculated from lasso with bootstrapping have the same rank; e.g. all variables with a selection frequency of 100% have rank 1. Therefore, more than one variable can be assigned to rank 1.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index; lasso, least absolute shrinkage and selection operator.

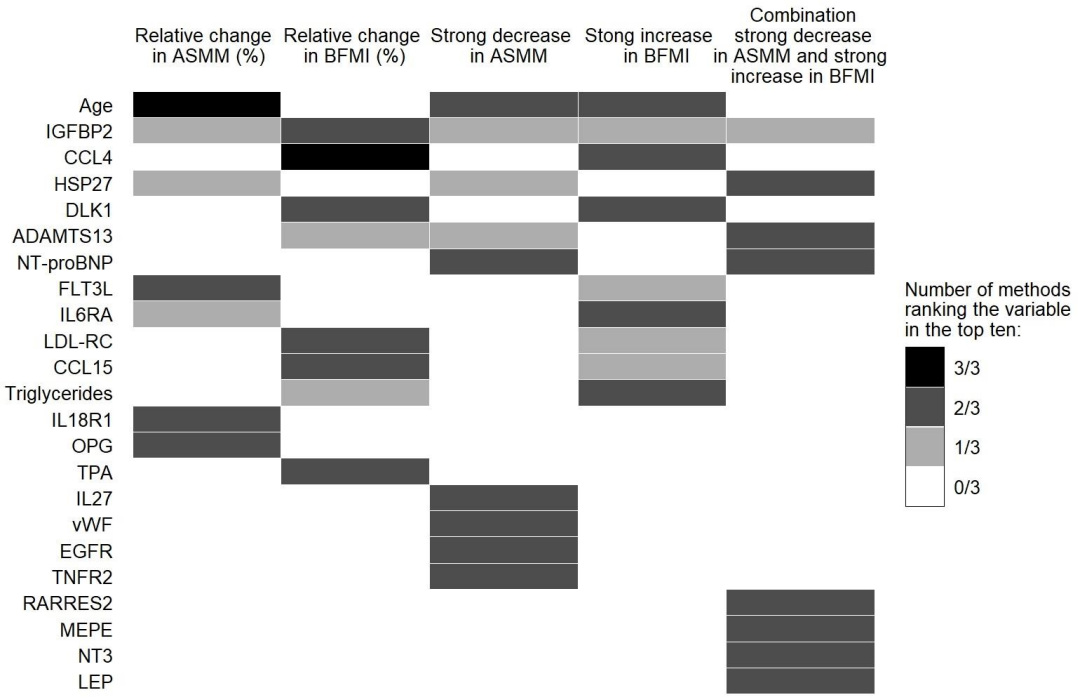

Figure S6: Sensitivity Analysis – Comparison of variables between the outcomes regarding the number of methods that ranked the variables in the top 10 – Longitudinal analysis

Only variables are included that were ranked in the top 10 in at least two of the three analysis methods (group least absolute shrinkage and selection operator with 100x bootstrapping, random forest, and support vector machine) in at least one of the five outcomes. Variables are primarily ordered descending according to the total number (sum of all outcomes) of methods that ranked the variable in the top 10, and secondary according to the outcome in the table from left to right based on the number of methods that ranked the variable in the top 10 for the outcome.

ASMM, appendicular skeletal muscle mass; BFMI, body fat mass index.

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Manuscript 3

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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

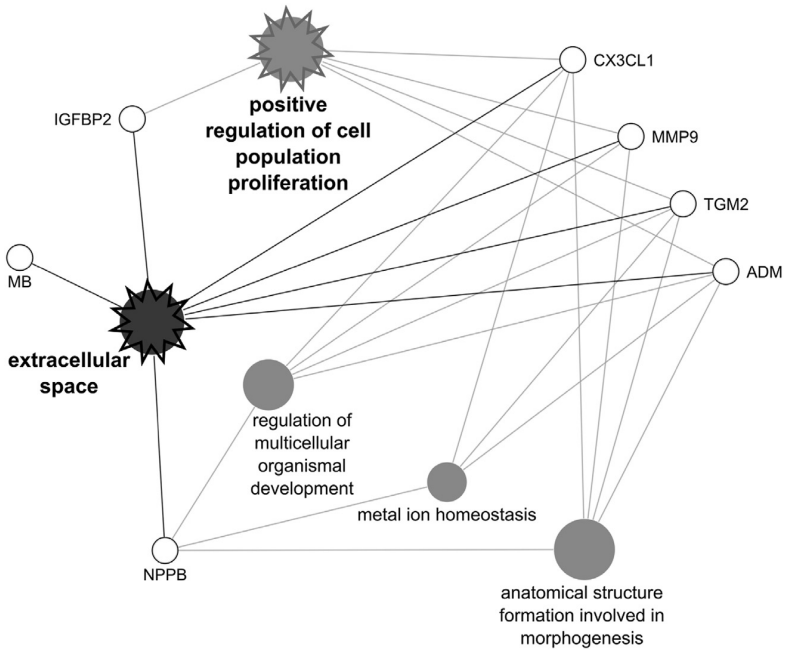

Fig. 1. Functionally grouped network of selected PhA-associated protein markers and their top five GO terms. GO terms are clustered in functional groups based on the kappa score, which considers the number of protein markers associated with two GO terms [29]. GO terms belonging to the same functional 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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Affidavit

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Eidesstattliche Versicherung

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List of publications

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