DEUTSCHES INSTITUT FÜR ERNÄHRUNGSFORSCHUNG POTSDAM-REHBRÜCKE
ABTEILUNG MOLEKULARE TOXIKOLOGIE

Relevance of Biomarkers for the Diagnosis of the Frailty Syndrome:
Focus on Parameters of Muscle Protein Turnover, Micronutrients and Oxidative Stress

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Kumulative Dissertation

zur Erlangung des akademischen Grades

"doctor rerum naturalium" (Dr. rer. nat.)

in der Wissenschaftsdisziplin "Toxikologie und Pharmakologie"

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Potsdam

vorgelegt von:

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Potsdam, September 2019

Disputation am 06. Dezember 2019

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Published online at the
Institutional Repository of the University of Potsdam:
https://doi.org/10.25932/publishup-44118
https://nbn-resolving.org/urn:nbn:de:kobv:517-opus4-441186

Für meine Eltern!

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1-MH	1-methylhistidne
3-MH	3-methylhistidine
3-NT	3-nitrotyrosine
AA	amino acid
CAF	C-terminal agrin fragment
Crea	creatinine
CRP	C-reactive protein
CT	computerized tomography
CVD	cardiovascular disease
d_3-	deuterated
DEXA	dual energy X-ray absorptiometry
eGFR	estimated glomerular filtration rate
GC(-MS)	gas chromatography (coupled to mass spectrometry)
HPLC	high performance liquid chromatography
IGF-1	insulin-like growth factor-1
IL-6	interleukin-6
LC	liquid chromatography
m/z	mass-to-charge ratio
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
n	number of participants in group of cohort/study population
N	number of participants in total cohort/study population
OPA	*o*-phthalaldehyde
OS	oxidative stress
PrCarb	protein carbonyls
RNS	reactive nitrogen species
ROS	reactive oxygen species
RS	reactive species
TNF-α	tumor necrosis factor-α
(UP)LC-MS/MS	(ultra-performance) liquid chromatography tandem mass spectrometry
VD	vitamin D

Abstract

Frailty and sarcopenia share some underlying characteristics like loss of muscle mass, low muscle strength, and low physical performance. Imaging parameters and functional examinations mainly assess frailty and sarcopenia criteria; however, these measures can have limitations in clinical settings. Therefore, finding suitable biomarkers that reflect a catabolic muscle state e.g. an elevated muscle protein turnover as suggested in frailty, are becoming more relevant concerning frailty diagnosis and risk assessment.

3-Methylhistidine (3-MH) and its ratios 3-MH-to-creatinine (3-MH/Crea) and 3-MH-to-estimated glomerular filtration rate (3-MH/eGFR) are under discussion as possible biomarkers for muscle protein turnover and might support the diagnosis of frailty. However, there is some skepticism about the reliability of 3-MH measures since confounders such as meat and fish intake might influence 3-MH plasma concentrations. Therefore, the influence of dietary habits and an intervention with white meat on plasma 3-MH was determined in young and healthy individuals. In another study, the cross-sectional associations of plasma 3-MH, 3-MH/Crea and 3-MH/eGFR with the frailty status (robust, pre-frail and frail) were investigated.

Oxidative stress (OS) is a possible contributor to frailty development, and high OS levels as well as low micronutrient levels are associated with the frailty syndrome. However, data on simultaneous measures of OS biomarkers together with micronutrients are lacking in studies including frail, pre-frail and robust individuals. Therefore, cross-sectional associations of protein carbonyls (PrCarb), 3-nitrotyrosine (3-NT) and several micronutrients with the frailty status were determined.

A validated UPLC-MS/MS (ultra-performance liquid chromatography tandem mass spectrometry) method for the simultaneous quantification of 3-MH and 1-MH (1-methylhistidine, as marker for meat and fish consumption) was presented and used for further analyses. Omnivores showed higher plasma 3-MH and 1-MH concentrations than vegetarians and a white meat intervention resulted in an increase in plasma 3-MH, 3-MH/Crea, 1-MH and 1-MH/Crea in omnivores. Elevated 3-MH and 3-MH/Crea levels declined significantly within 24 hours after this white meat intervention. Thus, 3-MH and 3-MH/Crea might be used as biomarker for muscle protein turnover when subjects did not consume meat 24 hours prior to blood samplings.

Plasma 3-MH, 3-MH/Crea and 3-MH/eGFR were higher in frail individuals than in robust individuals. Additionally, these biomarkers were positively associated with frailty in linear regression models, and higher odds to be frail were found for every increase in 3-MH and 3-MH/eGFR quintile in multivariable logistic regression models adjusted for several confounders. This was the first study using 3-MH/eGFR and it is concluded that plasma 3-MH, 3-MH/Crea and 3-MH/eGFR might be used to identify frail individuals or individuals at higher risk to be frail, and that there might be threshold concentrations or ratios to support these diagnoses.

Higher vitamin D_3, lutein/zeaxanthin, γ-tocopherol, α-carotene, β-carotene, lycopene and β-cryptoxanthin concentrations and additionally lower PrCarb concentrations were found in robust compared to frail individuals in multivariate linear models. Frail subjects had higher odds to be in the lowest than in the highest tertile for vitamin D_3 α-tocopherol, α-carotene, β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin, and had higher odds to be in the highest than in the lowest tertile for PrCarb than robust individuals in multivariate logistic regression models. Thus, a low micronutrient together with a high PrCarb status is associated with pre-frailty and frailty.

Zusammenfassung

Gebrechlichkeit (*englisch: frailty*) und Sarkopenie teilen einige zugrundeliegende Merkmale wie einen Verlust von Muskelmasse, eine geringe Muskelkraft und eine geringe körperliche Leistungsfähigkeit, welche durch einen erhöhten Muskelproteinumsatz entstehen können. Kriterien der Gebrechlichkeit und Sarkopenie werden hauptsächlich durch bildgebende Verfahren sowie funktionelle Untersuchungen gemessen, die in ihrer Durchführbarkeit im klinischen Alltag jedoch eingeschränkt sein können. Daher gewinnt das Finden geeigneter Biomarker zur Anzeige eines erhöhten Muskelproteinumsatzes (kataboler Muskelzustand) in Bezug auf Diagnose und Risikobewertung der Gebrechlichkeit zunehmend an Bedeutung.

3-Methylhistidin (3-MH) und die Verhältnisse 3-MH zu Kreatinin (3-MH/Crea) und 3-MH zu geschätzter glomerulärer Filtrationsrate (3-MH/eGFR) werden als solche möglichen Biomarker diskutiert und könnten folglich die Diagnose und Risikobewertung von Gebrechlichkeit unterstützen. Es herrscht jedoch eine gewisse Skepsis hinsichtlich der Zuverlässigkeit von 3-MH-Messungen, da 3-MH-Plasmakonzentrationen durch Fleisch- und Fischaufnahme beeinflusst werden können. Daher wurde der Einfluss von Ernährungsgewohnheiten (Mischkost oder vegetarisch) und einer Intervention mit Hähnchenfleisch auf Plasma-3-MH bei jungen und gesunden Personen untersucht. In einer weiteren Studie wurden die Querschnittsassoziationen von 3-MH, 3-MH/Crea und 3-MH/eGFR im Plasma mit dem *Frailty*-Status (*robust, pre-frail* und *frail*) untersucht.

Oxidativer Stress (OS) ist ein potentieller Faktor der zur Entwicklung von Gebrechlichkeit beiträgt, und sowohl hohe OS-Konzentrationen als auch niedrige Mikronährstoffkonzentrationen sind mit Gebrechlichkeit assoziiert. Daten zu simultanen Messungen von OS und Mikronährstoffen in Personen aller drei *Frailty*-Kategorien (*robust, pre-frail* und *frail*) fehlen jedoch. Aus diesem Grund wurden Querschnittsassoziationen von Proteincarbonylen (PrCarb), 3-Nitrotyrosin (3-NT) und mehrerer fettlöslicher Mikronährstoffe mit dem *Frailty*-Status bestimmt.

Eine validierte UPLC-MS/MS-Methode (*ultra-performance liquid chromatography tandem mass spectrometry*) zur simultanen Bestimmung von 3-MH und 1-MH (1-Methylhistidin als Marker für den Fleisch- und Fischkonsum) in Plasma wurde beschrieben und für die weiteren Analysen verwendet. Mischköstler wiesen höhere 3-MH-

und 1-MH-Konzentrationen in Plasma auf als Vegetarier. Die Intervention mit Hähnchenfleisch führte zu einem Anstieg von Plasma 3-MH, 3-MH/Crea, 1-MH und 1-MH/Crea bei Mischköstlern. Diese erhöhten 3-MH- und 3-MH/Crea-Spiegel sanken innerhalb von 24 Stunden nach der Intervention signifikant ab. Folglich stellen 3-MH und 3-MH/Crea potentielle Biomarker für den Muskelproteinumsatz dar, wenn Personen für 24 Stunden vor der Blutentnahme kein Fleisch verzehrt haben.

Gebrechliche Teilnehmer wiesen höhere Plasma 3-MH-, 3-MH/Crea- und 3-MH/eGFR-Werte auf als robuste Teilnehmer und zusätzlich waren diese Biomarker in linearen Regressionsmodellen positiv mit Gebrechlichkeit assoziiert. In multivariablen logistischen Regressionsmodellen (adjustiert für mehrere Confounder) waren gebrechliche Personen im Vergleich zu robusten Personen mit einer höheren Wahrscheinlichkeit in einer höheren 3-MH- und 3-MH/eGFR-Quintile. Diese erste Studie, die 3-MH/eGFR als Biomarker für Gebrechlichkeit untersucht hat, erlaubt die Schlussfolgerung, dass Plasma-3-MH, -3-MH/Crea und -3-MH/eGFR verwendet werden könnte, um gebrechliche Personen oder Personen mit einem erhöhten *Frailty*-Risiko zu identifizieren. Möglicherweise gibt es auch Schwellenwerte, die diese Diagnosen unterstützen können.

In multivariaten Regressionsanalysen wiesen robuste Personen höhere Vitamin D_3-, Lutein/Zeaxanthin-, γ-Tocopherol-, α-Carotin-, β-Carotin-, Lycopin- und β-Cryptoxanthin-Konzentrationen sowie niedrigere PrCarb-Konzentrationen auf als gebrechliche Personen. Zudem waren in multinomialen logistischen Regressionsanalysen gebrechliche Personen mit einer höheren Wahrscheinlichkeit sowohl in der niedrigsten Vitamin D_3-, α-Tocopherol-, α-Carotin-, β-Carotin-, Lycopin-, Lutein/Zeaxanthin- und β-Cryptoxanthin-Tertil als auch im höchsten PrCarb-Tertil zu finden als robuste Personen. Es wird daher geschlussfolgert, dass niedrige Mikronährstoffkonzentrationen zusammen mit hohen PrCarb-Konzentrationen mit Gebrechlichkeit und dessen Vorstufe (*pre-frailty*) assoziiert sind.

Introduction

The increase of the healthy lifespan and well-being by maintaining functional abilities and reducing the time of sickness and disability especially in the late years of life, is a great desire for all society. As the population becomes older and the life expectancy increases, the time span of healthy living decreases. While there will be an estimated increase of 1.8 % of the population in the European Union until 2070, the proportions of the age-groups 65+ years and 80+ years will increase from 19 % to 29 % and from 5 % to 13 %, respectively [1]. This progressive aging of our society is accompanied by an increased occurrence of age-related diseases, disabilities and subsequent dependency, all leading to an increasing social and health care burden.

During aging, the human body undergoes physiological, intrinsic as well as psychosocial changes, resulting in an attenuation of both the biological and physiological capacity against stressors. The decline in muscle mass and muscle strength, possibly accompanied by a decreased functionality and subsequent physical limitations can become pathological if progressively ongoing during aging. Muscle mass declines by 1-2 % annually after the age of 50 years, and muscle strength decreases annually by 1.5 % and 3 % after the ages of 50 years and 60 years, respectively [2], and by 3.6 % (men) and 2.8 % (women) in subjects older than 70 years [3]. This age-associated loss of muscle mass and function (strength and performance) is termed sarcopenia, which can further contribute to the development of the frailty syndrome [4, 5]. Oxidative stress (OS) and low micronutrient intake are contributing factors, beside others, to the pathogenesis of frailty and sarcopenia. Both conditions favor the development of disability and are further associated with morbidity and mortality [4-6]. In contrast to disability, frailty is a reversible geriatric condition and thus treatable e.g. by changes in lifestyle like nutrition and exercise [7, 8]. Therefore, the early identification and diagnosis of people with a higher risk to develop frailty and of those that are already frail are essential for both preventive and therapeutic approaches. There are several functional tests and imaging approaches to assess single frailty characteristics and muscle mass, respectively. These tests and approaches can have disadvantages for their use in the clinical routine, thus, the introduction of muscle-specific biomarkers for the diagnosis of frailty and its risk is needed [6, 9].

The Frailty Syndrome

Frailty is a multifactorial, geriatric syndrome which is caused by a dynamic, age-related decline of multiple physiological systems and which is characterized by a high vulnerability as well as a low biological and physiological reserve and resistance against stressors [10, 11]. Frail individuals forfeit dependencies in activities of daily living as well as quality of life, and suffer from a higher risk for falls, hospitalization, disability and death [5, 8, 12, 13]. Frailty can result in social isolation, due to less physical activity and immobility, as well as in cognitive or mental decline. This might further accelerate and solidify the frailty syndrome and its consequences. Frailty can possibly be treated by resistant and aerobic exercise, nutritional interventions (protein and calorie support), vitamin D (VD) supplementation and reduction of multi-medication [8, 14]. Therefore, the early diagnosis of frail individuals and those individuals at higher risk to become frail is important.

While there are several approaches to assess frailty characteristics, and subsequently to identify frailty in individuals, there is yet no consensus on a uniform operational definition for it [15]. The two main approaches to assess frailty, discriminate it either as a *physical frailty phenotype* [5], or as an accumulation of deficits by the *frailty index* [16]. Both approaches share common characteristics, but do not necessarily determine the same persons as frail [17, 18]. The *physical frailty phenotype* introduced by Fried and colleagues focuses mainly on impairments of the musculoskeletal system and the resulting consequences [5]. The single frailty criteria defined by Fried and their corresponding measurements are described in **Table 1**. Briefly, individuals exhibiting 3 or more of the 5 criteria are considered as frail; individuals exhibiting 1-2 criteria are considered as pre-frail; and those exhibiting none of these criteria are considered as robust or non-frail [5]. The other widely used measure of frailty is the *frailty index* that was introduced by Mitnitski *et al.*, describing frailty as an accumulation of age-associated health deficits, including symptoms, signs, functional impairments, and laboratory abnormalities [16], taking into account cognitive and social factors in contrast to Fried's criteria. Beside these two approaches, there exist several (about 25) other frailty scales or indexes (reviewed in [19-21]).

Table 1. Fried's criteria for physical frailty.

Characteristic	Measurement	Method
Slowness	Slow walking speed (stratified by age and gender)	Functional tests (15 feet walk time)
Physical inactivity	Low activity or low energy expenditure (stratified by gender)	Interview or questionnaire (Minnesota Leisure Time Activity questionnaire)
Weight loss or sarcopenia	Unintentionally ≥ 4.5 kg in the last year *Sarcopenia*: low muscle mass plus low muscle strength or low physical performance	Interview or questionnaire Imaging parameters and functional tests
Weakness	Hand grip strength (stratified by gender and BMI quartiles)	Dynamometer
Exhaustion	Being fatigued	Self-reporting/-assessment (CES–D Depression Scale)

Abbreviations: CES–D Depression Scale, Center for Epidemiologic Studies Depression Scale. Modified from [5].

The mean prevalence of pre-frailty and frailty, according to Fried's criteria, in community-dwelling subjects (≥ 65 years) from ten European countries was 42.3 % and 17.0 %, respectively [22]. The development, prevalence and incidence of frailty are dependent on several socio-demographic characteristics. Observational and longitudinal studies show that higher age, female sex, low education and low income are positively associated with a high frailty prevalence and incidence [5, 22-25].

While associations of socio-demographic characteristics with frailty are known, the mechanisms leading to frailty are not fully understood. There is only consensus that the development of the frailty syndrome relies on multiple causes [15], and it is described that frailty development underlies a feedback reaction of its possible causes and consequences, the so-called cycle of frailty (**Figure 1**) [5, 26]. A prominent contributor and at the same time a component of frailty can be sarcopenia, which is characterized by an age-related loss of muscle mass with an additional low muscle function (muscle strength or physical performance (**Table 1**)) [4, 7, 27, 28]. Frailty and sarcopenia base on a decreased muscle mass and muscle functionality, and additionally share characteristics like slowness, weakness and poor balance [9, 28, 29]. There is agreement that the frailty syndrome is more faceted than sarcopenia [8, 15].

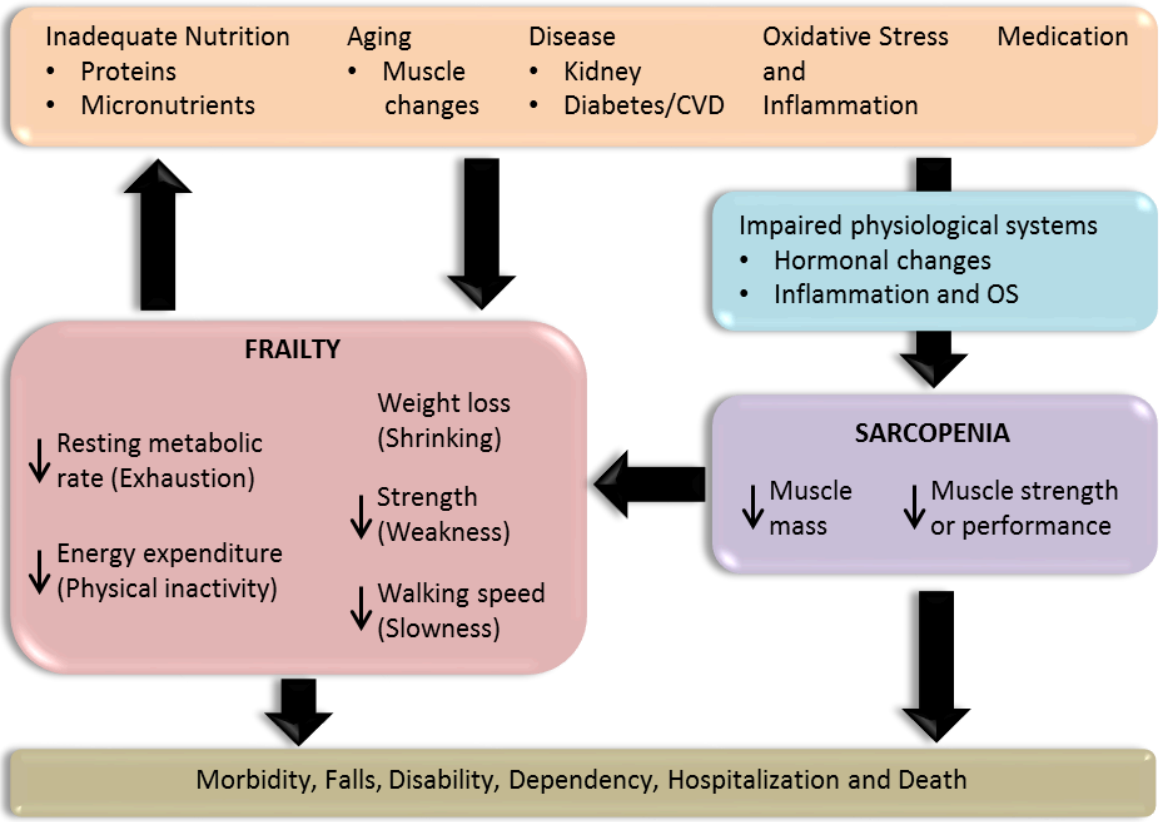

Figure 1. The frailty cycle. Possible feedback reactions between altered physiological conditions and resulting impaired physiological systems, sarcopenia, or clinical symptoms of frailty (Fried frailty criteria). Abbreviations: CVD, cardiovascular disease; OS, oxidative stress. Modified from [5, 26].

Maintaining muscle mass depends on a balanced muscle protein turnover, i.e. the functioning interaction between muscle protein synthesis (MPS, anabolic) and breakdown (MPB, catabolic). In the pathogenesis of frailty, alterations in muscle structure and function are described as key factors [30]. This is driven by an elevated muscle protein turnover or catabolic state of the muscle [31]. The causing factors might be (I) aging with its accompanying musculoskeletal changes, (II) inadequate nutritional intake of micronutrients or proteins, (III) age-associated OS, (IV) diseases like diabetes, cardiovascular disease (CVD) or impaired kidney function and (V) medication intake (**Figure 1**) [5, 26]. These can either result in impaired physiological systems, like hormonal changes and inflammation, which can contribute to the onset of sarcopenia; or might result in shrinking (weight loss) or physical limitations like weakness or slowness.

An inadequate intake of proteins and amino acids (AA) can lead to a reduced MPS and subsequently to a net MPB and thus to loss of muscle mass [32-35]. Decline of muscle mass and muscle function can occur as a consequence of age-related changes in i.e. muscle

size (atrophy), fiber types (type I versus type II; number of fibers), muscle contractile function (force per unit muscle area or mass), muscle structure and muscle composition (more fat infiltration) [36]. A decrease in muscle anabolic hormones like testosterone, estrogen, growth hormone and IGF-1 (insulin-like growth factor-1) and elevated pro-inflammatory cytokines i.e. interleukin-(IL-)6 and tumor necrosis factor-α (TNF-α) as well as C-reactive protein (CRP) levels can occur during aging and in several diseases and are associated with MPB and the loss of muscle mass and strength [31, 36].

Additionally, kidney function is associated with frailty [37] and multi-medication may contribute to the pathology of frailty [38]. OS was stated as a main contributor to frailty and additionally, inadequate intakes of micronutrients as well as low levels of micronutrients are linked to frailty. Age-associated OS and impairments in redox-homeostasis as well as in muscle structure, function and performance are key factors in the development of frailty [30, 36, 39].

Oxidative stress and micronutrients

Oxidative stress is a known prominent driver of the aging process and involved in the pathogenesis of several age-related diseases [40]. OS is defined as "*an imbalance between oxidants and anti-oxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage*" [41, 42]. The redox homeostasis is therefore dependent on the production of reactive species (RS) or oxidants, and the counteracting anti-oxidative defense mechanisms [41, 43] (**Figure 2**).

Reactive species are continuously generated under normal physiological conditions in the cell, can participate in redox signaling reactions [42, 44] and can be eliminated or neutralized by endogenous and exogenous antioxidant mechanisms [41, 45]. RS originate both, from endogenous processes and exogenous triggers as by-products during aerobic metabolism, enzyme reactions, and inflammation and due to UV-irradiation, radiation [41, 44]. RS are classified according to their chemical nature, the most important are reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can further be distinguished in free radicals (e.g. superoxide anion, hydroxyl-, peroxyl-, alkoxyl radical or nitric oxide and nitrogen dioxide) and chemically relatively stable non-radicals (hydrogen peroxide, organic hydroperoxide, singlet molecular oxygen or nitrite, peroxynitrite and -

nitrate) [46]. Beside ROS and RNS, there are also reactive chlorine/bromine, sulfur, carbonyl and selenium species [46, 47].

Low-grade OS has physiologic effects since it leads to a controlled redox signaling and thus contributing to normal physiological processes and redox homeostasis (oxidative eustress) and further maintaining a healthy condition [47, 48]. In contrast, high-grade OS leads to disturbed redox signaling and homeostasis, thus contributing to pathological processes, damaged biomolecules and disease development [47, 48]. OS may occur as consequence of a complex interplay of inflammatory and aging processes, inadequate nutrition, disease and medication, metabolism and environment, which either increase reactive species or decrease antioxidant concentrations or both [44, 49].

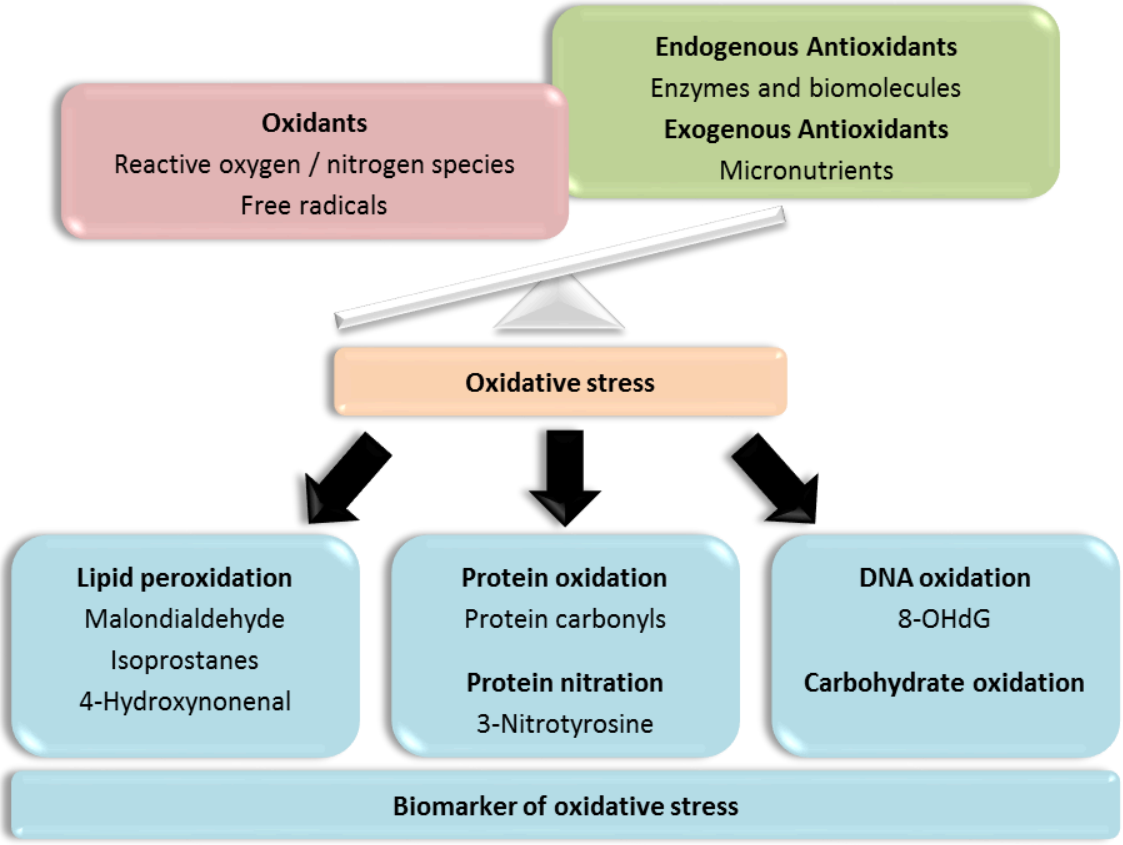

Figure 2. Concept of oxidative stress and common biomarkers. Abbreviation: 8-OHdG, 8-Hydroxy-deoxyguanosine. Modified from [49, 50].

Increased ROS and RNS formation can lead to chemical modifications (reversible as well as irreversible) of biomolecules like carbohydrates, lipids, proteins and DNA, resulting in impaired cellular functioning [42, 49]. Proteins and AA residues can undergo oxidation or nitration processes due to the attack of ROS and RNS, respectively. This attack can subsequently lead to the formation of protein carbonyls (PrCarb), 3-nitrotyrosine (3-NT),

respectively, and others, that can be used as biomarkers. Biomarkers of OS such as PrCarb, 3-NT, lipid hydroperoxides, malondialdehyde, isoprostanes as well as 8-hydroxy-deoxyguanosine, among others, can measure OS in different matrices (e.g. cells, blood and tissues) [50-54]. Increased concentrations of PrCarb and 3-NT, as biomarkers for protein modifications, were positively associated with age [55] as well as to several diseases like cancer, diabetes and neurodegenerative diseases [56-59]. Since there is no "gold standard" for analyzing OS in human studies, it is important to assess a set of different biomarkers, including oxidation products and antioxidants, to determine an individuals' "redox-status" [60].

ROS/RNS can be counteracted by the antioxidant defense system, which is distinguished into endogenous and exogenous systems [45]. Antioxidants are defined as "*any substance that delays, prevents or removes oxidative damage to a target molecule*" [45]. Prominent endogenously generated antioxidants are enzymes like superoxide dismutase, glutathione peroxidases and catalase, and anti-oxidative biomolecules like glutathione, thiols and uric acid. Additionally, there are several diet-derived micronutrients representing the exogenous antioxidants. These include both hydrophilic compounds like ascorbic acid (vitamin C) and polyphenols, as well as fat-soluble compounds like vitamin E (α- and γ-tocopherol) and carotenoids (α- and β-carotene, lycopene, lutein and zeaxanthin, and β-cryptoxanthin) [45].

Micronutrients appear in high amounts in fruits, vegetables, nuts, seeds and oils. Micronutrient concentrations are inversely associated with age [61] and a low micronutrient intake due to low fruits and vegetables intake are associated with several diseases like CVD, diabetes, metabolic syndrome, cognitive impairments and cancer [62-66]. In contrast, single micronutrient interventions and diets containing a mixture of micronutrients show possible beneficial effects on biomarkers of OS and disease [67]. Vitamin D has anti-oxidative properties as well, however, it is acting predominantly as a steroid hormone. VD is generated either from exposure to ultraviolet B radiation (VD_3) within the skin, or is derived from dietary intake (VD_3 in fatty fish and VD_2 in mushrooms), and both forms are activated the same way [68-70]. The activity of VD depends on the presence and its interaction with the VD receptor [68, 71], which appears in almost all human cells and tissues such as skeletal muscle, brain, breast, prostate, colon, parathyroid glands, pancreas and immune cells [68, 70]. VD deficiency is associated with immune

diseases and infections, as well as cancers, diabetes, the metabolic syndrome, and other cardiovascular risk factors [71, 72].

Link between frailty, oxidative stress and micronutrients

A lower risk for frailty was associated to both, a high fruit and vegetable consumption and a strong adherence to a Mediterranean diet in older individuals [73-76]. At the same time, a higher prevalence and risk for frailty was associated with an inadequate intake and status of vitamins and carotenoids, and a nutritional pattern low in vitamins A and E [77-80]. Additionally, biomarkers of OS were elevated in frail compared to non-frail subjects [81], and frailty was related to both higher OS and lower antioxidant levels [82]. A higher prevalence and incidence of frailty was associated with low VD levels [83, 84]. Furthermore, a decline in muscle mass and a poor physical performance in frail individuals were associated with low VD [85]. A low physical activity, weakness as well as slowness, which are main constituents of the frailty syndrome [83, 86] were linked to a sub-optimal VD status. Impaired muscle functions like weakness and muscle atrophy, as well as falls were also associated with VD deficiency [71, 72]. In both, VD deficient rat muscles and VD treated murine myoblasts (C2C12 cells) VD was related to redox homeostasis [87]. The expression of the VD receptor in human skeletal muscle decreases with age leading to a suppressed response to $1,25(OH)_2D$ (active form of VD) in muscle fibers of older people [88]. However, associations of the frailty syndrome with several simultaneously measured micronutrients and biomarkers of OS need to be investigated in large cohorts.

Frailty assessment tools and biomarkers

The assessments of frailty (and sarcopenia), more precisely reduced muscle mass as well as impaired muscle functionality are undertaken mainly by the use of imaging techniques and functional examinations (**Table 2**) [4-6, 89, 90]. Major approaches are e.g. DEXA (dual energy X-ray absorptiometry), MRI (magnetic resonance imaging), CT (computerized tomography), BIA (bioelectrical impedance analysis) and ultrasonography to measure muscle mass. Additionally, there are biomarker methods like body potassium (K) or deuterated creatine (d_3-creatine) dilution method estimating muscle mass.

Table 2. Measures of muscle mass and function, and their limitations for clinical application.

Measure	Limitation
***Muscle Mass* by imaging**	
DEXA	Availability, hydration status/body thickness, no intramuscular adipose tissue possible, differences between manufacturers
CT and MRI	Expensive, availability, radiation, highly qualified personnel
BIA	Dependent on body position, hydration status, food/beverage intake, general health condition, recent physical activity
Ultrasonography (muscle)	Highly qualified personnel, similar acoustic impedance of muscle and adipose tissue
Muscle function by physical examinations	
Grip strength	Several different protocols, reference population needed
Knee flexion/extension	Special equipment and training needed
SPPB	Compliance
Gait or walking speed	Inconsistent protocols and methodologies
Timed get-up-and-go test	Compliance and different protocols
Stair climb power	Compliance and different protocols

SPPB measures balance, gait speed, strength and endurance. Knee flexion/extension measured by power rig, straight-back chair or isokinetic dynamometer. Abbreviations: BIA, bioelectrical impedance analysis; CT, computerized tomography; DEXA, dual energy X-ray absorptiometry; K, potassium; MRI, magnetic resonance imaging; SPPB, short physical performance battery. Modified from [4-6].

Assessment tools for muscle strength and physical performance are grip strength measures, physical tests as well as questionnaires. However, those approaches and examinations can have drawbacks regarding clinical routine settings (availability, high costs, space requirements and highly qualified personnel), as well as regarding patients' compliance, being time-consuming procedures and space requirements for e.g. walking tests (**Table 2**) [4-6].

There are several biomarkers in biofluids like inflammatory cytokines, clinical parameters, hormones and biomarkers for oxidative damage that were investigated regarding muscle mass, muscle function, sarcopenia and frailty syndrome [9, 91]. However, these biomarkers relate also to several other diseases and might reflect a more general pathophysiological state of the body. Therefore, more muscle specific, objective and

elaborate biomarkers to determine an elevated muscle protein turnover are needed, and thus, focus has recently set to investigate and identify potential biomarkers in biofluids regarding muscle pathologic conditions.

Procollagen type III N-terminal peptide originates from proteolytic cleavage of procollagen III during muscle remodeling and, was positively associated with changes in lean body mass after exercise [92, 93]. C-terminal agrin fragment (CAF) originates from cleavage of agrin, which is important for the formation and stabilization of neuromuscular junction [94]. CAF was negatively associated with appendicular lean mass in pre-frail males, but not in women [95] and correlated negatively with neuromuscular fatigue in men and not in women [96]. Furthermore, in sarcopenic individuals elevated CAF concentrations were observed [97, 98]. Contrarily, CAF correlated positively with muscle cross-sectional area [93]. These proposed biomarkers for muscle status and sarcopenia need further validation, especially considering frail cohorts.

3-Methylhistidine

3-methylhistidine (3-MH; N-tau-methylhistidine, Nτ-methylhistidine) was first identified in 1954 in human urine and plasma samples [99, 100], followed by further investigations regarding its origin and metabolism. 3-MH is formed endogenously only in the muscle proteins actin and myosin [101, 102] by the post-translational methylation of histidine-residues of the peptide chains using S-adenosyl-methionine as methyl-group donor (**Figure 3**) [103].

In actin, the 3-MH amount (one 3-MH residue per actin molecule) was found to remain constant independent of the muscle type; contrarily in myosin the 3-MH amount is dependent on the type of muscle with higher amounts in white muscle than in red skeletal muscle as well as in smooth muscle [104]. While actin and myosin filaments are key components for the force generation in muscles, 3-MH does not seem to be involved in this process [104-106], and its function within muscles and the metabolism is still unclear. In 1970, Johnson and Perry showed that ATPase and actin-combining activities of myosin may not depend on 3-MH residues [104].

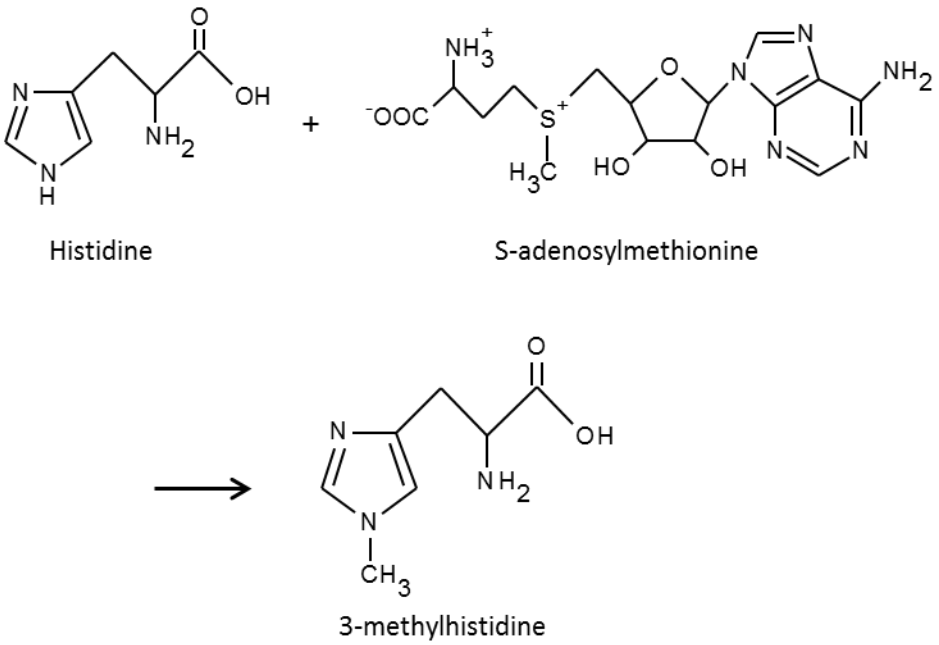

Figure 3. Synthesis of 3-methylhistidine. Transfer of a methyl group from S-adenosyl-methionine to histidine where the methyl group binds to a nitrogen atom of the imidazole ring.

3-MH is both, endogenously released into the blood circulation by muscle protein breakdown and exogenously taken up by dietary meat and fish (**Figure 4**). Meat and fish and their products contain either 3-MH itself or the 3-MH containing dipeptide balenine (synonym: ophidine; β-alanyl-Nτ-methyl-histidine) [107, 108], which can be degraded enzymatically by the enzyme carnosinase subsequently releasing 3-MH [108]. However, balenine can be neglected as source of 3-MH for humans since it was found mainly in whale and snake [108, 109]. Once 3-MH is released or taken up it is neither metabolized (e.g. reutilized for (muscle) protein synthesis) nor oxidized and thus becomes a component of the free AA pool in the plasma, and is then excreted quantitatively in the urine [110, 111].

In urine and plasma, 3-MH can be analytically determined and subsequently was suggested and used as a measure of muscle protein breakdown. Urinary excretion of 3-MH were used in human studies to identify an elevated muscle protein breakdown in (neuro)muscular diseases [112-115], muscle wasting diseases [116] as well as in aging [117].

Since 3-MH is dependent on the muscle mass of individuals, normalizing its concentrations to creatinine (Crea) resulting in the 3-MH-to-creatinine ratio (3-MH/Crea) is an approach to reduce inter-individual as well as intra-individual differences [118, 119] and was recommended to be measured in heterogeneous study populations [120].

Additionally, 3-MH/Crea was used to identify an elevated muscle protein breakdown [112, 114, 115]. Creatinine is produced in the muscle by hydrolysis of either creatine or creatinine-6-phosphate, and Crea concentrations in plasma and urinary concentrations represent one estimation of muscle mass [121]. Thus, 3-MH and 3-MH/Crea in plasma and urine originates endogenously only from muscle breakdown, indicating the suitability as potential biomarkers for (elevated) muscle protein turnover and subsequently for frailty assessment.

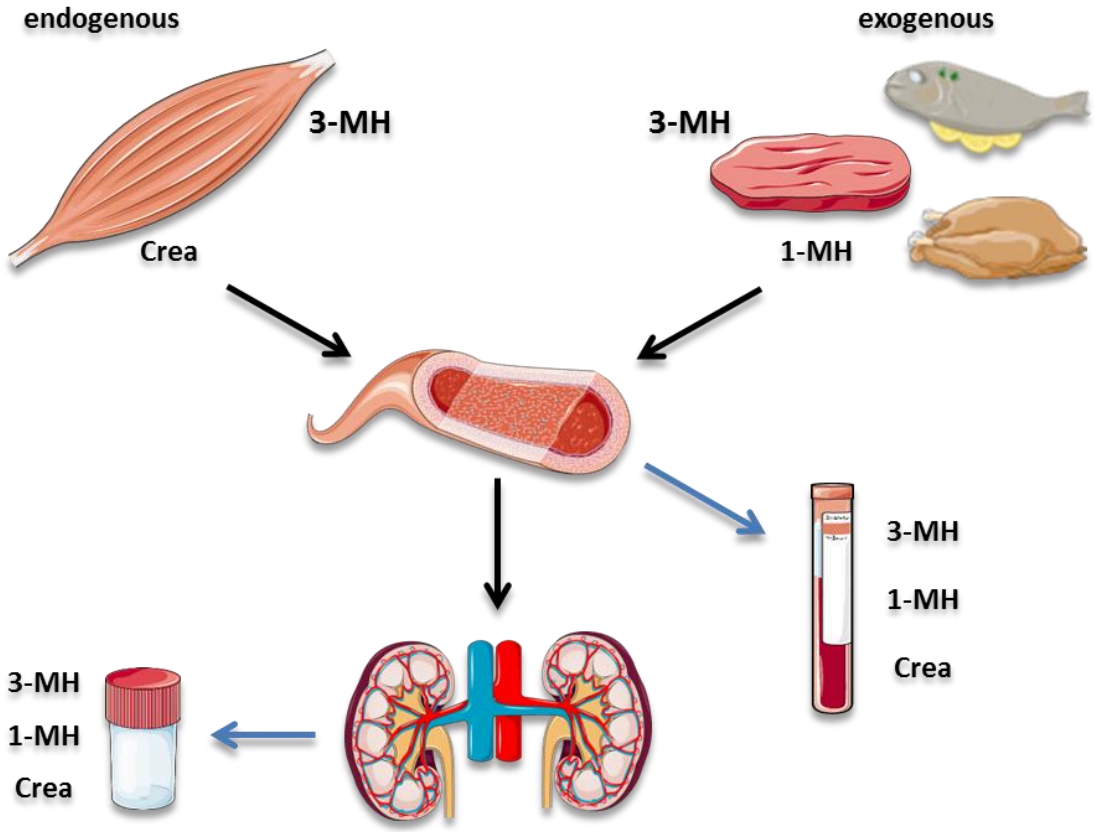

Figure 4. Sources and metabolism of 3-methylhistidine. Abbreviations: 3-MH, 3-methylhistidine; 1-MH, 1-methylhistidine; Crea, creatinine.

Contrarily, there is some skepticism concerning the reliability of 3-MH as a biomarker for muscle protein turnover since its concentration in plasma and urine is influenced by the dietary consumption of meat, fish and their products. Urinary examinations showed higher 3-MH and 3-MH/Crea concentrations after meat and fish administration [117, 118, 120, 122-125]. Similarly, plasma 3-MH concentrations were also elevated after meat intake [126].

3-MH half-life in urine was found to be about 12 hours [118], which is in accordance to the plasma half-life of about 12 hours [126]. However, after intravenous injection of labelled 3-MH its half-life in plasma was about 130 minutes [111].

Finally, to perform reliable 3-MH measurements without any exogenous impact, several authors recommended avoiding those foods for at least 2-3 days prior to sample collection [117, 123, 124]. However, investigations regarding this exogenous impact of meat and fish on plasma 3-MH concentrations are scarce and therefore, further intervention studies seemed worthy to be undertaken.

There is another form of methylhistidine. 1-methylhistidne (1-MH; N-pi-methylhistidine, N-π-methylhistidine; **Figure 5**) can function as biomarker for meat consumption and be assessed in parallel to 3-MH. 1-MH occurs as part of the dipeptide anserine (β-alanyl-Nπ-methyl-histidine) which is present in animal muscle but not in human [108, 109]. High amounts occur in chicken and to a lesser extent in beef and pork as well as in varying amounts in fish. Anserine is degraded by carnosinase and subsequently, the released 1-MH can be measured in human plasma and urine after meat and fish intake.

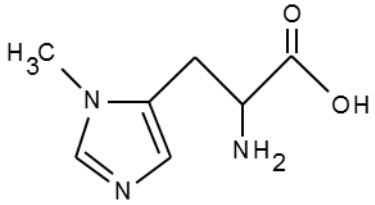

Figure 5. Structure of 1-methylhistidine.

Higher urinary excretions of 1-MH were shown in omnivorous compared to vegetarian individuals [127] and also after meat or fish consumption [118, 128, 129]. Additionally, the ratios of 1-MH-to-creatinine (1-MH/Crea) and 3-MH-to-1-MH (3-MH/1-MH) were able to display meat and fish consumption [118, 127-129]. None of these markers were influenced by other animal products like dairy or eggs [128, 129]. Thus, 1-MH, 1-MH/Crea and 3-MH/1-MH determinations seem to be suitable to display the exogenous influence of meat and fish on plasma 3-MH concentrations.

Additionally, kidney function seems to be a possible limiting factor for 3-MH as reliable biomarker since 3-MH is excreted by the kidney. In general, kidney function decreases with age, and can occur as a contributor or comorbidity of age-related diseases [130, 131]. Kidney diseases and low kidney functions were shown to be associated with

elevated 3-MH [132-134]. Kidney function can be calculated as the estimated glomerular filtration rate (eGFR) by using different equations, which include the creatinine concentration, age, height, sex and race of an individual [135-138]. Up to date, no effort was made to normalize plasma 3-MH concentrations to the corresponding eGFR of individuals resulting in the 3-MH-to-eGFR ratio (3-MH/eGFR), to minimize and control for possible kidney effects. Another factor influencing 3-MH concentrations and thus being a limitation for reliable 3-MH measurements is multi-medication of individuals as shown by urinary 3-MH and 3-MH/Crea levels [139, 140].

Methylhistidine detection and quantification by liquid chromatography-tandem mass spectrometry

For the detection and quantification of 3-MH as well as 1-MH in different sample matrices there are various analytical methods available. Analytical methods and devices used are AA analyzers [104, 111, 117, 118, 125, 141], ion-exchange chromatography in combination with ninhydrin derivatization and AA analyzers [142-144] and capillary electrophoresis techniques [145]. Furthermore, high-performance liquid chromatography (HPLC) including pre-column derivatization with fluorescamin or *o*-phthalaldehyde (OPA) and subsequent UV or fluorescence detection [133, 134, 146, 147], as well as gas chromatography-mass spectrometry (GC-MS) including derivatization [148-150] are techniques for the detection and quantification of methylhistidines in cells, tissues and bio fluids. These techniques may have some limitations considering extensive sample preparations, long run times and not being able to detect several analytes simultaneously from one sample. However, methylhistidines in biological fluids can be detected and quantified simultaneously by liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques using internal standards (isotope labelled) and without derivatization of the analytes [128, 129, 151-153].

LC-MS/MS is a technique where a liquid chromatography device is coupled to a tandem mass spectrometer (**Figure 6**). HPLC generally enables the separation of compounds according to their physical and chemical properties like hydrophobicity/polarity, molecular size and functional groups, and thus, due to their binding affinity and interaction with the stationary and mobile phase. Separation is followed by the detection of the molecules of interest by e.g. UV-vis, fluorescence or photo

diode array detectors. Since MS analysis provides a higher sensitivity and specificity than chromatographic detectors, the coupling of MS to LC has advantages compared to single HPLC measures alone [154].

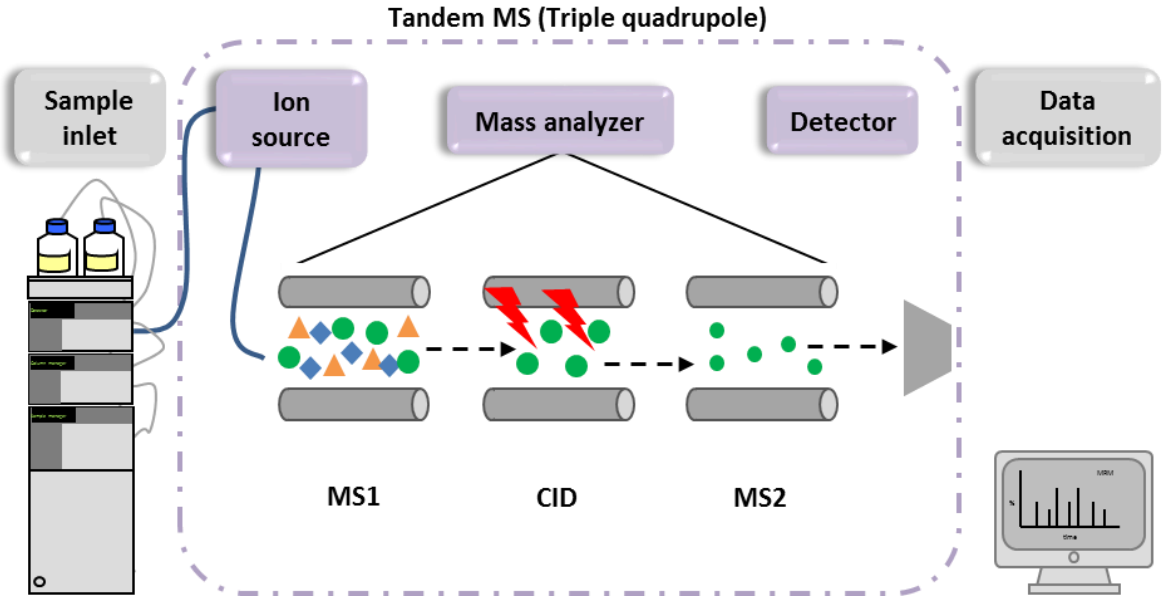

Figure 6. Scheme of LC-MS/MS flow. The ion source functions as ion generator; both MS1 and MS2 function as mass filters for ion selection; CID: collision induced dissociation or fragmentation; red flashes represent collision gas such as nitrogen or argon; the detector records ions of interest leaving the mass analyzer; data acquisition performed by special computer software for qualitative and quantitative assessment.

In principle, after substances are separated by LC, these are transferred to the MS. The MS then generates ions from these substances (inorganic and organic), then separates these ions according to their mass-to-charge ratio (m/z), and finally records m/z and m/z abundance qualitatively and quantitatively [155]. MS systems typically include an ion source, a mass analyzer, and a detector, and operate under a high vacuum.

Ionization of analytes is realized in the ion source by several different techniques like Electrospray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI), Atmospheric Pressure Photo-ionization (APPI) or Matrix-assisted Laser Desorption Ionization (MALDI). Generated ions are separated in the mass analyzer by different techniques such as (triple) quadrupole, time-of-flight (TOF) or Orbitrap.

A quadrupole mass analyzer consists of four rod electrodes (hyperbolic or cylindrically) to which different voltages can be applied. Ions of a specific m/z are able to pass between these rods to reach the detector, whereas other ions collide with the rods and do not pass to the detector. Thus, a quadrupole acts as kind of a mass filter. In triple

quadrupole MS (tandem MS or MS/MS), three quadrupoles are connected in series. Here, quadrupole 1 (MS1) and quadrupole 3 (MS2) function as mass filters and the quadrupole in between MS1 and MS2 can act as collision cell. In the collision cell, a collision gas (e.g. nitrogen or argon) can be applied to dissociate ions of a certain m/z which passed MS1, resulting in specific fragments. Specific footprints (fragments) of the analytes of interest are obtained by this collision-induced dissociation (CID). Triple quadrupole mass analyzers offer several modes of operation, where MS1 and MS2 can either scan certain m/z ratios or be fixed to certain m/z ratios, and additionally, the collision cell can apply collision gas or not. Based on the operational mode, generated ions and/or ion fragments of a specific m/z can then pass MS2 to reach the detector.

Detection happens by different detector techniques like electron multiplier or microchannel plates, resulting in ion chromatograms or mass spectra. Finally, analytes are quantified by external standard calibration, by internal standard calibration or by isotope dilution using isotope labeled internal standard. Isotope dilution is performed by adding a known amount of an isotope labelled internal standard to the sample during sample preparation, followed by the simultaneous assessment of both analyte and internal standard [154, 156, 157].

LC-MS/MS is used widely in research to measure AA (and related compounds), hormones like dopamine and serotonin, and biomarkers like advanced glycation end products, in the context of pathologies in humans and animal models [153, 158-160]. Over the last decades, LC-MS/MS methods have been established in clinical laboratory, biochemistry and endocrinology (reviewed in [154, 161, 162]).

Rationale and Aims

Frailty and age-associated losses of muscle mass, muscle strength and physical performance can either be prevented or treated by dietary interventions (protein, calorie or VD supplementation), exercise (resistant and aerobic), enhancing micronutrient status or reducing medication intake [8, 14]. Thus, the early diagnosis and the possible prediction of frailty is essential. Therefore, finding and evaluating elaborate and muscle specific biomarkers in bio fluids, which are able to display an elevated muscle protein breakdown or an altered muscle protein turnover are of major interest in this context. Up to date, no single biomarker alone seems to be suitable to identify frail individuals or individuals at a higher risk to become frail and it is suggested that a set of biomarkers is needed to achieve this goal [15]. Furthermore, associations of frailty with OS and micronutrient concentrations (i.e. redox-status) should be further evaluated to deepen our understanding of underlying mechanisms.

In this thesis, plasma 3-MH and its ratios 3-MH/Crea and 3-MH/eGFR are hypothesized to function as biomarkers that could support functional tests or that add to a set of biomarkers for the diagnosis or prediction of frailty. Additionally, it is hypothesized that biomarkers of OS are high, while simultaneously concentrations of micronutrients are low in frail individuals. Therefore, the following aims were to be accomplished in this thesis:

At first, a reliable technique for the simultaneous measurement of 3-MH and 1-MH in plasma was needed, and therefore, an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was developed and validated (**Publication 1**).

- The potential biomarkers 3-MH and 3-MH/Crea needed to be reexamined regarding the recommendation to avoid meat 3 days prior to blood sampling to obtain reliable measures. Furthermore, the use of plasma 1-MH and 1-MH/Crea to display such an exogenous meat intake needed to be evaluated. Therefore, an intervention study was realized and the following was investigated:
  (I) the associations of plasma 1-MH, 3-MH, and creatinine concentrations and 3-MH/Crea and 1-MH/Crea ratios with dietary habits in young omnivores and vegetarians (**Publication 1**)

(II) the impact of a white meat intervention on plasma 3-MH and 1-MH during a meat-free period in omnivores (**Publication 1**)

- Associations between the frailty status and plasma 3-MH concentrations and its ratios needed to be determined in a large cohort, including robust, pre-frail and frail individuals to evaluate, if 3-MH might be a suitable biomarker to display frail individuals and may be able to support frailty diagnosis or predict the frailty syndrome. Therefore, the following was investigated:

  (III) the cross-sectional relationship of the following plasma biomarkers with frailty status (robust, pre-frail and frail): 3-MH, 1-MH, Crea and eGFR as well as 3-MH/Crea, 3-MH/eGFR, 1-MH/Crea and 3-MH/1-MH (**Publication 2**)

  (IV) the capability of 3-MH, 3-MH/Crea and 3-MH/eGFR to identify frail individuals or individuals with higher odds to be frail according to biomarker quintiles (**Publication 2**)

- Associations of frailty status with micronutrients and biomarkers of OS in a large cohort including frail, pre-frail and robust individuals are lacking and therefore, the following was investigated in participants older than 65 years of the European FRAILOMIC Initiative:

  (V) the cross-sectional relationship of the fat-soluble vitamins A, D_3 and E, and carotenoids, as well as the OS biomarkers PrCarb and 3-NT with frailty status (**Publication 3**)

Publications

Publication 1

"The Influence of Dietary Habits and Meat Consumption on Plasma 3-Methylhistidine - A Potential Marker for Muscle Protein Turnover"

Bastian Kochlik, Christiana Gerbracht, Tilman Grune and Daniela Weber

Mol. Nutr. Food Res. 2018, 62, 1701062

DOI: 10.1002/mnfr.201701062

Journal of Molecular Nutrition and Food Research

Impact factor: 4.653 (2018)

Publication 2

"Associations of Plasma 3-Methylhistidine with Frailty Status in French Cohorts of the FRAILOMIC Initiative"

Bastian Kochlik, Wolfgang Stuetz, Karine Pérès, Catherine Féart, Jesper Tegner, Leocadio Rodriguez-Mañas, Tilman Grune and Daniela Weber

J. Clin. Med. 2019, *8*(7), 1010

DOI: 10.3390/jcm8071010

Journal of Clinical Medicine

Impact factor: 5.688 (2018)

Publication 3

"Associations of fat-soluble micronutrients and redox biomarkers with frailty status in the FRAILOMIC Initiative"

Bastian Kochlik, Wolfgang Stuetz, Karine Pérès, Sophie Pilleron, Catherine Féart, Francisco José García García, Stefania Bandinelli, David Gomez-Cabrero, Leocadio Rodriguez-Mañas, Tilman Grune and Daniela Weber

J Cachexia Sarcopenia Muscle. 2019

DOI: 10.1002/jcsm.12479

Journal of Cachexia, Sarcopenia and Muscle

Impact factor: 10.754 (2018)

Publication 1

"The Influence of Dietary Habits and Meat Consumption on Plasma 3-Methylhistidine - A Potential Marker for Muscle Protein Turnover"

Bastian Kochlik, Christiana Gerbracht, Tilman Grune and Daniela Weber

Mol. Nutr. Food Res. 2018, 62, 1701062

Own contribution

- Proportional design of the study concept
- Proportional preparation of the ethical approval application
- Proportional carrying out tests with participants
- Developing the concept for the manuscript
- Literature research
- Proportional sample preparation
- Sample measurement and evaluation
- Statistical analyses
- Preparation of figures and tables
- Preparation of the manuscript

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The Influence of Dietary Habits and Meat Consumption on Plasma 3-Methylhistidine—A Potential Marker for Muscle Protein Turnover

*Bastian Kochlik, Christiana Gerbracht, Tilman Grune, and Daniela Weber**

Scope: 3-Methylhistidine (3-MH) as a potential biomarker for muscle protein turnover is influenced by meat intake but data on the impact of meat on plasma 3-MH are scarce. We determined the association of plasma 3-MH, 1-methylhistidine (1-MH), and creatinine with dietary habits and assessed the impact of a single white meat intervention during a meat-free period.
Methods and results: Plasma 3-MH, 1-MH, and creatinine concentrations of healthy young omnivores ($n = 19$) and vegetarians ($n = 16$) were analyzed together with data on anthropometry, body composition, grip strength, and nutrition. After baseline measurements omnivores adhered to a meat-free diet for 6 days and received a defined administration of chicken breast on day four. At baseline, omnivores had higher plasma 3-MH and 1-MH concentrations than vegetarians. White meat administration led to a slight increase in plasma 3-MH in omnivores. The elevated 3-MH concentrations significantly declined within 24 h after white meat intake.
Conclusion: 1-MH concentrations in plasma seem to be suitable to display (white) meat consumption and its influence on 3-MH plasma concentration. 3-MH in plasma may be used as a biomarker for muscle protein turnover if subjects have not consumed meat in the previous 24 h.

1. Introduction

3-Methylhistidine (3-MH; *N*-tau-methylhistidine) seems to be a suitable biomarker to identify elevated muscle degradation or an unfavorable muscle protein turnover as shown in muscle wasting disease and elderly populations.[1–5] 3-MH is synthesized only in the muscle by the methylation of one histidine residue in actin and in varying amounts in myosin depending on the type of muscle.[6–8] 3-MH is released into the circulation during muscle degradation and then excreted quantitatively in the urine without being metabolized.[9,10] Thus, muscle protein degradation is the only endogenous source of 3-MH in human plasma. In healthy adults (20–70 years) the content of 3-MH in muscle and the 3-MH-to-creatinine excretion (3-MH/Crea) ratio remain constant.[2,4] Thus, we suggest that 3-MH might be a helpful biomarker in the assessment of muscle protein turnover, which is important in the diagnosis of frailty and sarcopenia. Up to date the diagnosis of sarcopenia and frailty is mainly performed by physical tests and imaging parameters.[11,12] However, both approaches have some drawbacks, including patients' compliance, availability in the clinical routine, and costs. Therefore, an objective and specific biomarker for the assessment of muscle protein turnover is needed. However, plasma 3-MH might be influenced by food intake. Meat, fish, and their products are the only exogenous sources of 3-MH and hence are able to influence plasma concentrations of 3-MH. The current recommendation to measure endogenously released 3-MH in plasma is to adhere to a meat-free diet for at least 3 days before blood sampling.[2] This is problematic when measuring samples of elderly patients, in the clinical routine and in large scale human studies.

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.201701062

DOI: 10.1002/mnfr.201701062

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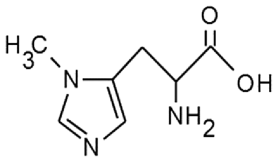
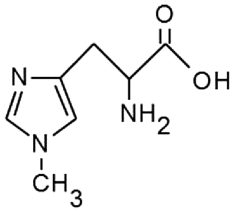
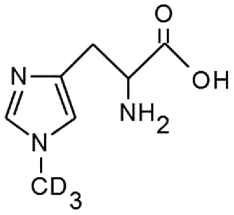
Berlin, Germany

Table 1. Structure and nomenclature of methylhistidines.

Structure			
Biochemical nomenclature[a]	1-Methylhistidine *N*-pi-methylhistidine	3-Methylhistidine *N*-tau-methylhistidine	3-Methyl-D_3-histidine *N*-tau-methyl-D_3-histidine
IUPAC nomenclature	(2S)-2-amino-3-(3-methylimidazol-4-yl) propanoic acid	(2S)-2-amino-3-(1-methylimidazol-4-yl) propanoic acid	

[a] Nomenclature used in this study.

Another form of methylhistidine, namely 1-methylhistidine (1-MH; *N*-pi-methylhistidine), exists besides 3-MH. 1-MH is mainly synthesized in muscle of animals but not in humans and occurs as part of the dipeptide anserine (β-alanyl-N1-methyl-histidine).[13–16] Therefore, it has been hypothesized that urinary 1-MH may function as a specific marker for meat consumption[17,18] and may indicate exogenous 3-MH.

In the literature there is some inconsistency about the nomenclature of methylhistidine metabolites in terms of numbering the methyl group of the imidazole ring of histidine.[16,19–21] In the present study, we prefer the biochemical names *N*-tau-methylhistidine and *N*-pi-methylhistidine which refer to 3-methylhistidine and 1-methylhistidine, respectively (**Table 1**), and which is in contrast to the IUPAC (International Union of Pure and Applied Chemistry) nomenclature but allows better comparison to previous literature in this field.

Creatinine, its urinary excretion and its plasma concentrations are associated with muscle metabolism[22–24] and smaller interindividual variations of methylhistidines (MH) have been shown when adjusted to creatinine.[25,26]

The aim of this pilot study was to investigate 1) the associations of plasma 1-MH, 3-MH, and creatinine with dietary habits in young omnivores and vegetarians, and 2) the impact of a single white meat intervention on plasma 1-MH and 3-MH during a meat-free period in omnivores. Further, we aimed to display exogenous 3-MH and to reexamine the recommendation of avoiding all meat for 3 days before blood sampling.

2. Experimental Section

This study was conducted with each participant's understanding and informed written consent. Ethical clearance was given by the ethics committee of the University of Potsdam, Potsdam, Germany (permission number 36/2016). This study was conducted in accordance with the Declaration of Helsinki (1964).

2.1. Study Population and Study Design

Healthy participants (absence of chronic or acute diseases) were recruited for the study by bulletin advertisements at the University of Potsdam and the German Institute of Human Nutrition. The participants were divided according to their nutritional habits into a vegetarian and an omnivorous group.

Inclusion criteria for both groups were: age between 20–30 years and a BMI of 20–30 kg m^{-2}. An additional inclusion criterion for the vegetarian group was ovo-lacto vegetarian eating behavior. Exclusion criteria for both groups were: pregnancy, lactating period, chronic medication (except oral contraceptive), and chronic diseases. For the vegetarian group the additional exclusion criteria were consuming meat, fish, and such products.

The study scheme is shown in **Figure 1**. After baseline, the omnivore group followed an ovo-lacto vegetarian diet for 6 days, that is, no meat, fish, and such products were consumed (Figure 1B). On day three (d3) a blood sample was taken. After blood sampling on day four (d4), the participants received a single bolus of 160 g cooked chicken breast together with a mixed salad and bread. This amount of 160 g chicken meat contains about 35.52 g protein (22.2% protein) resulting in 126.5 μmol 3-MH (3.56 μmol 3-MH g^{-1} protein).[27] An additional blood sample was taken 3 h after white meat intervention (d4.1). Further blood collections were performed on day five (d5) and day six (d6) during the meat-free period. Then the participants were allowed to resume their normal eating habits for 7 days followed by a final blood sampling on day 13 (d13).

All anthropometric measurements and blood samplings were performed by trained and qualified study nurses. Plasma preparation and biomarker analysis were performed by trained and qualified scientists.

2.2. Nutritional Data

Before baseline measurement, the participants were instructed to eat according to their usual dietary habits and fill out a 3-day dietary record. The intake of energy, protein, and meat was evaluated using PRODI Software Version 6.5 (Nutri-Science GmbH, Hausach, Germany).

2.3. Measurements of Anthropometry and Body Composition

Anthropometrics and body composition were assessed at baseline by using a SECA Stadiometer 274, a nonelastic tape measure and a SECA mBCA 515 with integrated scale for the

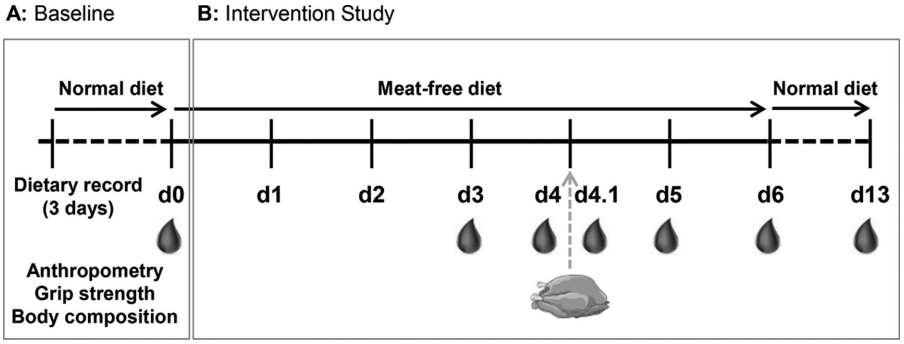


Figure 1. Study Design. A) Baseline examination and blood sampling in omnivores ($n = 19$) and vegetarians ($n = 16$). B) Time course of dietary conditions and time points of blood samplings and white meat intervention for the omnivores.

bioelectrical impedance analysis (BIA; Seca GmbH & Co. KG, Hamburg, Germany). Physical activity level (PAL) was self-reported by participants during the BIA measurement. PAL was categorized according to participants' lifestyle and profession: ≤1.2 (inactive), 1.4, 1.6, 1.8, and ≥2.0 (highly active).

2.4. Measurement of Grip Strength

Grip strength was measured at baseline by using a Jamar Plus Digital Hand Dynamometer (Patterson Medical, Sammons Preston Bolingbrook, IL, USA) and carried out according to Roberts et al.[28]

2.5. Blood Samples

Blood samples (4 mL) were collected by venipuncture into EDTA vacutainers (Sarstedt, Numbrecht, Germany). Blood samples were kept at 4 °C until centrifugation (EBA 200, Hettich Zentrifugen, Tuttlingen, Germany) for 10 min at 2700 g and room temperature to separate the plasma. Plasma samples were stored at −80 °C until analysis and thawed immediately before preparation for analysis.

2.6. Analysis of Plasma Methylhistidines

The simultaneous measurement of 3-MH and 1-MH (μmol L^{-1}) in plasma was carried out by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) using an Acquity Ultra Performance LC system coupled to a Waters Quattro Premier XE mass spectrometer (both Waters Corporation, Milford, MA, USA) and the Waters MassLynx Software (Version 4.1). UPLC conditions recommended by the column manufacturer were modified as described below in section 2.6.2.

2.6.1. Materials

Standards used were 1-MH and 3-MH (both Sigma Aldrich, Taufkirchen, Germany) and deuterated 3-methylhistidine (d_3-3-MH; Cambridge Isotope Laboratories Inc., Andover, MA, USA) as internal standard.

Reagents used were water, methanol, acetonitrile (all LC-MS grade; Merck, Darmstadt, Germany), ammonium formate (for MS; Sigma Aldrich, Taufkirchen, Germany), and formic acid (eluent additive for MS; Honeywell, Offenbach, Germany).

1-MH and 3-MH stock solutions were prepared in water and then mixed to a standard mixture followed by dilutions in various concentrations (0.24 μM–120 μM) with water and treated like plasma samples for quantification.

2.6.2. Sample Preparation and UPLC-MS/MS Analysis

Internal standard (2.4 μM; 25 μL) was added to plasma samples (25 μL), vortex mixed, and incubated for 1 h at 7 °C; then 150 μL methanol was added to precipitate proteins, vortex mixed, and incubated for 2 h at 7 °C; samples were centrifuged for 10 min at 4 °C and 16 500 × g (Biofuge primo R; Heraeus, Thermo Fisher Scientific, Germany). The supernatant was consequently centrifuged for 10 min at 4 °C and 16 500 × g; then the supernatant was diluted with an equal volume of water; transferred to an autosampler vial; kept at 15 °C in the autosampler and 4 μL of the sample was injected into the UPLC-MS/MS system. Analyte separation was carried out on an Intrada Amino Acid column (3 μm, 50 × 3 mm; Imtakt USA, Portland, OR, USA) at a column temperature of 37 °C. Eluents A (0.25% formic acid in ACN v/v) and B (0.25% formic acid in 100 mM ammonium formate v/v) were used at a flow rate of 0.8 mL min^{-1}. Analytes were eluted at 7.0 min (3-MH and d_3-3-MH) and 7.3 min (1-MH) from the column with a stepwise gradient (initial: 95% A; min 1–3: 95–85% A; min 3–8: 85–20% A; min 8–9: 0% A; min 9–10: 95% A). Electrospray ionization operated in positive mode (ESI+), and MS/MS settings were as follows—capillary voltage: 0.75 kV, cone voltage: 20 V, cone gas flow: 100 L h^{-1}, source temperature: 120 °C, desolvation temperature: 400 °C, desolvation gas flow: 850 L h^{-1}. Three mass transitions with optimal collision energies (CE) each were defined for MRM analysis of 1-MH, 3-MH, and d_3-3-MH. Mass transitions of the quantifier ion of 1-MH (m/z 170 > 96, CE: 20 V), 3-MH (m/z 170 > 124, CE: 16 V), and d_3-3-MH (m/z 173 > 127, CE: 16 V) were used for quantification. Mass transitions of the qualifier ions were as follow: 1-MH (m/z 170 > 126, CE: 18 V; m/z 170 > 109, CE: 16 V), 3-MH (m/z 170 > 109, CE: 18 V; m/z 170 > 96, CE: 30 V) and d_3-3-MH (m/z 173 > 112, CE: 18 V; m/z 173 >

99, CE: 30 V). Quantification of 1-MH in plasma was performed by external standard calibration and quantification of 3-MH in plasma was performed by standard isotope-dilution approach.

In total, 148 plasma samples were randomly prepared in batches of 20 samples and 6-point standard calibration (0.015–7.5 μM 1-MH and 3-MH including 0.15 μM IS) was used for quantification. Two quality control samples (pooled plasma samples; $n = 5$ measurements) were measured between batches.

2.6.3. Method Validation

Linearity was assessed by triplicate measurement of six concentration levels (0.015–7.5 μM) of a standard mixture of 1-MH, 3-MH, and d_3-3-MH dissolved in water or spiked to pooled plasma.[29] The same set of samples was used to determine the *matrix effect* on 1-MH, 3-MH, and d_3-3-MH by comparing the slopes obtained from calibration curves in water with those in plasma.[29,30] *Recoveries* were calculated by spiking six plasma samples with standard mixtures of 1-MH, 3-MH, and d_3-3-MH with two different concentrations (0.075 μM and 1.5 μM) before and after sample extraction. Subsequently, peak areas of the analytes in the pre-spiked samples were compared with peak areas in the post-spiked samples. Further, on three different days a set of six plasma samples was prepared (see Section 2.6.2) in duplicate, measured in triplicate, and quantities were calculated to determine *inter-assay* and *intra-assay variations* for 1-MH and 3-MH in plasma.[31] For quantification three 6-point calibration mixtures (0.015–7.5 μM for 1-MH and 3-MH including 0.15 μM IS) were prepared and measured in triplicate. For 3-MH, peak areas were normalized to peak areas of the corresponding IS, plotted against the applied concentration, and the resulting calibration curve used to quantify sample concentrations. 1-MH quantification was performed by external standard calibration due to a lack of internal standard. The same sets of calibration mixtures were used to estimate the *theoretical limit of detection (tLOD)*. The *limit of quantification (LOQ)* was defined as the lowest concentration of the calibration curve.[29,32] *tLOD* was estimated using the formula:

$$tLOD = t_\alpha \times S_{\bar{x}}, \quad (1)$$

with t_α according to Student's *t*-test using $n - 1$ measurements and $S_{\bar{x}}$ as an estimate of the true standard deviation of the distribution of sample means.[32]

Stability of processed samples and calibration mixtures was determined by measuring concentrations of the same set of samples and calibration mixtures kept for 24 or 48 h at −20 °C. Autosampler *stability* was assessed by measuring concentrations of six samples and by a set of calibrators kept for 36 h in the autosampler (at 15 °C).

2.7. Analysis of Plasma Creatinine

Creatinine (μmol L^{-1}) in plasma was measured by using the ABX Pentra Creatinine CP kit in a Pentra 400 (ABX Diagnostics, Montpellier, France) device. The analysis was based on the enzymatic Jaffé method and was carried out according to the manufacturer's instructions.[33,34]

Table 2. Characteristics of the study population.

	Omnivores	Vegetarians	*P*
n (% female)	19 (84.2)	16 (87.5)	—
Age [years]	26.3 ± 2.4	26.4 ± 2.9	n.s.
Assessment before baseline measurement			
Energy intake [kcal d^{-1}]	1973 ± 563	1986 ± 484	n.s.
Protein intake [g d^{-1}]	75.1 ± 33.0	62.9 ± 20.7	n.s.
Meat intake [g d^{-1}]	106.5 ± 79.7	0 ± 0	—
Meat intake [g] (at d1)	93.9 ± 126.1	0 ± 0	—
At baseline			
BMI [kg m^{-2}]	22.0 ± 2.10	21.9 ± 2.10	n.s.
FMI [kg m^{-2}]	5.38 ± 1.75	6.25 ± 1.58	n.s.
FFMI [kg m^{-2}]	16.6 ± 2.00	15.6 ± 1.26	n.s.
SMM [kg]	21.4 ± 5.39	20.6 ± 3.94	n.s.
PAL	1.64 ± 0.08	1.65 ± 0.09	n.s.
Grip strength [kg]	31.7 ± 9.27	30.8 ± 8.08	n.s.

Values are given as (means ± SD) or (n [%]). FMI, fat-mass index; FFMI, fat-free mass index; SMM, skeletal muscle mass; PAL, physical activity level; n.s., not significant.

2.8. Statistical Analysis

For all analyses, $p < 0.05$ was considered statistically significant. Student's *t*-test was used to compare intergroup differences and one-way ANOVA (post hoc Tukey test) was used to compare intragroup differences. Normal distribution of data was checked groupwise for each time point by Kolmogorov–Smirnov test. Two outliers of 1-MH from time points d3 and d4.1 were excluded. One sample is missing in the omnivore group on d3. This results in data from $n = 19$ omnivores at d0, d4, d4.1, d5, d6, and d13 and $n = 18$ omnivores at d3 for 3-MH and creatinine. For 1-MH, data from $n = 19$ omnivores at d0, d4, d5, d6, and d13, $n = 18$ omnivores at d4.1 and $n = 17$ omnivores at d3 were analyzed. Finally, data of $n = 16$ vegetarians at baseline was assessed for 3-MH, 1-MH, and creatinine. IBM SPSS Statistics Version 20 (Release 20.0.0) was used for statistical analyses.

3. Results

3.1. Baseline and Intervention Study

In total, 35 participants were enrolled into the study and grouped according to their eating behavior into an omnivorous ($n = 19$) or a vegetarian ($n = 16$) group. Characteristics of both groups are shown in **Table 2**. Both groups were similar regarding age, anthropometric data, body composition, grip strength, energy, and protein intake and PALs.

At baseline, plasma 3-MH, 1-MH, and creatinine concentrations as well as the 1-MH/Crea ratio were higher in omnivores than in vegetarians ($p < 0.05$; **Table 3** and **Figure 2**).

Table 3. Influence of the dietary habit on plasma methylhistidine concentrations and creatinine ratios in omnivores and vegetarians at baseline.

	Omnivores	Vegetarians	*P*
3-MH [μM]	3.03 ± 0.85	2.46 ± 0.59	0.032
1-MH [μM]	4.86 ± 7.11	0.54 ± 0.09	0.016
Creatinine [μM]	80.7 ± 10.9	71.1 ± 9.7	0.010
3-MH/Crea ratio	0.037 ± 0.008	0.035 ± 0.007	n.s.
1-MH/Crea ratio	0.055 ± 0.079	0.008 ± 0.001	0.013

Plasma concentrations of 3-MH, 1-MH, creatinine, and corresponding MH/Crea ratios of omnivores and vegetarians at baseline. Values are given as (means ± SD). n.s., not significant.

Additionally, we observed a positive correlation between 1-MH and meat intake ($r = 0.483$; $p = 0.036$) in omnivores as well as between 3-MH and both skeletal muscle mass ($r = 0.648$; $p = 0.007$) and grip strength ($r = 0.504$; $p = 0.046$) in vegetarians.

Plasma 3-MH and 1-MH concentrations and both MH/Crea ratios declined to a basal level within 3–4 days on a meat-free diet in omnivores ($p < 0.05$; **Figure** 3A,B). Plasma concentrations and MH/Crea ratios in omnivores were similar between d3 and d4 on a meat-free diet and thus may represent endogenously released 3-MH and 1-MH in plasma. Further, 3-MH and 1-MH plasma concentrations and MH/Crea ratios in omnivores on d4 were similar to those in vegetarians at baseline (Figure 3C–F). Plasma creatinine was significantly higher in omnivores on d4 than in vegetarians at baseline ($p = 0.038$; data not shown).

An increase in plasma 3-MH ($p < 0.001$), 1-MH ($p < 0.001$), and both MH/Crea ratios ($p < 0.001$) was found 3 h after white meat intervention (d4.1; **Figure** 4A). The elevated 3-MH concentrations and 3-MH/Crea ratios in plasma declined significantly within 24 h after this intervention (d5). The increased 1-MH concentrations and 1-MH/Crea ratios in plasma fell to basal levels after 48 h (d6). Further, we observed that plasma 3-MH and 3-MH/Crea on d0, d5, and d13 were similar (Figure 4C,E). Regarding plasma 1-MH and 1-MH/Crea we found higher values on d5 compared to d0 and d13 ($p < 0.05$; Figure 4D,F). The relative amount of endogenously released methylhistidines at baseline, d5, and d13 were 79, 82, and 80 for 3-MH (**Table 4**) and 12, 6, and 14 for 1-MH, respectively (data not shown).

3.2. Method Validation

Calibration curves of a standard mixture of 1-MH, 3-MH, and d_3-3-MH in water and plasma extracts showed *linearity* in the range of 0.015–7.5 μM ($r^2 > 0.999$; Table 2). The *tLOD* was estimated to be 6.0 nM and 5.3 nM for 1-MH and 3-MH, respectively. The *LOQ*, defined as the lowest concentration of the calibration curve, was shown to be 15 nM for both analytes. The *matrix effect*, calculated by comparing the slopes obtained from calibration curves in water with those in plasma, was between 102.62% and 111.12% for 3-MH and 1-MH, respectively. *Recovery* values, showing analyte signal loss during sample preparation, varied between 85.95% (1-MH) and 90.52% (3-MH) for low-concentration spiked samples and 107.83% (3-MH) and 112.06% (1-MH) for high-concentration spiked samples. *Intra-assay* and *inter-assay variation coefficients* of 1-MH and 3-MH concentrations in plasma were <10% for both analytes. *Stability* of 1-MH and 3-MH concentrations in plasma extracts varied between 82.98%–101.29% (36 h autosampler at 15 °C), 99.75–107.77% (24 h at −20 °C), and 85.27–104.08% (48 h at −20 °C).

4. Discussion

Our results show that plasma 3-MH concentrations and 3-MH/Crea ratios only slightly increase in omnivores after a defined single white meat intervention. The elevated plasma 3-MH concentrations and 3-MH/Crea ratios significantly

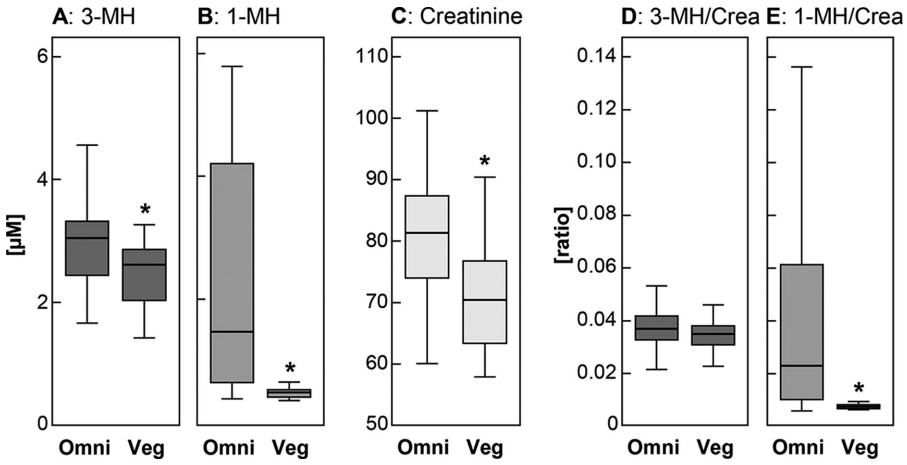


Figure 2. Influence of dietary habits on plasma methylhistidine concentrations and creatinine ratios in omnivores and vegetarians at baseline. A,B) Plasma 3-MH and 1-MH (one extreme value is not shown in the figure but included in the statistical analysis). C) Plasma concentrations of creatinine. D,E) Ratios of 3-MH/Crea and 1-MH/Crea (one extreme value is not shown in the figure but included in the statistical analysis). Values are given as (means ± SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. *$p < 0.05$. Omni, omnivores/omnivore group; Veg, vegetarians/vegetarian group.

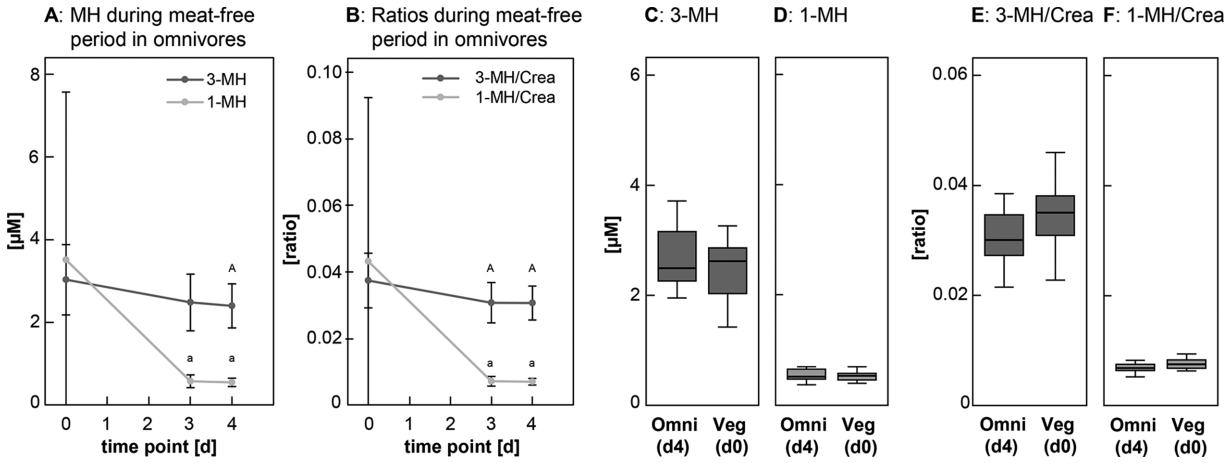


Figure 3. Influence of a meat-free diet on plasma methylhistidine concentrations and creatinine ratios. A) Plasma concentrations of 3-MH and 1-MH (one extreme value at d0 is not shown in the figure but included in the statistical analysis); 3-MH (A = d0, B = d3, C = d4); 1-MH (a = d0, b = d3, c = d4). B) Ratios of 3-MH/Crea and 1-MH/Crea (one extreme value at d0 is not shown in the figure but included in the statistical analysis); 3-MH/Crea (A = d0, B = d3, C = d4); 1-MH/Crea (a = d0, b = d3, c = d4). C,D) Plasma concentrations of 3-MH and 1-MH in omnivores on d4 and in vegetarians at baseline. E,F) Ratios of 3-MH/Crea and 1-MH/Crea in omnivores on d4 and in vegetarians at baseline. Values are given as (means ± SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. A+B: A, a: $p < 0.05$. Omni, omnivores/omnivore group; Veg, vegetarians/vegetarian group.

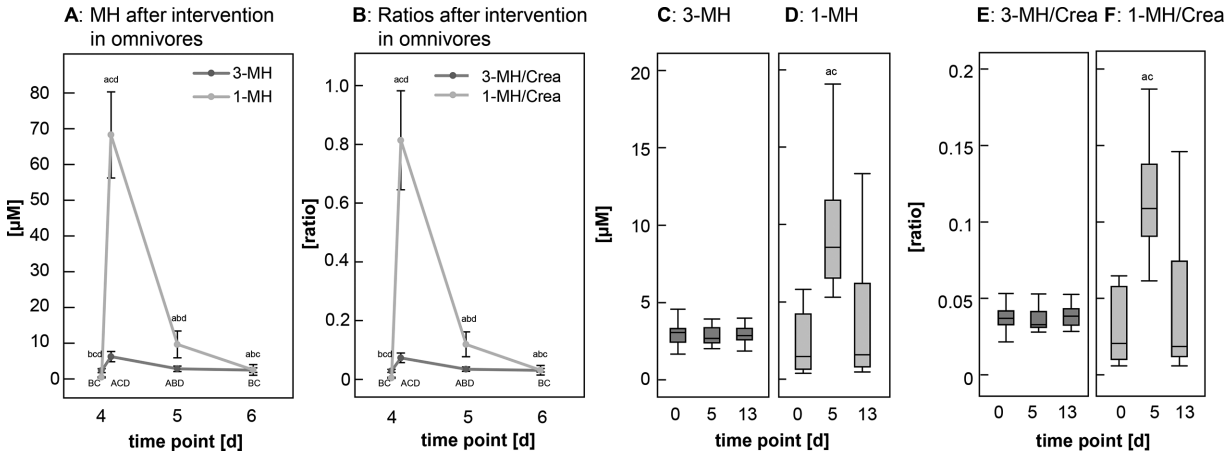


Figure 4. Influence of a single white meat administration and a general meat-containing diet on plasma methylhistidine concentrations and creatinine ratios in omnivores. A) Plasma concentrations of 3-MH and 1-MH; 3-MH (A = d4, B = d4.1, C = d5, D = d6); 1-MH (a = d4, b = d4.1, c = d5, d = d6). B) Ratios of 3-MH/Crea and 1-MH/Crea; 3-MH/Crea (A = d4, B = d4.1, C = d5, D = d6); 1-MH/Crea (a = d4, b = d4.1, c = d5, d = d6). C,D) Plasma concentrations of 3-MH and 1-MH at baseline, d5 and d13; 3-MH (A = d0, B = d5, C = d13); 1-MH (a = d0, b = d5, c = d13). E,F) Ratios of 3-MH/Crea and 1-MH/Crea at baseline, d5, and d13; 3-MH (A = d0, B = d5, C = d13); 1-MH (a = d0, b = d5, c = d13). Values are given as (means ± SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. A+B) repeated measures ANOVA with Bonferroni post hoc test: BC, ACD, ABD, bcd, acd, abd, abc: $p < 0.05$; D+F) ac: $p < 0.05$.

Table 4. Endogenous and exogenous 3-MH in plasma of omnivores.

Time point	d0	d4	d5	d13
3-MH_{total} [% (μM)]	100 (3.03 ± 0.85)	100 (2.39 ± 0.53)	100 (2.92 ± 0.73)	100 (3.00 ± 0.71)
$3\text{-MH}_{endogenous}$ [% (μM)]	**79** (2.39)	**100** (2.39)	**82** (2.39)	**80** (2.39)
$3\text{-MH}_{exogenous}$ [%(μM)]	21 (0.63)	0 (0.00)	18 (0.53)	20 (0.60)

The total absolute concentration of 3-MH at d4 represents endogenous 3-MH, which is used to calculate the exogenous 3-MH amounts on d0, d5, and d13 (μm $3\text{-MH}_{exogenous}$ = μm 3-MH_{total} − μm $3\text{-MH}_{endogenous}$). Total absolute 3-MH concentrations on d0, d4, d5, and d13 are set to 100% and the relative amounts of endogenous and exogenous 3-MH were calculated. Values are given as (% [mean ± SD]) or (% [mean]).

Table 5. Method validation parameters.

Parameter	1-MH	3-MH
Linearity [μM][a]	0.015–7.5 ($r^2 = 0.9993$)	0.015–7.5 ($r^2 = 0.9999$)
tLOD [nM][b]	6.0	5.3
LOQ [nM][c]	15	15
Matrix effect [%][d]	111.12 ± 2.8	102.62 ± 2.8
Intra-assay variation [%][e]	8.5 ± 4.8	4.0 ± 2.2
Inter-assay variation [%][e]	7.5 ± 10.9	6.6 ± 12.7

[a] Standard calibration mixtures measured in triplicate in water and plasma; [b] Repeated injection of LOQ concentration of standard calibration mixtures on three different days followed by using peak areas (1-MH) or analyte-to-IS-peak area ratios (3-MH) and Equation (1) to calculate tLOD; [c] Defined as the lowest concentration of the calibration curve; [d] Comparison of slopes obtained from standard curves in water and plasma; [e] % CV of concentrations of six plasma samples prepared in duplicate and measured in triplicate on three different days.

declined within 24 h. We assume that about 80% of plasma 3-MH is of endogenous origin after an overnight fast of omnivores. Further, we suppose that plasma 1-MH is able to reflect an exogenous uptake of 3-MH in plasma. Furthermore, we found that plasma 1-MH concentrations and 1-MH/Crea ratios are strongly influenced by white meat consumption. Moreover, we observed plasma 3-MH and 1-MH concentrations and both MH/Crea ratios to be higher in omnivores than in vegetarians at baseline.

The higher plasma 3-MH and 1-MH levels in omnivores at baseline reflect the general meat consumption of omnivores which is confirmed by a positive correlation of 1-MH and meat intake. Additionally, we observed a significant positive correlation between 3-MH and both skeletal muscle mass and grip strength in vegetarians showing the association of 3-MH with muscle status.

Methylhistidines are assessed primarily in the urine; however, the sampling of 24 h urine can be prone to errors especially in an elderly study population.[14,26] Therefore, our aim was to measure 3-MH, 1-MH, and creatinine in plasma samples which is more practical, more objective, and not biased by patients' compliance. However, for the interpretation of 3-MH, information on the subjects' previous meat consumption is required since the influence of meat intake on urinary 3-MH excretion is well described.[14,15,17,18,22,27,35] Contrarily, the impact of meat consumption on plasma 3-MH has hardly been investigated. The purpose of our study was therefore to generate novel data on plasma levels of 3-MH, 1-MH, and creatinine regarding dietary influences.

Our findings of elevated plasma 3-MH due to an omnivorous diet and a white meat administration during a meat-free period are in accordance with previous examinations in urine and plasma. Urinary 3-MH excretion was significantly higher in subjects on omnivorous diets and increased due to interventions with different types and amounts of meat during meat-free periods.[18,22,27] On the other hand, urinary 3-MH excretion did not increase after the administration of 100 g of different types of meat.[15] This may be due to lower amounts of meat in comparison to the present study. A dose-dependent increase of 3-MH has previously been shown after the intake of different meats (100 g) or capsules (20 mg, 60 mg, or 120 mg of 3-MH).[36] Additionally, the half-life of 3-MH in plasma was found to be about 12 h.[36] In contrast, another study observed a half-life of only about 130 min after an intravenous injection of ^{14}C-labeled 3-MH.[10]

These findings of meat consumption on 3-MH urinary excretion and plasma levels led to the recommendation not to eat any meat 3 days before sample collection. We found that increased plasma 3-MH and creatinine ratios significantly declined within 24 h after white meat administration. Additionally, we found similar plasma 3-MH and 3-MH/Crea ratios in fasted omnivores and 24 h after white meat administration. Further, plasma 3-MH at d0, d5, and d13 represent about 80% of endogenously released 3-MH in omnivores. We suggest that avoiding meat consumption 3 days before blood sampling may not be necessary. Furthermore, we recommend that participants avoid meat consumption for at least 24 h before blood sampling.

To adhere to strict dietary recommendations for 3 days or to give correct statements on previous meat intake may be difficult, especially in elderly patients. Therefore, we additionally measured 1-MH in plasma to reexamine this recommendation and to assess whether samples can be excluded on the basis of 1-MH values indicating that subjects consumed meat in the previous 24 h. 1-MH is independent of the human muscle metabolism and it is more sensitive to meat consumption, especially to white meat, than 3-MH.[14,17,18] 1-MH has been described as suitable urinary biomarker for meat consumption. We suggest that plasma 1-MH can also be used as a marker for exogenous 3-MH in plasma. Previously, Myint et al. were able to distinguish between omnivores and vegetarians based on urinary 1-MH excretion.[17] Cross et al. found a dose-dependent increase in 1-MH excretion in omnivores on a red meat diet and elevated excretion levels on an omnivorous compared to a lacto-vegetarian diet.[18] Further studies showed that the impact of different meat sources on urinary 1-MH was strong and that an omnivorous diet led to higher urinary 1-MH than 3-MH excretion.[14,15,17] In accordance with these findings, our results show the same impact of dietary habits and white meat consumption on plasma 1-MH. Hence, by the measurement of plasma 1-MH, reflecting general and white meat consumption, data from participants may be excluded or adjusted when assessing muscle protein turnover.

The strong influence of the chicken meat intervention on plasma 1-MH compared to a small influence on 3-MH in this study may have occurred due to varying contents of methylhistidines in different types of meat. White meat, that is, chicken, contains high amounts of 1-MH and anserine, whereas the amount of 3-MH is rather low.[13–16]

Interestingly, we found a low basal level of 1-MH in plasma although neither 1-MH nor anserine is synthesized in human muscle. One explanation could be that anserine is present in non-muscle tissues[16] and thus anserine degradation may lead to low 1-MH plasma concentrations.

Creatinine excretion and serum concentrations have previously been discussed as useful markers for muscle mass,[22–24,37] despite being influenced by meat and protein intake.[25,38] Urinary creatinine was shown to correlate positively with urinary 3-MH excretion ($r > 0.7$).[22,25] Similarly, we found a positive correlation between plasma creatinine and plasma 3-MH in omnivores ($r = 0.622$; $p = 0.004$) and vegetarians ($r = 0.642$; $p = 0.007$). Additionally, plasma creatinine was significantly correlated with skeletal muscle mass ($r = 0.577$; $p = 0.010$) and grip strength ($r = 0.482$;

$p = 0.037$) in omnivores and with skeletal muscle mass ($r = 0.607$; $p = 0.013$) in vegetarians. Interestingly, we observed neither an influence of chicken intake on creatinine nor a correlation of creatinine with meat and protein intake confirming the dependence of plasma creatinine on muscle metabolism.

Previously, urinary MH/Crea ratios have been described to have smaller interindividual variations[26] and this led to the recommendation to prefer the MH/Crea ratios in the assessment of muscle protein breakdown in heterogeneous populations.[25] In our study, the 1-MH/Crea ratio is significantly higher in omnivores than in vegetarians and both ratios are significantly increased after the administration of chicken to omnivores. Increased 3-MH/Crea ratios declined significantly and returned to values in the same range as before the intervention (d4: 0.0306 ± 0.0051 μM vs d5: 0.0362 ± 0.0075 μM) 24 h after white meat intervention. The same was true for 1-MH/Crea. These results lead to the suggestions that plasma 1-MH/Crea ratios can detect short-term white meat consumption and that plasma 3-MH/Crea ratios may depict reliable data concerning muscle protein turnover in participants who did not consume meat in the previous 24 h.

One limitation of our study is that we did not correct for exercise and nutrition during the intervention study. Thus, we cannot exclude our results being biased since there may be an influence of exercise and protein intake on muscle metabolism[39,40] leading to a varying release of 3-MH into the blood and resulting in altered urinary 3-MH and 3-MH/Crea.[39,41,42] Future studies should include participants' physical activity and data on nutrition to evaluate the influence of these factors. Alternatively, further studies may include avoiding exercise and may implement a standardized diet in the study design. In our study, we normalized methylhistidine concentrations to creatinine levels to minimize the influence of muscle mass. Another limitation is that we did not draw blood samples in smaller intervals after the white meat administration to provide more details concerning the bioavailability of methylhistidines from white meat. Our study investigated only young and healthy subjects and thus a real association between plasma 3-MH and muscle degradation as occurring in elderly or in a muscle wasting condition cannot be assessed here. Future studies in elderly populations diagnosed with muscle wasting diseases, sarcopenia or frailty and also regarding dietary influences will be needed to validate the suitability of plasma 3-MH as a biomarker for muscle protein turnover.

In conclusion, here we provide novel data on the impact of dietary habits and white meat intake on plasma levels of 3-MH and 1-MH as well as on MH/Crea ratios in young and healthy omnivores and vegetarians. Our results confirm that white meat intake only has a weak impact on plasma 3-MH but a strong impact on plasma1-MH in omnivores. Hence, we suggest that plasma 1-MH and the 1-MH/Crea can be used both as biomarkers for meat consumption by signaling short-term meat consumption, and to display exogenous 3-MH in plasma. Furthermore, our data suggest that plasma 3-MH and 3-MH/Crea may be used as biomarkers for muscle protein turnover when subjects are on a meat-free diet, but this must be further validated in another study population. Finally, we suggest that blood sampling for 3-MH measurements should at least be performed in subjects which did not consume meat in the previous 24 h.

Abbreviations

1-MH, 1-methylhistidine; 3-MH, 3-methylhistidine; Crea, creatinine; FA, formic acid

Acknowledgements

This work was supported by NutriAct-Competence Cluster Nutrition Research Berlin-Potsdam funded by the Federal Ministry of Education and Research (FKZ: 01EA1408A-G). We thank the Human Study Center of the German Institute of Human Nutrition Potsdam-Rehbruecke, namely the trustee, the examination unit, the data hub personnel, and the participants for the provision of their data, and the head of the HSC, Manuela Bergmann, for the substantial contribution to the study by leading the underlying processes of data generation. We also thank Katrin Sprengel from the Department of Clinical Nutrition of the German Institute of Human Nutrition Potsdam-Rehbruecke for analyzing creatinine. B.K. designed the study, performed the 1-MH, 3-MH analyses, prepared the manuscript, and prepared the figures. C.G. critically discussed the manuscript. T.G. designed the study. D.W. designed and supervised the study and prepared the manuscript. All authors have approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomarkers, intervention studies, meat, methylhistidine, muscle protein turnover

Received: December 22, 2017
Revised: March 8, 2018
Published online: April 19, 2018

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Publication 2

"Associations of Plasma 3-Methylhistidine with Frailty Status in French Cohorts of the FRAILOMIC Initiative"

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J. Clin. Med. 2019, *8*(7), 1010

Own contribution

- Developing the concept for the manuscript
- Literature research
- Proportional sample preparation
- Sample measurement and evaluation
- Statistical analyses
- Preparation of figures and tables
- Preparation of the manuscript

Journal of
Clinical Medicine

MDPI

Article

Associations of Plasma 3-Methylhistidine with Frailty Status in French Cohorts of the FRAILOMIC Initiative

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Received: 12 June 2019; Accepted: 8 July 2019; Published: 10 July 2019

Abstract: Frailty and sarcopenia are characterized by a loss of muscle mass and functionality and are diagnosed mainly by functional tests and imaging parameters. However, more muscle specific biomarkers are needed to improve frailty diagnosis. Plasma 3-methylhistidine (3-MH), as well as the 3-MH-to-creatinine (3-MH/Crea) and 3-MH-to-estimated glomerular filtration rate (3-MH/eGFR) ratios might support the diagnosis of frailty. Therefore, we investigated the cross-sectional associations between plasma 3-MH, 3-MH/Crea and 3-MH/eGFR with the frailty status of community-dwelling individuals (>65 years). 360 participants from two French cohorts of the FRAILOMIC initiative were classified into robust, pre-frail and frail according to Fried's frailty criteria. General linear models as well as bivariate and multiple linear and logistic regression models, which were adjusted for several confounders, were applied to determine associations between biomarkers and frailty status. The present study consisted of 37.8% robust, 43.1% pre-frail and 19.2% frail participants. Frail participants had significantly higher plasma 3-MH, 3-MH/Crea and 3-MH/eGFR ratios than robust individuals, and these biomarkers were positively associated with frailty status. Additionally, the likelihood to be frail was significantly higher for every increase in 3-MH (1.31-fold) and 3-MH/GFR (1.35-fold) quintile after adjusting for confounders. We conclude that 3-MH, 3-MH/Crea and 3-MH/eGFR in plasma might be potential biomarkers to identify frail individuals or those at higher risk to be frail, and we assume that there might be biomarker thresholds to identify these individuals. However, further, especially longitudinal studies are needed.

Keywords: frailty; aging; methylhistidine; biomarker; human study; muscle protein turnover

1. Introduction

Frailty as multifactorial geriatric syndrome, including sarcopenia as a contributor as well as a component [1], is associated with a higher risk for falls, hospitalization, disability, and death [2] and is thus becoming an increasing social and health care burden. Frailty and sarcopenia are both linked to a reduction of muscle quantity (e.g., muscle mass), and muscle quality (e.g., muscle function) [3] due to a catabolic state with an elevated muscle protein turnover [4]. Muscle mass

and functionality are mainly diagnosed using functional tests and imaging parameters, which might have limitations regarding patients' compliance, availability in the clinical routine, costs as well as being time-consuming procedures [2,5,6]. Both frailty and its components (physical performance and function, muscle mass and strength) are associated with numerous biomarkers for inflammation (interleukine-6, C-reactive protein and tumor necrosis factor α) and oxidative stress (protein carbonyls); with hormones (insulin-like growth factor-1, testosterone, dehydroepiandosterone and vitamin D); and with antioxidants (carotenoids and vitamin E) [3,7,8]. However, these biomarkers are not specific for muscle pathology, reflecting more the general impairment of the metabolism or an insufficient nutritional status. Therefore, finding more muscle specific biomarkers for the assessment of muscle protein turnover and frailty is of increasing interest, especially since it was stated that a single biomarker alone is not sufficient for the assessment of frailty and its possible risk [3,7,9]. P3NP (procollagen type III N-terminal peptide) might function as marker for muscle remodeling and muscle mass, however, this was only shown in post-menopausal women and P3NP levels might be influenced by fibrosis of non-skeletal muscle tissues containing collagen [10]. CAF (C-terminal agrin fragment)/Agrin negatively correlates with lean mass, however, this was only shown in men and CAF seems to be specific only for degradation of neuromuscular junctions [11]. Several myokines might act as markers for muscle function or dysfunction [3]; however, all these compounds need further investigation, especially in respect to the frailty syndrome. In addition, a new approach is estimating muscle mass, using an oral administration of deuterated creatine (D3-creatine) and the resulting urinary D3-enrichment of creatinine [12,13]. However, this approach depends on the accessibility of D3-creatine, which might be expensive and challenging for clinical routine, and requires further validation and evaluation in the context of frailty.

Plasma 3-methylhistidine (3-MH; N-tau-methylhistidine) seems to be a potential biomarker to display an elevated muscle protein turnover [14–16]. 3-MH is formed in the muscle by the post-translational methylation of histidine residues in actin and myosin [17–19]. During muscle degradation, 3-MH is released, then not further metabolized, followed by quantitative excretion in the urine [20,21]. Additionally, serum creatinine (Crea) has previously been discussed as useful marker for muscle mass [22,23]. Normalizing 3-MH to Crea concentrations (3-MH/Crea) showed smaller inter-individual variations and therefore, these ratios are recommended for the assessment of muscle protein breakdown in heterogeneous populations [24]. The amount of 3-MH in the muscle and the excretion of 3-MH/Crea remained constant in healthy subjects (20–70 years) [14,25]. Contrarily, 3-MH excretion was increased in muscle wasting diseases [14–16,25,26].

For reliable measures of 3-MH, several factors, both exogenous and endogenous may be limiting. Meat and fish should not be consumed for about 24 hours prior to blood samplings since they can influence 3-MH plasma concentrations in humans [27,28]. Plasma 3-MH concentration depends on the kidney function [20,29,30], which decreases with age [31,32] and can be expressed by the estimated glomerular filtration rate (eGFR) [33]. We suppose that plasma 3-MH concentrations might be influenced by medication intake and multi-morbidity since frailty is related to multi-medication [34–36] and associated with multi-morbidity [37]. Additionally, urinary 3-MH and 3-MH/Crea excretion is influenced by medication intake [38,39]. Currently, plasma 1-methylhistidine (1-MH; N-pi-methylhistidine) and the 1-MH/Crea and 3-MH/1-MH ratios were shown to be useful for displaying meat and fish consumption [28,40,41].

We hypothesize that in the future, plasma 3-MH and 3-MH/Crea may add to clinical and functional tests or to a set of biomarkers for the diagnosis of frailty or for the prediction of frailty risk, when fully validated. The normalization of 3-MH to eGFR (3-MH/eGFR ratio), i.e., considering kidney function, might be a further possibility in this context. Therefore, we aim to investigate (I) the cross-sectional relationship of the following plasma biomarkers with frailty status (robust, pre-frail and frail): 3-MH, 1-MH, Crea and eGFR as well as 3-MH/Crea, 3-MH/eGFR, 1-MH/Crea and 3-MH/1-MH; and (II) the capability of 3-MH, 3-MH/Crea and 3-MH/eGFR to identify frail individuals (a diagnostic biomarker)

or individuals with higher odds to be frail according to biomarker quintiles (a predictive biomarker), in two French cohorts of the FRAILOMIC initiative.

2. Methods

2.1. Study Population and Cohorts

In this study we investigated 360 individuals older than 65 years from two population-based French cohorts on aging, the Bordeaux sample of the Three-City Study (3-C) [42], and the Aging Multidisciplinary Investigation cohort (AMI) [43], from the FRAILOMIC initiative. The FRAILOMIC initiative aims to identify and validate classical and 'omics'-based biomarkers that predict the risk of frailty, detect frailty and assess the progression of frailty [5]. Both cohorts are described in more detail elsewhere [42,43]. Study protocols of 3-C and AMI cohorts were approved by the Ethical Committee of the University Hospital of Kremlin-Bicêtre [42] and by the Ethics Committee of the University Hospital of Bordeaux [43], respectively, according to the principles of the Declaration of Helsinki and all participants signed a written consent.

2.2. Participant Characteristics

For the present analysis we retained the following participants' information: sex, age (years), body mass index (BMI; kg/m^2), cohort affiliation, education (i.e., low (no schooling or primary education) and intermediate-to-high educated (secondary and/or vocational and higher education) [44]), multi-morbidity (≥2 of the following diseases: hypertension, angina pectoris, myocardial infarction, congestive heart failure, stroke, diabetes mellitus, cancer and depression), intake of medication (amount per day), and intake of meat and fish (both servings per day; assessed by a food frequency questionnaire recording the usual intake during the last year [45]), as well as occurrence of frailty criteria. The measures of characteristics are described in more detail elsewhere [42–44]. At the time of blood assessment, these participants were representative of elderly people from Bordeaux [46]. Fasting plasma samples were stored at −80 °C until analysis and shipped on dry ice. Frailty status was defined using Fried's frailty criteria [2]. The harmonization of these criteria across cohorts is described in detail elsewhere [44,47]. Participants were classified into robust, pre-frail and frail. Briefly, participants exhibiting ≥3 of the 5 following criteria were considered as frail: slowness, low energy expenditure, shrinking, weakness, and self-reported exhaustion; while those exhibiting 1 to 2 of these criteria were considered as being pre-frail.

2.3. Biomarker Analyses

Biomarker analyses were conducted on 360 participants with available plasma samples (174 in 3-C and 186 in AMI). Plasma 3-MH and 1-MH concentrations (µmol/L) were determined simultaneously by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS; Acquity Ultra Performance LC system, Quattro Premier XE mass spectrometer, MassLynx Software Version 4.1 (all Waters Corporation, Milford, MA, USA)) according to Kochlik et al. [28].

The eGFR, as an estimation of kidney function, was calculated according to Levey et al. [33], taking into account sex, age and plasma creatinine concentrations of the participants, as follows:

$$\text{Female} : \leq 62\ \mu\text{mol/L creatinine} : \ eGFR = 144 \times (Crea/0.7)^{-0.329} \times 0.993^{Age} \quad (1)$$

$$\text{Female} : > 62\ \mu\text{mol/L creatinine} : \ eGFR = 144 \times (Crea/0.7)^{-1.209} \times 0.993^{Age} \quad (2)$$

$$\text{Male} : \leq 80\ \mu\text{mol/L creatinine} : \ eGFR = 141 \times (Crea/0.9)^{-0.411} \times 0.993^{Age} \quad (3)$$

$$\text{Male} : > 80\ \mu\text{mol/L creatinine} : \ eGFR = 141 \times (Crea/0.9)^{-1.209} \times 0.993^{Age} \quad (4)$$

Four ratios were calculated: 3-MH/Crea, 1-MH/Crea, 3-MH/eGFR and 3-MH/1-MH.

2.4. Statistical Analyses

Demographic characteristics are described using means ± standard deviation (SD) for continuous variables and frequencies (% (n)) for categorical variables. Pearson's chi-squared test (categorical variables) and general linear models (GLM; continuous variables) assessed differences in characteristics between frailty groups.

When necessary, biomarker concentrations and ratios were logarithmically (Ln) transformed to achieve normal distribution and are described by geometric means with 95% confidence intervals (95% CI). Differences in biomarker concentrations and ratios between the three frailty groups were assessed by simple GLMs. Bivariate correlation analyses (Pearson correlation coefficient *r*) between each biomarker and age, BMI, intake of medication, meat and fish were performed (Supplementary Table S1).

Furthermore, pre-frailty and frailty as a factor (i.e., independent variable) for the biomarkers 3-MH and its ratios to Crea, eGFR and 1-MH (i.e., dependent variables) were assessed in bivariate (simple) and multiple linear regression models adjusted for cohort, sex, age, BMI, education, multi-morbidity, and medication, meat and fish intake. Multivariable-adjusted linear regression models are: *Model 1*: frailty status, cohort, sex, age, BMI and education; *Model 2*: frailty status, cohort, sex, age, BMI, education, multi-morbidity, and medication, meat and fish intake.

Additionally, simple (odds ratio (OR) with 95% CI) and multinomial (adjusted OR (AOR) with 95% CI) logistic regression analyses were applied to assess associations of biomarker quintiles (i.e., independent categorical variables; biomarker quintiles shown in Supplementary Table S2) with frailty status (i.e., dependent variable; robust as reference group). Multivariable-adjusted logistic regression models are: *Model 1*: biomarker quintile, cohort, sex, age, BMI and education; *Model 2*: biomarker quintile, cohort, sex, age, BMI, education, multi-morbidity, and intake of medication, meat and fish. These logistic regression analyses were performed, to obtain ORs and AORs to be rather frail or pre-frail compared robust for each increase in biomarker quintile.

Statistically significant differences were considered present at $p < 0.05$. All statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA; Version 20.0.0). For figure preparation, Microsoft PowerPoint was additionally used.

3. Results

The present study included 360 participants with a mean age of 78.8 ± 6.4 years and 49.4% being females, 51.7% being low educated, 44.6% consuming meat at least daily and 43.7% consuming fish at least twice or thrice a week (Table 1). Frailty status was distributed as follows: 37.8% were robust, 43.1% were pre-frail and 19.2% were frail. Frail participants were significantly older, more often females, more likely to be obese, low educated, and reported consuming more medications than robust participants. Additionally, frailty prevalence according to cohort affiliation and intake of meat and fish are shown in Table 1, and prevalence of frailty criteria among participants are shown in Supplementary Table S3.

Frail participants had significantly higher plasma 3-MH concentrations than robust participants, and had significantly higher 3-MH/Crea ratios compared to robust and pre-frail participants in simple GLMs (Table 2 and Supplementary Figure S1). Frail and pre-frail participants had significantly higher 3-MH/eGFR ratios (Table 2 and Supplementary Figure S1) and had significantly lower eGFRs than robust participants (Table 2). For 1-MH, Crea, 1-MH/Crea and 3-MH/1-MH no differences between the three groups were found (Table 2).

Table 1. Sociodemographic, clinical and dietary characteristics by frailty status among 360 participants from the 3-City Bordeaux and AMI cohorts involved in the FRAILOMIC initiative.

Characteristic	Total	Robust	Pre-Frail	Frail	*p*-Value
N, % (*n*)	100 (360)	37.8 (136)	43.1 (155)	19.2 (69)	
Sex, % (*n*)					<0.001 #
Female	49.4 (178)	34.6 (47)	56.1 (87)	63.8 (44)	
Male	50.6 (182)	65.74 (89)	43.9 (68)	36.2 (25)	
Age, years	78.8 ± 6.4	75.9 ± 6.0 [a]	79.6 ± 5.8 [b]	83.0 ± 5.8 [c]	<0.001
BMI, kg/m^2	27.0 ± 4.5	26.7 ± 3.1	27.0 ± 4.4	27.7 ± 6.6	0.390
BMI groups, % (n)					0.003 #
<25 kg/m^2	33.5 (119)	28.4 (38)	35.7 (55)	38.8 (26)	
25–29.9 kg/m^2	44.8 (159)	56.0 (75)	42..2 (65)	28.4 (19)	
≥30 kg/m^2	21.7 (77)	15.7 (21)	22.1 (34)	32.8 (22)	
Cohort, % (n)					<0.001 #
3-C	48.3 (174)	27.9 (38)	63.9 (99)	56.6 (37)	
AMI	51.7 (186)	72.1 (98)	36.1 (56)	46.6 (32)	
Education, % (n)					0.036 #
low	51.7 (186)	58.1 (79)	43.9 (68)	56.5 (39)	
intermediate-high	48.3 (174)	41.9 (57)	56.1 (87)	43.5 (30)	
Medication (n/day)	5.38 ± 3.30	4.39 ± 2.88 [a]	5.34 ± 3.08 [b]	7.43 ± 3.64 [c]	<0.001
Meat servings, % (n)					0.001#
≤3 per week	18.2 (64)	12.5 (17)	18.3 (28)	30.2 (19)	
4–6 per week	37.2 (131)	30.9 (42)	44.4 (68)	33.3 (21)	
≥7 per week	44.6 (157)	56.6 (77)	37.3 (57)	36.5 (23)	
Fish servings, % (*n*)					0.201 #
<1 per week	15.1 (53)	13.2 (18)	13.9 (21)	22.2 (14)	
1 per week	38.3 (134)	45.6 (62)	35.8 (54)	28.6 (18)	
2–3 per week	43.7 (153)	39.0 (53)	47.7 (72)	44.4 (28)	
≥4 per week	2.9 (10)	2.2 (3)	2.6 (4)	4.8 (3)	

Data are shown as mean ± SD or % (n). BMI = body mass index. BMI: N = 355, n = 134 robust, n = 154 pre-frail, n = 67 frail; Meat servings: N=352, n=136 robust, n=153 pre-frail, n=63 frail; Fish servings: N = 350, n = 136 robust, n = 151 pre-frail, n = 63 frail. # Differences between frailty groups for categorical variables determined by Pearson's chi-squared test, $p < 0.05$. [a, b, c] Differences between frailty groups for continuous variables determined by simple GLM, $p < 0.05$.

Table 2. Plasma biomarker concentrations [µmol/L] and ratios by frailty status among 360 participants of the 3-City Bordeaux and AMI cohorts involved in the FRAILOMIC initiative.

Biomarker	Robust (*n* = 136)	Pre-Frail (*n* = 155)	Frail (*n* = 69)	*p*-Value
3-MH	4.72 (4.40; 5.07) [a]	5.16 (4.82; 5.52) [a,b]	5.72 (5.18; 6.32) [b]	0.006
1-MH	5.28 (4.46; 6.25)	5.42 (4.64; 6.35)	5.57 (4.39; 7.06)	0.930
Crea	86.70 (80.52; 92.89)	94.42 (88.62; 100.21)	97.70 (88.88; 106.52)	0.074
3-MH/Crea	0.059 (0.055; 0.063) [a]	0.060 (0.056; 0.064) [a]	0.067 (0.063; 0.071) [b]	0.011
1-MH/Crea	0.063 (0.054; 0.074)	0.060 (0.052; 0.070)	0.061 (0.048; 0.076)	0.899
eGFR	70.54 (67.67; 73.41) [a]	60.96 (58.27; 63.65) [b]	58.42 (54.33; 62.52) [b]	<0.001
3-MH/eGFR	0.069 (0.062; 0.077) [a]	0.089 (0.080; 0.099) [b]	0.102 (0.087; 0.120) [b]	<0.001
3-MH/1-MH	0.894 (0.763; 1.048)	0.950 (0.819; 1.103)	1.026 (0.819; 1.287)	0.601

Results are shown as geometric mean (95% CI). eGFR in (ml/min/1.73 m^2). Crea, 3-MH/Crea, 1-MH/Crea, eGFR and 3-MH/eGFR: N = 359, n = 136 robust, n = 155 pre-frail, n = 68 frail. [a, b] Differences between frailty groups determined by simple GLM, $p < 0.05$.

Simple linear regression models (Table 3) showed significant positive associations of 3-MH (β = 0.096 (95% CI = 0.035; 0.157)), 3-MH/Crea (β = 0.004 (0.001; 0.007)) and 3-MH/eGFR (β = 0.196 (0.100; 0.292) with frailty. Significant associations were confirmed for 3-MH and 3-MH/eGFR after

adjusting for cohort, sex, age, BMI, education, multi-morbidity, and intake of medication, meat and fish (*Models 1* and *2*); and for 3-MH/Crea after adjusting for cohort, sex, age, BMI and education (*Model 1*). Additionally, 3-MH/eGFR was significantly positively associated with pre-frailty (β = 0.248 (0.103; 0.393) in the simple but not in the adjusted models. No associations with pre-frailty and frailty were found for 3-MH/1-MH ratio at all (Table 3).

Table 3. Associations of plasma 3-MH concentrations and, 3-MH/Crea, 3-MH/eGFR and 3-MH/1-MH ratios with pre-frailty and frailty (robust as reference group) assessed by multiple linear regression models.

Biomarker	Pre-Frail (*n* = 155) vs. Robust (*n* = 136)		Frail (*n* = 69) vs. Robust (*n* = 136)	
	B (95% CI)	*p*-Value	B (95% CI)	*p*-Value
3-MH	0.089 (−0.001; 0.178)	*0.053*	0.096 (0.035; 0.157)	0.002
Model 1	0.068 (−0.031; 0.167)	*0.180*	0.107 (0.033; 0.181)	0.005
Model 2	0.066 (−0.034; 0.166)	*0.194*	0.083 (0.005; 0.162)	0.038
3-MH/Crea	0.001 (−0.003; 0.005)	*0.578*	0.004 (0.001; 0.007)	0.006
Model 1	0.001 (−0.004; 0.005)	*0.795*	0.004 (0.000; 0.008)	0.029
Model 2	0.001 (−0.004; 0.005)	*0.756*	0.003 (−0.001; 0.007)	0.092
3-MH/eGFR	0.248 (0.103; 0.393)	*0.001*	0.196 (0.100; 0.292)	<0.001
Model 1	0.137 (−0.020; 0.294)	*0.087*	0.170 (0.055; 0.285)	0.004
Model 2	0.130 (−0.028; 0.287)	*0.106*	0.136 (0.013; 0.260)	0.031
3-MH/1-MH	0.061 (−0.159; 0.281)	*0.586*	0.069 (−0.068; 0.206)	0.320
Model 1	0.128 (−0.117; 0.373)	*0.304*	0.046 (−0.123; 0.215)	0.593
Model 2	0.146 (−0.098; 0.390)	*0.239*	0.052 (−0.129; 0.234)	0.570

Simple: biomarker as the dependent variable and frailty status as covariate; 3-MH/Crea, 3-MH/eGFR: $N = 359$, $n = 136$ robust, $n = 155$ pre-frail, $n = 68$ frail. *Model 1*: biomarker as the dependent variable and frailty status, cohort, sex, age, BMI and education as covariates; 3-MH, 3-MH/1-MH: $N = 355$, $n = 134$ robust, $n = 154$ pre-frail, $n = 67$ frail; 3-MH/Crea, 3-MH/eGFR: $N = 354$, $n = 134$ robust, $n = 154$ pre-frail, $n = 66$ frail. *Model 2*: biomarker as dependent variable and frailty status, cohort, sex, age, BMI, education, multi-morbidity, and intake of medications, meat and fish as covariates; $N = 346$, $n = 134$ robust, $n = 150$ pre-frail, $n = 62$ frail. Statistical significant associations at $p < 0.05$.

The results were confirmed by subsequent multiple logistic regression analyses (Table 4). The likelihood to be frail compared to be robust was significantly higher for every increase in quintile of 3-MH (OR = 1.39 (95% CI = 1.12; 1.71)), 3-MH/Crea (1.24 (1.01; 1.53)) and 3-MH/eGFR (1.56 (1.25; 1.94)) in simple logistic regression models. Positive associations remained significant for 3-MH (AOR = 1.31 (1.01; 1.70)) and 3-MH/eGFR (1.35 (1.03; 1.77)) after adjusting for cohort, sex, age, BMI, education, multi-morbidity, and intake of medication, meat and fish (*Models 1* and *2*). No associations of increasing biomarker quintiles with the likelihood to be pre-frail were found, except for 3-MH/eGFR (OR = 1.30 (1.10; 1.54)) in the simple logistic regression.

Table 4. Likelihood (simple and multivariable-adjusted odds ratios with 95 % CI) to be pre-frail and frail (robust as control group) with increasing biomarker quintiles.

Biomarker Quintiles	Pre-Frail (n = 155) vs. Robust (n = 136)		Frail (n = 69) vs. Robust (n = 136)	
	OR (95% CI)	p-Value	OR (95% CI)	p-Value
3-MH	1.11 (0.95; 1.31)	*0.198*	1.39 (1.12; 1.71)	0.003
Model 1	1.07 (0.89; 1.28)	*0.494*	1.31 (1.03; 1.67)	0.029
Model 2	1.09 (0.90; 1.32)	*0.382*	1.31 (1.01; 1.70)	0.046
3-MH/Crea	1.02 (0.86; 1.20)	*0.855*	1.24 (1.01; 1.53)	0.043
Model 1	1.01 (0.83; 1.22)	*0.942*	1.18 (0.92; 1.51)	0.185
Model 2	1.02 (0.84; 1.24)	*0.857*	1.21 (0.93; 1.58)	0.161
3-MH/eGFR	1.30 (1.10; 1.54)	*0.002*	1.56 (1.25; 1.94)	<0.001
Model 1	1.16 (0.96; 1.41)	*0.127*	1.33 (1.04; 1.71)	0.025
Model 2	1.18 (0.97; 1.43)	*0.109*	1.35 (1.03; 1.77)	0.030
3-MH/1-MH	1.04 (0.89; 1.23)	*0.603*	1.10 (0.90; 1.35)	0.365
Model 1	1.10 (0.92; 1.32)	*0.292*	1.13 (0.89; 1.44)	0.310
Model 2	1.11 (0.92; 1.34)	*0.269*	1.17 (0.90; 1.51)	0.246

Simple: frailty status as dependent variable and biomarker quintiles as covariate; 3-MH/Crea, 3-MH/eGFR: N = 359, n = 136 robust, n = 155 pre-frail, n = 68 frail. *Model 1*: frailty status as dependent variable and biomarker quintiles, cohort, sex, age, BMI and education as covariates; 3-MH, 3-MH/1-MH: N = 355, n = 134 robust, n = 154 pre-frail, n = 67 frail; 3-MH/Crea, 3-MH/eGFR: N = 354, n = 134 robust, n = 154 pre-frail, n = 66 frail. *Model 2*: frailty status as dependent variable and biomarker quintiles, cohort, sex, age, BMI, education, multi-morbidity, and intake of medications, meat and fish as covariates; N = 346, n = 134 robust, n = 150 pre-frail, n = 62 frail. Statistical significant odds ratios (95% CI) at $p < 0.05$.

4. Discussion

In the present study, we aimed to determine: (I) the cross-sectional associations of plasma 3-MH concentrations, and 3-MH/Crea and 3-MH/eGFR ratios with frailty status, and (II) the potential capability of these biomarkers to identify frail individuals or individuals with a higher likelihood to be frail in a French population (≥65 years). Here we provide novel data showing differences in 3-MH, 3-MH/Crea and 3-MH/eGFR between robust, pre-frail and frail participants, as well as cross-sectional associations of these biomarkers with the frailty status of participants.

The distribution of the different frailty status in our population is similar to previously published prevalence of pre-frail (42.3%) and frail (17.0%) community-dwelling subjects (>65 years) from ten European countries [48]. Additionally, our results are in accordance with previous studies showing older age, female sex, and lower education as well as lower kidney function (shown by lower eGFR) to be positively associated with higher frailty prevalence and incidence [2,34–36,48–52].

Current research focuses on potential biomarkers that are linked to different pathologic muscle states and the main objective of our analyses was to find relevant biomarkers that might help to identify frail individuals. Plasma 3-MH and different 3-MH ratios might be potential biomarkers for the assessment of frailty due to its muscle-specific metabolism [17,20], and the fact that muscle protein degradation is the only endogenous source for 3-MH in human plasma. However, investigations on plasma 3-MH, especially in frail populations, are lacking.

In the present study, higher plasma 3-MH and 3-MH/Crea were found in frail and pre-frail participants compared to robust participants, assuming an elevated muscle protein turnover in these participants. Due to its renal excretion, the plasma 3-MH concentration is linked to the kidney function [20,29,30]. In the present study, we normalized plasma 3-MH concentrations to the eGFR (3-MH/eGFR ratio) to surpass kidney function limitations. We observed that frail participants are more likely to exhibit higher 3-MH/eGFR ratios compared to robust participants. Thus, we assume a potential use of 3-MH, 3-MH/Crea and 3-MH/eGFR in the assessment of frail individuals. However, this has to be evaluated and confirmed in future studies.

For reliable 3-MH analyses, there is a recommendation to not consume meat (and fish), as those products contain 3-MH, three days prior to blood samplings. In contrast, we previously showed that

3-MH and 3-MH/Crea in plasma may be used as biomarkers if subjects stay meat-free for 24 h prior to blood sampling [28]. Additionally, plasma 1-MH, 1-MH/Crea and 3-MH/1-MH were previously shown as biomarkers for meat consumption [28,40,41]. In the present study, 1-MH, 1-MH/Crea and 3-MH/1-MH were similar between the three frailty groups and furthermore, there was only a weak positive correlation of 1-MH with fish intake and no correlations between 3-MH, 3-MH/Crea and 3-MH/eGFR, and meat or fish intake (Supplementary Table S1). Thus, if there was an impact of meat or fish intake on plasma 3-MH and the corresponding 3-MH ratios in our participants, this impact seems to be similar for all three frailty groups. Nevertheless, we controlled for meat and fish intake in the statistical analyses to consider this potential confounder.

Plasma 3-MH concentrations might also be influenced by medication intake and multi-morbidity of the participants, although, to the best of our knowledge, the impact of medication intake and multi-morbidity on plasma 3-MH is not known. Multi-medication was associated with a higher prevalence of frailty and higher risk for frailty in older adults [34–36], although the causality is not clear. Thorough investigations on the role of medication intake and of different drugs on the potential biomarkers should be investigated in further studies but were out of scope in this study. In the present study, we controlled for medication intake and multi-morbidity in the statistical analyses in regard of possible influences on plasma 3-MH as well as kidney function.

We are able to show significant positive associations between the biomarkers 3-MH, 3-MH/Crea and 3-MH/eGFR and the frailty status in bivariate and multivariate linear regression models. Additionally, significantly higher likelihoods to be frail for higher 3-MH and 3-MH/eGFR quintiles were observed in logistic regression models, after adjusting for multi-morbidity and medication as well as meat and fish intake. Thus, we suggest that 3-MH and 3-MH/eGFR might be suitable biomarkers for the assessment of frail individuals or individuals that have higher odds to be frail. Furthermore, we assume that there might be threshold concentrations and ratios for these biomarkers, for identifying frail individuals or individuals at risk for frailty. However, this has to be evaluated in further, especially longitudinal studies. Additionally, the biomarkers should be tested in combination with other parameters regarding frailty and muscle pathology to evaluate if a set of biomarkers is able to reliably assess frailty.

There are some limitations of our study. First, no conclusion on causality is possible because of the cross-sectional design. Longitudinal studies are needed to further investigate the potential of these biomarkers in the prediction or diagnosis of frailty. Second, data on meat and fish consumption were only provided by food frequency questionnaires; whereas 24h recalls, dietary protocols and, as was previously shown, determinations of some metabolites in urine [53] would more precisely reflect meat and fish intake, and thus the dietary impact on the potential biomarkers. Third, kidney function was calculated as eGFR by using creatinine concentrations, age and sex, which provides only an estimation of kidney function. Furthermore, information on kidney diseases was missing, and should be considered in further studies. Fourth, multi-medication may reflect a general unhealthy status of the participants with multiple comorbidities possibly affecting frailty development, and thus, may reflect the multifactorial pathology of frailty and represents another limitation in the use of 3-MH and its different ratios.

The main strength of our study is that we were the first to assess associations of plasma 3-MH and 3-MH ratios in a cohort, including a frailty classification into robust, pre-frail and frail by harmonized frailty criteria used in the two cohorts. Furthermore, we used a broad spectrum of parameters, including demographic and nutritional data as well as multi-morbidity and medication intake to perform analyses in the context of frailty and possible associations with 3-MH and its ratios as potential biomarkers. Analyzing plasma 3-MH and 1-MH by the same, trained persons within one laboratory, which limits possible variances related to operators, methods and analytical instruments, is a further strength of this study.

5. Conclusions

In the present study, we show for the first time, that higher plasma 3-MH concentrations as well as higher 3-MH/Crea and 3-MH/eGFR ratios are positively associated with frailty, even after adjusting for several confounders, in two French cohorts of adults aged 65 years and older. We therefore conclude that 3-MH, 3-MH/Crea and 3-MH/eGFR in plasma might be potential biomarkers to identify frail individuals or individuals with higher odds to be frail. Furthermore, we suggest that there might be threshold 3-MH concentrations and 3-MH/eGFR ratios by which frail individuals or those at risk for frailty can be identified. These thresholds should be determined in different cohorts, and all results need to be evaluated in further studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/7/1010/s1, Figure S1: (A) plasma 3-methylhistidine concentrations, (B) 3-methylhistidine-to-creatinine ratios and (C) 3-methylhistidine-to-eGFR ratios by frailty status among 360 participants of the 3-City Bordeaux and AMI cohorts involved in the FRAILOMIC initiative, Table S1: Correlations (Pearson correlation coefficient r) between plasma biomarker concentrations (µmol/L) or ratios and study characteristics among 360 participants from the 3-City Bordeaux and AMI cohorts involved in the FRAILOMIC initiative, Table S2: Quintiles of 3-MH concentrations and 3-MH/Crea, 3-MH/eGFR and 3-MH/1-MH ratios; Table S3: Prevalence of frailty criteria among 360 participants from the 3-City Bordeaux and AMI cohorts involved in the FRAILOMIC initiative.

Author Contributions: The following contributions were made by the authors: conceptualization, B.K., T.G. and D.W., data formal analysis, J.T., investigation, B.K., K.P., and C.F.; methodology, B.K. and W.S.; writing—original draft preparation, B.K. and D.W.; writing—review and editing, T.G., L.R.-M., W.S., K.P. and C.F.; project administration and funding acquisition, L.R.-M., K.P. and C.F.

Funding: This work was supported by the European Union's Seventh Framework Programme (FP7; grant number 305483, FRAILOMIC Project). The 3-C Study is conducted under a partnership agreement between the Institut National de la Santé et de la Recherche Médicale (INSERM), Victor Segalen – Bordeaux2 University and Sanofi-Synthélabo. Fondation pour la Recherche Médicale funded the preparation and beginning of the study. The 3-C Study is sponsored by the Caisse Nationale Maladie des Travailleurs Salariés, Direction Générale de la Santé, Conseils Régionaux of Aquitaine and Bourgogne, Fondation de France, Ministry of Research-INSERM Program Cohortes et collections de données biologiques, Fondation Plan Alzheimer (FCS 2009-2012), and Caisse Nationale pour la Solidarité et l'Autonomie (CNSA) and "Programme Longévité et vieillissement" (COGICARE 07-LVIE 003 01). The AMI project was funded by AGRICA (CAMARCA, CRCCA, CCPMA PREVOYANCE, CPCEA, AGRI PREVOYANCE), la Mutualité Sociale Agricole (MSA) de Gironde, la Caisse Centrale de la Mutualité Sociale Agricole (CCMSA).

Conflicts of Interest: Catherine Féart received fees for conferences from Danone Research and Nutricia not related to the present work. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. No further conflicts of interest are declared.

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Publication 3

"Associations of fat-soluble micronutrients and redox biomarkers with frailty status in the FRAILOMIC Initiative"

Bastian Kochlik, Wolfgang Stuetz, Karine Pérès, Sophie Pilleron, Catherine Féart, Francisco José García García, Stefania Bandinelli, David Gomez-Cabrero, Leocadio Rodriguez-Mañas, Tilman Grune and Daniela Weber

J Cachexia Sarcopenia Muscle. 2019

Own contribution

- Developing the concept for the manuscript
- Literature research
- Statistical analyses
- Preparation of figures and tables
- Preparation of the manuscript

ORIGINAL ARTICLE

Journal of Cachexia, Sarcopenia and Muscle (2019)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/jcsm.12479

Associations of fat-soluble micronutrients and redox biomarkers with frailty status in the FRAILOMIC initiative

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Abstract

Background A poor fat-soluble micronutrient (FMN) and a high oxidative stress status are associated with frailty. Our aim was to determine the cross-sectional association of FMNs and oxidative stress biomarkers [protein carbonyls (PrCarb) and 3-nitrotyrosine] with the frailty status in participants older than 65 years.

Methods Plasma levels of vitamins A (retinol), D_3, E (α-tocopherol and γ-tocopherol) and carotenoids (α-carotene and β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin), PrCarb, and 3-nitrotyrosine were measured in 1450 individuals of the FRAILOMIC initiative. Participants were classified into robust, pre-frail, and frail using Fried's frailty criteria. Associations between biomarkers and frailty status were assessed by general linear and logistic regression models, both adjusted for cohort, season of blood sampling, gender, age, height, weight, and smoking.

Results Robust participants had significantly higher vitamin D_3 and lutein/zeaxanthin concentrations than pre-frail and frail subjects; had significantly higher γ-tocopherol, α-carotene, β-carotene, lycopene, and β-cryptoxanthin concentrations than frail subjects, and had significantly lower PrCarb concentrations than frail participants in multivariate linear models. Frail subjects were more likely to be in the lowest than in the highest tertile for vitamin D_3 (adjusted odds ratio: 2.15; 95% confidence interval: 1.42–3.26), α-tocopherol (2.12; 1.39–3.24), α-carotene (1.69; 1.00–2.88), β-carotene (1.84; 1.13–2.99), lycopene (1.94; 1.24–3.05), lutein/zeaxanthin (3.60; 2.34–5.53), and β-cryptoxanthin (3.02; 1.95–4.69) and were more likely to be in the highest than in the lowest tertile for PrCarb (2.86; 1.82–4.49) than robust subjects in multivariate regression models.

Conclusions Our study indicates that both low FMN and high PrCarb concentrations are associated with pre-frailty and frailty.

Keywords Fat-soluble micronutrients; Carotenoids; Frail; Protein carbonyls; 3-Nitrotyrosine

Received: 22 January 2019; Accepted: 24 June 2019
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Introduction

Frailty, a geriatric syndrome caused by an age-related dynamic process affecting multiple physiological systems[1,2], is associated with a higher risk for falls, hospitalization, disability, and death[3] and its prevalence increases with age and is more common in women.[3–6] Age-associated oxidative stress (OS) and impairments in redox homeostasis as well as

impairments in muscle structure, function, and performance are key factors in the development of frailty.[7–9] Fortunately, frailty might be reversed by exercise and decelerated by nutritional interventions.[10]

Higher fruit and vegetable consumption and higher adherence to a Mediterranean diet were associated with a lower risk for frailty in older individuals.[11–13] Additionally, a suboptimal vitamin and carotenoid intake and/or status as well as a micronutrient pattern low in vitamins A and E were associated with a higher prevalence and risk for frailty.[14–17] Furthermore, a suboptimal vitamin D (VD) status was shown to be related with low physical activity, weakness, and slowness, which are main constituents of the frailty syndrome,[18,19] with reduced muscle mass and poor physical performance in frail subjects[20] and with a higher prevalence and incidence of frailty.[19,21] Moreover, VD is linked to redox homeostasis as shown in both VD deficient rat muscles and VD-treated murine myoblast C2C12 cells.[22]

Some fat-soluble micronutrients (FMN) can counteract OS, which is associated with several age-related diseases and the aging process itself. OS can be monitored by biomarkers such as protein carbonyls (PrCarb) and 3-nitrotyrosine (3-NT),[23–25] and some OS biomarkers have been shown to be elevated in frail subjects.[26] Both higher OS and lower antioxidant parameters are associated with frailty.[27]

To the best of our knowledge, no study explored the association of frailty with FMN and OS biomarkers simultaneously in a large cohort so far. We therefore investigated the cross-sectional relationship of plasma vitamins A, D_3, E, carotenoids, PrCarb, and 3-NT with frailty status (robust, pre-frail, and frail) of participants aged >65 years in the European FRAILOMIC initiative.

Materials and methods

Study population and cohorts

In this study, we investigated 1450 individuals out of 1636 participants from the FRAILOMIC database by excluding subjects with missing values for frailty status (n = 114) and VD_3 (n = 74). The FRAILOMIC initiative aims to identify and validate classical and 'omics'-based biomarkers that predict the risk of frailty, detect frailty, and assess the progression of frailty.[28] Participants of the FRAILOMIC initiative come from four population-based European cohorts of older adults: the Bordeaux sample of the Three-City Study (France),[29] the Aging Multidisciplinary Investigation cohort (Gironde, France),[30] the Toledo Study for Healthy Aging (TSHA, Toledo, Spain),[31] and the Invecchiare in Chianti (InCHIANTI) study (Chianti geographical area, Tuscany, Italy).[32] These cohorts were described more in detail elsewhere.[14] Study protocols of all cohorts were approved by Ethical Committees according to the principles of the Declaration of Helsinki and all participants signed a written consent. Three-City Study, Aging Multidisciplinary Investigation, TSHA, and InCHIANTI were approved by the Ethical Committee of the University Hospital of Kremlin-Bicêtre,[29] Ethics Committee of the University Hospital of Bordeaux,[30] Clinical Research Ethics Committee of the University Hospital of Toledo,[31] and Ethical Committee of the Italian National Research Council on Aging,[32] respectively.

Frailty classification

Participants were classified into robust, pre-frail, and frail using criteria by Fried *et al.*[3] The harmonization of criteria across cohorts was described in detail elsewhere.[14,31] Briefly, participants exhibiting ≥3 of the five following criteria were considered as frail: slowness, low energy expenditure, shrinking, weakness, and self-reported exhaustion; while those exhibiting 1 to 2 of these criteria were considered as pre-frail.

Participant characteristics

Participants' information included gender, age (years), weight (kg), height (cm), body mass index (BMI; kg/m^2), smoking (current smoker), and global cognitive performance (Mini-Mental State Examination). The assessment of characteristics is described elsewhere.[14,29–32]

Biomarker analyses

All analyses were carried out at the Department of Nutritional Toxicology (University of Jena, Germany) between 2010 and 2013.

Vitamin D_3 in plasma samples was measured by the high-performance liquid chromatography (HPLC) method described by Pilleron *et al.*[14] VD_3 was detected in all samples; in contrary, only 20/1430 samples (1.4%) revealed values above the limit of detection for VD_2. Therefore, only VD_3 is described and included in the statistical analyses. Analysis of retinol, α-tocopherol and γ-tocopherol, α-carotene and β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin in plasma samples was performed by the HPLC method described by Stuetz *et al.*[33] and Weber *et al.*[34] PrCarbs and protein bound 3-NT in plasma samples were measured by non-commercial in-house ELISA methods as described by Weber *et al.*[34,35]

Statistical analyses

Demographic characteristics are described using means ± standard deviation for continuous variables (age, weight, height, BMI, and Mini-Mental State Examination) and frequencies (%) for categorical variables (gender, frailty status,

DOI: 10.1002/jcsm.12479

and smoking). Differences in characteristics between frailty groups and between cohorts were assessed by general linear models (GLMs; continuous variables) and Pearson's χ^2 test (categorical variables). When necessary, biomarker concentrations were transformed to achieve normal distribution using logarithmic (LN) transformation and are described by geometric means with 95% confidence intervals. Differences of biomarkers between frailty groups were assessed by simple (frailty status as only factor) and adjusted (covariates included cohort, season of blood sampling, gender, age, height, weight, and smoking) GLMs. Additionally, simple [odds ratio (OR); frailty status as only factor] and multiple adjusted [adjusted OR (AOR); covariates included cohort, season of blood sampling, gender, age, height, weight, and smoking] logistic regression analysis using tertiles (Supporting Information, *Table* S1) of FMN and OS biomarkers were applied: ORs and AORs of the lowest tertile and the median tertile vs. the highest tertiles for FMN and, vice versa for OS markers, in pre-frail and frail compared with control groups were calculated. For a clearer presentation of the results, ORs and AORs are not shown for median vs. highest tertiles for FMN, and vice versa for OS biomarkers. Statistically significant differences were considered to be present at $P < 0.05$. All statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA; Version 20.0.0). Figures were prepared by using SPSS Version 20.0.0 and Microsoft Office Power Point 2007.

Results

Study characteristics for both the total sample and the three frailty groups are shown in *Table* 1. In our study, 41.7% and 22.1% of the participants were pre-frail and frail, respectively, and 65.7% of the frail participants were women. Frail participants (81.4 ± 6.3 years) were significantly older than pre-frail (78.0 ± 6.0 years) and robust participants (74.6 ± 5.9 years), and also significantly lighter ($P = 0.012$) and shorter ($P < 0.001$) than robust participants, while BMI was not associated with frailty status. Additionally, characteristics and biomarker concentrations were different between the individual FRAILOMIC cohorts (Supporting Information, *Tables* S2 and S3).

Fat-soluble micronutrient and OS concentrations differed significantly between the frailty groups (*Table* 2 and *Figure* 1). Significantly higher VD_3 (*Figure* 1A) and lutein/zeaxanthin (*Figure* 1B) concentrations were observed in robust participants compared with pre-frail and frail participants, and in pre-frail compared with frail participants (all $P < 0.01$), in simple GLMs. Furthermore, robust and pre-frail participants had higher γ-tocopherol, α-carotene, β-carotene, lycopene, and β-cryptoxanthin (*Figure* 1C) concentrations than frail subjects (all $P \leq 0.02$); pre-frail participants had higher α-tocopherol concentrations than frail subjects ($P = 0.002$); and robust subjects had significantly lower PrCarb concentrations than frail and pre-frail participants ($P < 0.001$; *Figure* 1D). In multivariate analyses, β-cryptoxanthin was higher in robust than in pre-frail and frail participants and in pre-frail compared with frail participants. Furthermore, VD_3 and β-carotene concentrations were higher in robust than in pre-frail and frail participants (all $P \leq 0.001$); lycopene and lutein/zeaxanthin concentrations were higher in robust and pre-frail participants compared with frail subjects (all $P \leq 0.02$); α-tocopherol concentrations were higher in pre-frail participants than in frail subjects ($P = 0.013$); and lower PrCarb concentrations were found in robust compared with frail and pre-frail participants ($P = 0.006$). No association was observed between retinol or 3-NT and frailty status.

Results from GLMs were confirmed by logistic regression analyses (*Table* 3). Pre-frail and frail participants were more likely to be in the lowest than in the highest tertile for VD_3 and lutein/zeaxanthin than robust participants. Frail participants were more likely to be in the lowest than in the highest tertile for α-tocopherol, γ-tocopherol, α-carotene, β-carotene, lycopene, and β-cryptoxanthin than robust participants. Subsequently, multivariable adjustment showed that pre-frail and frail compared with robust participants were more likely to be in the lowest than in the highest tertiles for VD_3 (AOR: 1.98; 95% confidence interval: 1.42–2.76 and 2.15; 1.42–3.26), β-carotene (1.56; 1.06–2.28 and 1.84; 1.13–2.99), and especially for lutein/zeaxanthin (1.38; 1.00–

Table 1 Study characteristics by frailty groups

	Total	Robust	Pre-frail	Frail	*P*
N, % (*n*)	100 (1450)	36.1 (524)	41.7 (605)	22.1 (321)	—
Females, % (*n*)	55.9 (811)	48.1 (252)	57.5 (348)	65.7 (211)	<0.001#
Age, years	77.5 ± 6.5	74.6 ± 5.9[a]	78.0 ± 6.0[b]	81.4 ± 6.3[c]	<0.001
Weight, kg	70.6 ± 13.7	72.0 ± 12.0[a]	69.9 ± 14.0[b]	69.7 ± 15.5[b]	0.012
Height, cm	160.0 ± 9.6	161.5 ± 9.5[a]	160.0 ± 9.5[b]	157.6 ± 9.6[c]	<0.001
BMI, kg/m^2	27.6 ± 4.6	27.6 ± 3.9	27.3 ± 4.6	28.0 ± 5.6	0.066
Smoker, % (*n*)	5.0 (73)	5.6 (29)	5.6 (34)	3.1 (10)	0.209#
MMSE, points	25.6 ± 4.1	26.5 ± 3.1[a]	26.1 ± 3.3[a]	22.9 ± 5.7[b]	<0.001

All results reported as means ± standard deviation or % (*n*). BMI, body mass index; MMSE, Mini-Mental State Examination. Superscript letters indicate statistical significant differences between frailty groups by unadjusted GLM.
#Differences between frailty groups determined by Pearson's χ^2 test. $P < 0.05$.

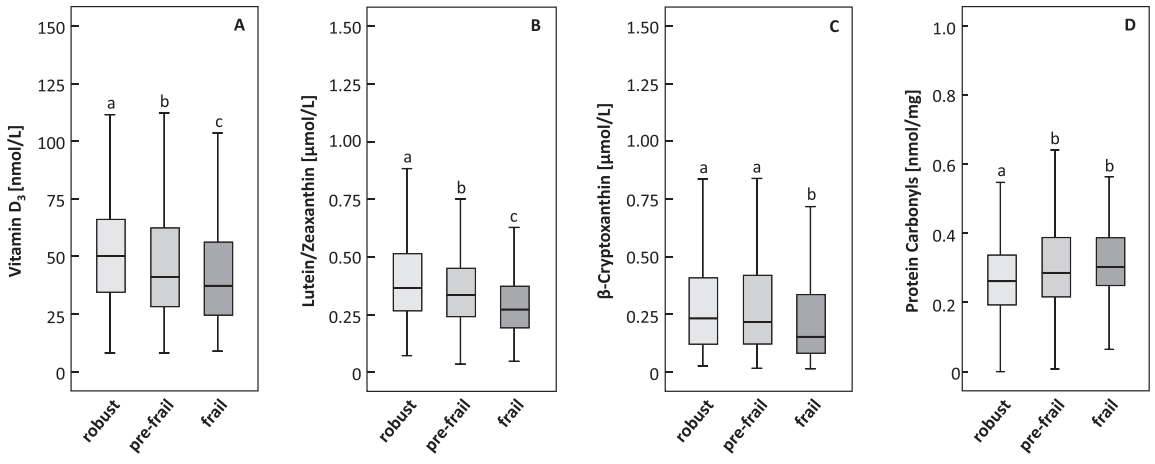
DOI: 10.1002/jcsm.12479

Table 2 Plasma concentrations of biomarkers by frailty groups

Biomarker	FRAILOMIC	Robust	Pre-frail	Frail	*P*
Vitamin D_3, nmol/L	42.0 (40.8–43.2)	47.2 (45.0–49.5)[a]	40.9 (39.1–42.7)[b]	36.6 (34.4–38.9)[c]	<0.001
adjusted		46.6 (44.0–9.5)[a]	39.5 (37.2–41.8)[b]	37.5 (34.9–40.2)[b]	<0.001
Retinol, μmol/L	1.88 (1.85–1.91)	1.87 (1.82–0.91)	1.90 (1.86–1.94)	1.87 (1.82–1.93)	0.577
adjusted		1.84 (1.78–0.90)	1.87 (1.81–1.93)	1.86 (1.79–1.93)	0.757
α-Tocopherol, μmol/L	29.4 (29.0–29.9)	29.2 (28.5–9.9)[a,b]	30.3 (29.6–30.9)[a]	28.3 (27.4–29.2)[b]	0.002
adjusted		28.8 (27.9–0.7)[a,b]	29.4 (28.5–30.3)[a]	27.2 (26.1–28.3)[b]	0.013
γ-Tocopherol, μmol/L	1.19 (1.16–1.22)	1.21 (1.16–1.26)[a]	1.22 (1.18–1.27)[a]	1.10 (1.04–1.16)[b]	0.005
adjusted		1.18 (1.12–1.25)	1.17 (1.11–1.24)	1.12 (1.05–1.19)	0.405
α-Carotene, μmol/L	0.12 (0.12–0.13)	0.13 (0.12–0.14)[a]	0.12 (0.12–0.14)[a]	0.11 (0.10–0.12)[b]	0.020
adjusted		0.12 (0.11–0.13)[a]	0.10 (0.10–0.11)[a,b]	0.10 (0.09–0.11)[b]	0.018
β-Carotene, μmol/L	0.41 (0.40–0.43)	0.44 (0.41–0.47)[a]	0.43 (0.40–0.46)[a]	0.35 (0.32–0.39)[b]	<0.001
adjusted		0.42 (0.39–0.45)[a]	0.36 (0.34–0.39)[b]	0.34 (0.31–0.37)[b]	0.001
Lycopene, μmol/L	0.36 (0.34–0.37)	0.40 (0.37–0.42)[a]	0.36 (0.34–0.39)[a]	0.29 (0.26–0.31)[b]	<0.001
adjusted		0.35 (0.32–0.38)[a]	0.33 (0.30–0.35)[a]	0.28 (0.25–0.30)[b]	0.002
Lutein/zeaxanthin, μmol/L	0.33 (0.32–0.34)	0.36 (0.35–0.38)[a]	0.33 (0.32–0.34)[b]	0.27 (0.25–0.28)[c]	<0.001
adjusted		0.34 (0.32–0.36)[a]	0.32 (0.30–0.33)[a]	0.27 (0.25–0.29)[b]	<0.001
β-Cryptoxanthin, μmol/L	0.20 (0.19–0.21)	0.22 (0.21–0.24)[a]	0.21 (0.20–0.23)[a]	0.16 (0.14–0.18)[b]	<0.001
adjusted		0.21 (0.20–0.23)[a]	0.17 (0.16–0.19)[b]	0.14 (0.13–0.16)[c]	<0.001
Protein carbonyls, nmol/mg	0.31 (0.30–0.31)	0.26 (0.25–0.27)[a]	0.30 (0.29–0.32)[b]	0.31 (0.30–0.33)[b]	<0.001
adjusted		0.26 (0.25–0.27)[a]	0.27 (0.25–0.28)[a]	0.30 (0.28–0.32)[b]	0.006
3-Nitrotyrosine, pmol/mg	7.22 (6.94–7.51)	7.64 (7.15–8.16)	6.98 (6.56–7.42)	7.01 (6.45–7.63)	0.103
adjusted		7.49 (6.90–8.14)	7.93 (7.32–8.60)	7.13 (6.45–7.88)	0.202

All results reported as geometric means (95% confidence interval). Superscript letters indicate statistical significant differences between frailty groups by GLM (unadjusted; *adjusted* for cohort, season of blood sampling, gender, age, height, weight, and smoking status); unadjusted model: $n = 1448$, except PrCarb ($n = 1446$), 3-NT ($n = 1441$), and VD_3 ($n = 1450$); *adjusted* model: $n = 1448$, except PrCarb ($n = 1429$), 3-NT ($n = 1441$), and VD_3 ($n = 1450$). $P < 0.05$.

Figure 1 Plasma concentrations of (A) vitamin D_3, (B) lutein/zeaxanthin, (C) β-cryptoxanthin, and (D) protein carbonyls by frailty groups. Superscript letters indicate statistical significant differences between frailty groups by unadjusted GLM; $P < 0.05$.



1.91 and 3.60; 2.34–5.53) and β-cryptoxanthin (1.70; 1.20–2.41 and 3.02; 1.95–4.69). Furthermore, frail participants were more likely to be in the lowest than in the highest tertile for α-tocopherol (2.12; 1.39–3.24), α-carotene (1.69; 1.00–2.88), and lycopene (1.94; 1.24–3.05) than robust participants. In contrast, for OS markers, ORs and AORs to be in the highest than in the lowest tertiles were calculated. Pre-frail and frail participants were associated with a higher likelihood to be in the highest than in the lowest tertile for PrCarb. For frail participants, this association was confirmed in the adjusted logistic regression model (2.86; 1.82–4.49). No associations were found between retinol and 3-NT tertiles and frailty status in both logistic regression models.

Discussion

In our study, we aimed to determine cross-sectional associations of FMNs and OS biomarkers simultaneously with the

DOI: 10.1002/jcsm.12479

Table 3 Odds ratios of pre-frail and frail participants (robust participants as reference) related to biomarker tertiles

	Pre-frailty	Frailty
Vitamin D_3	1.96 (1.46–2.63)*	2.83 (1.99–4.01)*
adjusted	1.98 (1.42–2.76)*	2.15 (1.42–3.26)*
Retinol	0.88 (0.66–1.17)	0.98 (0.70–1.37)
adjusted	0.93 (0.67–1.28)	0.82 (0.55–1.22)
α-Tocopherol	0.82 (0.62–1.09)	1.54 (1.09–2.19)*
adjusted	1.12 (0.81–1.54)	2.12 (1.39–3.24)*
γ-Tocopherol	0.95 (0.72–1.27)	1.62 (1.15–2.28)*
adjusted	1.04 (0.76–1.42)	1.46 (0.98–2.19)
α-Carotene	0.96 (0.72–1.28)	1.48 (1.05–2.07)*
adjusted	1.40 (0.92–2.13)	1.69 (1.00–2.88)
β-Carotene	1.10 (0.82–1.46)	1.75 (1.24–2.47)*
adjusted	1.56 (1.06–2.28)*	1.84 (1.13–2.99)*
Lycopene	1.28 (0.96–1.71)	2.36 (1.66–3.36)*
adjusted	1.18 (0.83–1.68)	1.94 (1.24–3.05)*
Lutein/zeaxanthin	1.58 (1.18–2.12)*	4.09 (2.84–5.87)*
adjusted	1.38 (1.00–1.91)*	3.60 (2.34–5.53)*
β-Cryptoxanthin	1.11 (0.83–1.49)	2.09 (1.48–2.93)*
adjusted	1.70 (1.20–2.41)*	3.02 (1.95–4.69)*
Protein carbonyls	1.83 (1.37–2.44)*	2.90 (2.00–4.21)*
adjusted	1.21 (0.87–1.68)	2.86 (1.82–4.49)*
3-Nitrotyrosine	0.79 (0.59–1.05)	0.72 (0.52–1.02)
adjusted	1.31 (0.94–1.82)	0.90 (0.59–1.36)

All results reported as odds ratios (95% confidence interval). Multivariate logistic regression models (unadjusted; *adjusted* for cohort, season of blood sampling, gender, age, height, weight, and smoking status); T3 was the reference for vitamin D_3, retinol, α-tocopherol, γ-tocopherol, α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin, and T1, was the reference for PrCarb and 3-NT; unadjusted model: $n = 1448$, except PrCarb ($n = 1445$), 3-NT ($n = 1441$), and VD_3 ($n = 1450$); *adjusted* model: $n = 1431$, except PrCarb ($n = 1432$), 3-NT ($n = 1428$), and VD_3 ($n = 1433$).
*$P < 0.05$.

frailty status of participants older than 65 years, and we demonstrated both differences in concentrations between robust, pre-frail, and frail individuals and associations of FMN and PrCarb with pre-frailty and frailty.

The prevalences of pre-frailty (41.7%) and frailty (22.1%) are in accordance to previously shown prevalences of 42.3% and 17.0%, respectively, in community-dwelling individuals from 10 European countries.[4] Our findings confirm previously published associations of frailty with age and female gender.[4–6]

A higher risk for frailty has previously been related to a low intake of micronutrients and possibly resulting lower micronutrient status. Low intakes of vitamin E or VD led to higher ORs to be frail than non-frail, after adjusting for several confounders, in 802 subjects (>65 years) from the InCHIANTI cohort.[15] Women (>65 years) in the lowest quartile of serum carotenoids had a higher risk of becoming frail during a 3 year follow-up period in the Women's Health and Aging Study I (WHAS I).[17] Lower VD, retinol, α-carotene, β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin plasma concentrations were found in frail compared with non-frail women of the WHAS I and WHAS II, and the ORs of being frail were significantly higher for women in the lowest quartile compared with the top three quartiles for total carotenoids, α-tocopherol, and VD.[16] The strongest association was found for β-carotene, lutein/zeaxanthin, and total carotenoids, after adjusting for age, sociodemographic status, smoking, and BMI.[16] Using FRAILOMIC initiative data, Pilleron *et al*. observed a significant relationship between a circulating micronutrient pattern, which is low in vitamins A and E, and high in carotenoid concentrations, and a higher prevalence of frailty.[14] However, all these studies did not make the distinction between frail and pre-frail, considering pre-frail as robust.

The lack of association between retinol and frailty status, found in our study, was previously reported in participants of the FRAILOMIC initiative and the InCHIANTI cohort.[14,15] This might be due to the homeostatically regulated retinol metabolism, and furthermore, circulating retinol is considered a poor relevant marker of vitamin A status.[36]

Beside low micronutrient concentrations possibly leading to frailty, VD itself may play a role in the pathogenesis of frailty. Vitamin D, beside its endocrine/indirect effects on muscle function, can act directly on skeletal muscle via the VD receptor, contributing to a normal muscle structure and metabolism.[37] A low VD status was related to a reduced muscle mass and an impaired physical performance in 127 pre-frail and frail Dutch participants (>65 years).[20] Vogt *et al*. observed that participants (>65 years) having baseline VD levels <37.5 nmol/L compared with ≥75 nmol/L were more likely to become pre-frail (2.4; 1.17–5.03) and pre-frail/frail combined (2.5; 1.23–5.22), after a 3 year follow-up.[19] Our findings of an association between low VD_3 and pre-frailty and frailty prevalence differ from those found by Pilleron *et al*.[14] who observed no association between VD_3 and frailty status in the same sample cohort. This was possibly due to the fact that Pilleron *et al*. used different cut-offs for VD_3 and included the pre-frail participants in the robust group. In our analyses, tertiles of VD_3 were chosen to be able to compare groups with similar sample size. The lowest VD_3 tertile (<33.7 nmol/L) in our study meets the definition of a deficient or even severely deficient VD_3 status, depending on the references used.[38]

Low FMN concentrations might be due to a low intake of fruits, vegetables, nuts, seeds, and oils, which could potentially originate from a decreased ability for older persons to go shopping or prepare meals themselves. A low VD status may occur due to less exposure to sunlight and less physical activity, which might be a result of frailty itself. The reason for low concentrations of some carotenoids may lie in their food source. Elderly persons may experience physiological changes in the gastrointestinal tract leading to reflux, heartburn, or constipation that may result in a decreased intake of fruits and vegetables. Our observations might just reflect a low intake of these fruits and vegetables; unfortunately, we are not able to adjust our models for dietary intake.

Inadequate micronutrient intake and plasma concentrations may result in higher OS leading to an impaired muscle

function and performance and contributing to frailty. A low dietary intake of a combination of vitamins A, E, B_6, and B_{12}, folate, selenium, and zinc led to a lower oxidative capacity and reduced muscle function and physical activity in aged male C57/BL6J mice.[39] In contrast, a high intake of fruits and vegetables was previously associated with low biomarkers of OS in 296 healthy, middle-aged men[40] and a lower risk of frailty in older individuals (>60 years).[13] In a previous analysis of the TSHA cohort, higher PrCarb concentrations were observed in frail compared with non-frail individuals but no association was observed with age.[26] In contrast, higher plasma PrCarb were previously observed in older adults (61–85 years) compared with young subjects (21–40 years)[24] supported by an age-dependent increase in PrCarb levels in 80 healthy persons (18–85 years).[25] In our study, there was a significant positive correlation between age and PrCarb ($r = 0.196$; $P < 0.001$; not shown) but also a negative correlation between age and 3-NT ($r = -0.225$; $P < 0.001$; not shown) in robust participants. In frail subjects, no correlations between age and OS markers were found. In addition, an association between higher OS levels and frailty was reported,[27] but only one study on frailty used PrCarb as OS marker, and therefore, no comparisons with other studies are possible. However, PrCarb was two-fold lower and 3-NT was two-fold higher compared with subjects from a general population in the MARK-AGE study.[41] The FMN concentrations on the other hand were comparable with those found in the MARK-AGE study, except for lycopene that was two-fold higher in MARK-AGE.[33]

Due to the cross-sectional design of our study, we cannot conclude whether frailty leads to a low micronutrients/high PrCarb status or if a low micronutrients/high PrCarb status leads to frailty. Furthermore, data regarding socio-economic status and income were not available and, therefore, are missing in the multivariate-adjusted models. Strengths of our study are the large sample size including frailty classification into robust, pre-frail, and frail and the harmonized frailty criteria used in all four cohorts. Especially the possibility to include the pre-frail group is a novel feature in a study with such a large sample size. Additionally, participants from different European countries were analyzed; thus, our study results reflect a broad range of the society and different lifestyles. Furthermore, the broad spectrum of parameters, including nutritional biomarkers, antioxidants, and OS biomarkers, are a unique feature of this study. The high-quality blood analyses were performed by the same trained persons in one laboratory, thus limiting variability related to operators, methods, and analytical instruments.

From our study, we conclude that both low concentrations of several single FMN (VD_3, β-carotene, lutein/zeaxanthin, and β-cryptoxanthin) and high concentrations of PrCarb are associated with pre-frailty and frailty in four European cohorts of adults aged 65 years and older. Thus, we suggest that following a diet rich in FMN, subsequently leading to higher micronutrient and lower OS concentrations, may support the prevention of frailty. Further large-scale longitudinal and intervention studies are needed to investigate the role of FMN on the frailty risk and the potential mediating effect of OS.

Acknowledgements

This work was supported by the European Union's Seventh Framework Programme (FP7-HEALTH; grant number 305483, FRAILOMIC Project).

The 3-C Study is conducted under a partnership agreement between the Institut National de la Santé et de la Recherche Médicale (INSERM), Victor Segalen–Bordeaux2 University, and Sanofi-Synthélabo. Fondation pour la Recherche Médicale funded the preparation and beginning of the study. The 3-C Study is sponsored by the Caisse Nationale Maladie des Travailleurs Salariés, Direction Générale de la Santé, Conseils Régionaux of Aquitaine and Bourgogne, Fondation de France, Ministry of Research-INSERM Program Cohortes et collections de données biologiques, Fondation Plan Alzheimer (FCS 2009-2012), and Caisse Nationale pour la Solidarité et l'Autonomie (CNSA) and 'Programme Longévité et vieillissement' (COGICARE 07-LVIE 003 01). The AMI project was funded by AGRICA (CAMARCA, CRCCA, CCPMA PREVOYANCE, CPCEA, and AGRI PREVOYANCE), la Mutualité Sociale Agricole (MSA) de Gironde, and la Caisse Centrale de la Mutualité Sociale Agricole (CCMSA). The InCHIANTI study baseline (1998–2000) was supported as a 'targeted project' (ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336); the Follow-up 1 (2001–2003) was funded by the U.S. National Institute on Aging (Contracts: N.1-AG-1-1 and N.1-AG-1-2111); the Follow-ups 2 and 3 (2004–2010) were financed by the U.S. National Institute on Aging (Contract: N01-AG-5-0002) and supported in part by the Intramural research program of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland. The TSHA cohort was funded by grants PI07/90637, PI10/01532, and CB16/10/00456 from the Instituto de Salud Carlos III (Ministerio de Economía y Competitividad, Spain), 03031-00 from the Instituto de Ciencias de la Salud de Castilla la Mancha, and PI2010/020 from FISCAM.5.

The authors certify that they comply with the Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle.[42]

Online supplementary material

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

Table S1 Tertiles of fat-soluble micronutrients and oxidative stress markers

Table S2 Study characteristics by individual FRAILOMIC cohorts

Table S3 Plasma concentrations of biomarkers by the FRAILOMIC cohorts

Conflict of interest

C.F. received fees for conferences from Danone Research and Nutricia not related to the present work. No further conflicts of interest are declared.

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Discussion

Biomarker analyses in the context of risk assessment and diagnosis of diseases, gain more and more relevance, not only in the frailty syndrome, since they have several advantages compared to functional examinations. Rising numbers of newly conceptualized large-scale studies and Initiatives to find suitable biomarkers concerning muscle-related diseases like sarcopenia and frailty reflect this growing relevance. There are for example the FRAILOMIC Initiative [6], the BIOSPHERE (BIOmarkers associated with Sarcopenia and PHysical frailty in ElderRly pErsons) study [163] or the SPRINTT (Sarcopenia and Physical fRailty IN older people: multi-componenT Treatment strategies) study [164].

For **Publications 2** and **3**, data from the FRAILOMIC Initiative (FRAILOMIC) were investigated. FRAILOMIC is an EU-funded "*international, large-scale, multi-endpoint, community- and clinic-based research study*", which aims "*to develop validated measures of both classic and 'omics'-based laboratory biomarkers*" [6]. These biomarkers should further be used to assess "*the risk of an older individual developing frailty (risk biomarkers) as well its identification (diagnostic biomarkers), clinical course (prognostic biomarkers) and likely response to treatment (predictive biomarkers)*" [6]. In this thesis, individuals originating from four cohorts of FRAILOMIC (n = 1633, ≥ 65 years) were used. These four cohorts were the Bordeaux sample of the Three-City Study (3-C, France) [165], the Aging Multidisciplinary Investigation cohort (AMI, Gironde, France) [166], the Toledo Study for Healthy Aging (TSHA, Toledo, Spain) [167] and the Invecchiare in Chianti Study (InCHIANTI, Chianti geographical area, Tuscany, Italy) [168]. FRAILOMIC cohorts provide data regarding frailty, demographics, cognition, lifestyle, medication, physical examinations, comorbidities as well as clinical and biological biomarker data [26] that were used in **Publications 2** and **3**. Additionally, FRAILOMIC cohorts provided a large sample size including harmonized Fried criteria for the classification of individuals into robust, pre-frail and frail that is a beneficial feature in such large studies like **Publication 2** and **3**. Thus, leading to more specific results and interpretations regarding biomarker associations with the frailty syndrome, and therefore, representing a novelty in this thesis.

A biomarker is defined as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* [169]. Biomarkers can be applied

to monitor health status and for the diagnosis of diseases. Thus, biomarkers can be used as (I) a diagnostic tool for the identification of individuals with a disease or abnormal condition, (II) a tool for classifying a disease state, (III) a prognosis tool for a disease, or for (IV) the prediction and monitoring of a response to an intervention. A biomarker can further be *"any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"* [170].

Biomarkers should fulfill some requirements to be suitable (**Table 3**) [169, 171]. These include e.g. tissue or disease specificity including a strong association with the disease or its outcome in clinical studies. Biomarkers should have none or a low degree of bias by internal or external confounders and a validated method for the accurate and reproducible detection and quantification of the biomarker is also required. Further criteria are listed in **Table 3**.

Table 3. Criteria and requirements for an ideal biomarker.

Criteria	Description
Validation	Accurate, specific, sensitive and reproducible method for the detection and quantification
Specificity	Tissue and/or disease specific; strong associations with the disease or its outcome in clinical studies
Sensitivity	Discrimination of diseased from healthy; small changes should be detected
Confounder	No to low bias by possible endogenous or exogenous confounders
Application	Feasible, reasonable costs, high sample throughput; simple and fast sample preparation; minimal or non-invasive

Modified from [169, 171].

Suitable plasma biomarkers for frailty or an elevated muscle protein turnover are still lacking and need to be evaluated and validated, thus 3-MH and its ratios needed to be investigated regarding accurate and reproducible quantification, reliable measures, possible confounders and their ability to identify frailty (**Publications 1** and **2**). 1-MH was analytically validated and then evaluated as a biomarker of meat consumption comparing omnivores and vegetarians (**Publication 1**) and was subsequently was used to display meat/fish or exogenous 3-MH intake (**Publication 2**). Redox biomarkers and micronutrients are well described in the context of several diseases including frailty and analytic techniques are validated. These biomarkers were investigated in this thesis together with the more specific biomarker 3-MH.

Analysis of amino acids and methylhistidines in plasma

In principle, AA in biofluids can be analyzed by different methods including ion-exchange chromatography (on cation-exchange column) including post-column derivatization with ninhydrin [172, 173], OPA [174] or fluorescamine [175] followed by detection in AA analyzers, or reversed-phase HPLC using pre-column derivatization with e.g. OPA [176] or 9-fluorenylmethyl chloroformate [177]. Additional methods are thin-layer chromatography including ninhydrin derivatization [178, 179], solid-phase extraction followed by derivatization and analysis by GC [180, 181] or GC-MS [182, 183] and capillary electrophoresis [184].

While AA analyzers were first recommended for routine applications due to their high reliability, HPLC methods gained higher importance in AA analysis due to shorter analysis times, lower cost of instrumentation and maintenance, higher sensitivity and flexibility [185]. Nowadays, MS/MS and LC-MS/MS methods attract more and more attention in the AA analysis. AA analyzers, HPLC and GC-MS methods use derivatized samples and may have disadvantages like long run times, extensive sample preparations and interfering derivatives compared to MS/MS or LC-MS/MS methods, which have been shown to be suitable for measuring underivatized AA [158, 186-189]. Quantification of underivatized AA from a biological sample may result in fewer errors introduced by derivatization like derivative instability, side reactions and reagent interferences [186]. Coupling LC with MS/MS can improve both target AA and dipeptide analysis due to high resolution and analytical specificity while providing a high sensitivity over a broad concentration range. Additionally, LC-MS/MS is a reproducible and accurate technique due to the possible use of internal standard, and is able to detect different AA with concentrations varying over a thousand-fold concentration range in the same sample [189, 190]. There is a need to be able to measure several analytes of interest in parallel within a single sample and a need for a high sample throughput. LC-MS/MS applications provide such high sample throughputs and simultaneous analyses of several AA and dipeptides in a single run using internal standards [189, 190].

For 1-MH and 3-MH, which have the same parent ion m/z, the accurate detection and subsequent quantification are achieved after separation based on different optimized product masses or fragmentation patterns [152, 189]. Both 3-MH and 1-MH concentration in plasma and in urine samples can be measured by several different analytical methods.

While methylhistidine analyses are primarily performed in 24-hour urine samples, there are limitations regarding accurate and complete sampling especially in an elderly study population [118, 119]. Thus, the first aim within this work was to establish an UPLC-MS/MS method to measure 3-MH and 1-MH simultaneously in plasma samples. However, note that there exists an inconsistent nomenclature of both 3-MH and 1-MH in the literature since there are different denotations of their structures. The numbering of atoms, in this case the nitrogen atom within the imidazole ring of histidine that binds the methyl-group is different between biochemists and chemists (International Union of Pure and Applied Chemistry) [149, 191]. The nomenclature in this thesis refers to the historically more commonly used nomenclature of biochemists and thus allows better comparability with the literature.

Publication 1 described a reliable, fast and simple UPLC-MS/MS method for the simultaneous measurement of 3-MH and 1-MH in plasma samples. Here, chromatographic separation of both MHs was achieved and standard isotope dilution method was performed for quantification by the use of deuterated 3-MH (d_3-3-MH; **Figure 6**) as internal standard.

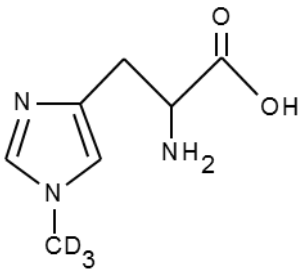

Figure 7. Structure of deuterated 3-methylhistidine.

The method in **Publication 1** provides a fast and simple sample preparation without derivatization, a subsequent short analysis time of only 10 min. per sample and requires only 25 µL of plasma per sample. Thus, a high sample throughput in a short time is possible as required for **Publication 2** measuring 360 samples. Previous run times for LC-MS/MS methods were 24 min. for detecting both MHs [152], 30 min. for detecting 52 AA including both MHs [153], 35.5 min. for 31 AA including both MHs [189] and about 13 min. for both MH (using LC-QTOF-MS) [192] in plasma. Reported sample volumes of these methods differ from only 10 µL [153], to 30 µL [192], to 50 µL [152] and even to 100 µL [189]. Furthermore, the method established in **Publication 1**, showed linearity ($r^2 > 0.999$) over a 500-fold

concentration range (0.015-7.5 μM) for MHs in water as well as in plasma extracts. The *limit of detection* was estimated to be 6.0 nM and 5.3 nM for 1-MH and 3-MH, respectively, and the *limit of quantification* was shown to be 15 nM for both MHs. *Intra-assay* and *inter-assay variations* were < 10 % for both MHs. In practical approaches, 3-MH and 1-MH concentrations in plasma were quantified and omnivores were distinguished from vegetarians by 1-MH (**Publication 1**) as well as frail participants from robust participants by 3-MH (**Publication 2**) using this UPLC-MS/MS method.

Influence of meat and fish on 3-methylhistidine analyses in plasma

3-MH plasma concentrations and urinary 3-MH excretion are related to the dietary meat and fish consumption of individuals. Therefore, for reliable analyses and interpretations of 3-MH as potential biomarker of muscle protein breakdown, it was recommended that intake of these foods two to three days prior to sample collection should be avoided [117, 123] or detailed information about meat and fish intake was needed. However, complying with dietary restrictions and recalling correct information about dietary meat and fish intake can be challenging for elderly individuals in clinical settings as well as in large-scale human studies. Most studies which investigated the impact of meat and fish intake on 3-MH and further provided the basis for these recommendations, analyzed 3-MH excretion in urine [117, 118, 122-125, 193], whereas studies considering the influence of meat or exogenous 3-MH intake on plasma 3-MH concentrations [126] are lacking.

Results from **Publication 1** suggest that a general influence of an omnivorous diet on plasma 3-MH concentrations can be assumed by higher plasma 3-MH levels observed in omnivores compared with individuals on a vegetarian diet. Interestingly, no differences were observed for 3-MH/Crea ratios (**Publication 1**). Creatinine concentrations and excretions are described as markers for muscle mass [121, 123, 194, 195], although both might be influenced by meat or protein intake [120, 125]. However, the administration of chicken did not increase plasma creatinine concentrations and plasma creatinine did neither correlate with meat and protein intake at baseline in **Publication 1** nor with meat and fish servings in **Publication 2**.

Higher urinary 3-MH excretions were observed both, in participants on meat diets compared to participants on vegetarian diets [125], and in omnivores (n = 87) compared to

vegetarians (n = 36) [127], which is in accordance with results in plasma of **Publication 1**. Additionally, urinary 3-MH excretion positively correlated with red meat, poultry, fish as well as total meat intake [127]. However, plasma 3-MH and 3-MH/Crea neither correlated with total meat intake in **Publication 1** nor with servings of meat and fish in **Publication 2**.

Plasma 3-MH and 3-MH/Crea of omnivores reached apparently basal levels after three days on a vegetarian diet. These plasma 3-MH and 3-MH/Crea levels were similar to those levels of vegetarians at baseline, suggesting endogenous 3-MH (**Publication 1**). This is in accordance to previous results shown in urine. Urinary 3-MH and 3-MH/Crea excretions of males (n = 6; 28-36 years) reached baseline levels after two to three days on a vegetarian diet [117]. Urinary 3-MH excretion was found to be higher in subjects (14 men; age: 20-30 years) on a 4-day meat diet with beef and chicken as main protein source, than on a subsequent 7-day meat-free diet [123]. Furthermore, a decline in 3-MH excretion until the third day on this meat-free diet was observed, subsequently, assuming endogenous 3-MH excretion after three days on a meat-free diet. A decline in urinary 3-MH and 3-MH/Crea ratios during three days on a meat-free diet, followed by an increase after meat or meat soup consumption in healthy subjects (n = 6; age: 25-60 years) was determined [124]. However, these studies are limited regarding small sample sizes [117, 124, 125], study populations (only gender-specific [117, 123], only obese subjects [125] or a wide age-range [124]) as well as missing detailed information on the amount of ingested meat or fish [117, 123-125].

In **Publication 1**, a white meat intervention with ~160 g chicken breast led to a rapid increase of plasma 3-MH and 3-MH/Crea followed by a significant decline of both biomarkers within 24 hours. Accordingly, plasma 3-MH was increased after the intake of 100 g of beef or chicken, with a more pronounced impact by chicken, in 30 healthy subjects (aged 24-43 years) [126]. Additionally, plasma 3-MH declined after 24 hours and remained constant [126]. Furthermore, ingestion of capsules containing pure 3-MH led to maximum 3-MH concentrations within 60-90 min. compared with meat ingestion that led to a maximum after 180 min. Plasma 3-MH half-life was shown to be around 12 hours [126]. Unfortunately, intervals of blood drawings after the white meat administration in **Publication 1** were not narrow enough, thus, detailed information on bioavailability cannot be provided.

An increased urinary 3-MH excretion during a 3-day intervention with chicken, beef or fish, followed by a return to basal 3-MH excretion levels within two to three days on a vegetarian diet was observed in five subjects (age: 22-30 years) [122]. However, only a small sample size (n = 5) with one person per meat source were investigated, and varying amounts of meat (chicken: 465 g; beef: 245 g) and fish (plaice: 227 g or 454 g raw fresh wt.; cod: 570 g raw frozen wt.) were applied [122]. Consumption of 250 g of beef tartar led to an increase in urinary 3-MH excretion in seven healthy subjects (age: 21-59 years) on a meat-free diet, followed by the quantitative excretion within two days [193]. An increase in 3-MH excretion was shown after the intake of 280-320 g beef, whereas an ovo-lacto-vegetarian diet did not increase 3-MH excretion [118]. Additionally, an urinary 3-MH half-life of 12.6 hours were stated [118], which is in accordance with the half-life in plasma [126]. However, high amounts of beef were administered to the individuals [118, 122, 193]. In a multi-crossover intervention study, higher 3-MH excretion levels were observed after consuming meat diets compared with diets containing dairy protein or grain protein in 30 subjects [129]. Here, no detailed information on the amount of meat intake, only as percentage of protein amount, is given. In contrast, no increases in 3-MH and balenine excretion occurred after the ingestion of either 100 g of beef, pork, chicken, eel, or 150 g of tuna [147].

The increase of plasma 3-MH after a white meat intervention was weak compared with increases in plasma 1-MH (**Publication 1**). Therefore, one might suggest that small amounts of ingested meat or fish may not affect or only weakly affect plasma 3-MH concentrations. A dose-dependent relationship between meat (pork: 116-316 g; chicken: 100-287 g) or fish (plaice: 108-301 g) intake with urinary 3-MH [118], as well as a dose-dependent increase of 3-MH excretion (vegetarian < 60 g/d < 120 g/d < 420 g/d red meat) in male individuals (age: 24-74 years) [142] was shown. There also was a dose-dependent increase in plasma 3-MH by administrations of varying 3-MH amounts (meats, or capsules containing 20 mg, 60 mg or 120 mg of 3-MH) [126].

In **Publication 1**, about 160 g of chicken breast were administered to the participants, which reflects the general meat intake per person per day in Germany (~60 kg meat per year per capita) [196]. This is more representative compared to some previous studies, which administered large and varying amounts of meats to their participants [118, 122, 193]. Since there might be a decline in meat and fish consumption in older adults,

intake of these foods might be less prominent in populations that are at higher frailty risk. Individuals of the Seniors-ENRICA cohort (N = 2982; > 60 years) consumed 35.0 (standard deviation: 34.0) g/day processed meat, 31.9 (28.2) g/day red meat and 33.8 (28.3) g/day poultry [197]. Consumption of animal products like red meat, poultry, fish, eggs and dairy and a general omnivorous dietary habit declined with increasing age in individuals of the Adventist Health Study-2 (N = 51,082) [198]. Thus, the influence of meat and fish on plasma 3-MH might not be significant in advanced-aged individuals. Furthermore, data of **Publication 1** suggests that avoidance of meat 24 hours prior to blood sampling might be sufficient for reliable 3-MH measurements. This is a major improvement in clinical as well as research settings regarding the diagnosis of elevated muscle protein turnover, risk of frailty or frailty itself. Additionally, in research settings like in **Publication 2** statistical controlling for meat and fish intake can be performed or 1-MH analysis might be an alternative to display exogenous 3-MH in plasma, instead of gathering data on the actual meat and fish consumption or avoiding eating those foods prior to sample collection.

1-MH, as part of anserine, also occurs in meat and fish (and their products). Plasma and urinary 1-MH were more influenced by meat and fish intake than 3-MH. Results of **Publication 1** led to the suggestion that plasma 1-MH concentrations and 1-MH/Crea ratios are able to be used as biomarkers for recent (< 24 hours) white meat intake, which is in accordance with previous findings in urine [118, 127, 129, 142, 192] as well as in plasma [128, 192].

In **Publication 1**, omnivores were distinguished from vegetarians since they had higher plasma 1-MH concentrations and 1-MH/Crea ratios. Additionally, positive correlations of plasma 1-MH concentrations with total meat intake (**Publication 1**) and of 1-MH and 1-MH/Crea with servings of fish (**Publication 2**) were found. This is in accordance with previous results in urine, which showed that 1-MH excretion was higher in omnivores than in vegetarians [127, 142] and that 1-MH excretion increased after beef (280-320 g), pork (116-316 g), chicken (100-287 g) or fish (108-301 g) intake [118, 129]. Furthermore, 1-MH excretion correlated positively with meat intake regardless of meat source [127] and 1-MH excretion was dose-dependently associated with meat and fish intake [118, 142]. Furthermore, both 1-MH and anserine excretion increased after tuna (150 g) and chicken (100 g) intake [147]. Interestingly, almost no influence on urinary 1-MH excretion after the intake of 100 g of beef, pork, or eel was observed.

According to the results for 3-MH and 3-MH/Crea in plasma, 1-MH and 1-MH/Crea increased rapidly after the administration of white meat to omnivores during a vegetarian diet (**Publication 1**). Here, increases of plasma 1-MH and 1-MH/Crea were higher compared to those of 3-MH and 3-MH/Crea, and increased plasma 1-MH and 1-MH/Crea declined back to basal levels after 48 hours. Similarly, the increase of urinary 1-MH excretion was also more pronounced compared to 3-MH after beef [118] as well as red meat, poultry and fish intake [127], and 1-MH was excreted rapidly within 20-40 hours [147]. Furthermore, 1-MH half-life was calculated to be 11.7 hours [118]. One reason for the stronger increase in plasma 1-MH compared to plasma 3-MH after white meat intake might result from different amounts of methylhistidines found in different meat and fish sources. White meat sources contain higher amounts of 1-MH or anserine compared to 3-MH or balenine [107, 108, 118, 147], and anserine is rapidly converted to 1-MH during digestion [147]. Thus, 1-MH might be more bioavailable than 3-MH.

Low concentrations of plasma 1-MH were found in vegetarian individuals, although no source of 1-MH and anserine was ingested (**Publication 1**). 1-MH can be released from degraded anserine of non-muscle tissues where rather high amounts of anserine occur [108]. Similar 1-MH, 1-MH/Crea and 3-MH/1-MH levels were observed in robust, pre-frail and frail individuals of **Publication 2**. This led to the assumption that meat or fish intake had the same influence on plasma 3-MH and its ratios in all three groups. Nevertheless, adjustment of the statistical tests for meat and fish intake as potential confounders was performed.

Possible confounders for 3-methylhistidine as biomarker

Beside the influence of 3-MH-containing foods, there are possible other confounders for 3-MH (shown in **Figure 8**) that will be discussed in the following section.

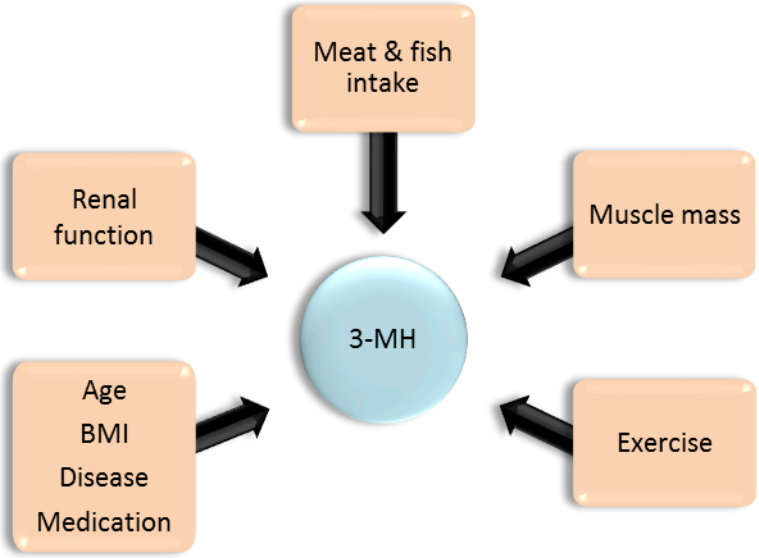

Figure 8. Possible confounders for 3-methylhistidine as biomarker for muscle protein turnover. Blue circle represents 3-MH measured in plasma; orange boxes represent possible confounders for 3-MH. Abbreviations: 3-MH, 3-methylhistidine; BMI, body mass index.

The 3-MH amount in skeletal muscles of healthy individuals (4-65 years) remained constant at around 3.6 µmol/g mixed muscle as did the excretion of 3-MH/Crea [117]. Similar 3-MH contents for skeletal muscle of around 3.3 µmol/g fat-free mass were observed in healthy subjects (N = 5, age: 43-67 years) [199]. In contrast, smaller amounts of skeletal muscle 3-MH were found in healthy individuals (N = 13, age: 10-60 years) compared to studies above, however, these amounts did not differ within the study population [200]. Differing 3-MH amounts found in these studies might be due to different analytical methods used to measure 3-MH, different measured muscle types or appearing muscle diseases. Individuals with Duchenne muscular dystrophy had significantly lower amounts of 3-MH/g muscle than their healthy counterparts [200]. Furthermore, there might be an age-related impact on the 3-MH release from muscle, although results are not consistent. Higher interstitial 3-MH concentrations were observed in old (≥ 70 years) compared to young (25-30 years) individuals [201]. In contrast, plasma 3-MH concentrations were similar between healthy individuals aged around 25 years (n = 27) and aged around 75 years (n = 84), whereas significantly higher plasma 3-MH occurred in centenarians (n = 71) [202]. 3-MH/Crea excretion was not different between healthy young (n = 8, age: 18-25 years) and advanced-aged (n = 11, age: 67-91 years) individuals [203]. In **Publication 2**, plasma 3-MH was positively associated with age, while no correlation was observed for plasma 3-MH/Crea.

3-MH occurs in the blood after the degradation of muscle tissue and is quantitatively excreted in the urine [110, 111]. It was previously calculated that more than 90 % of urinary 3-MH originates from skeletal muscle [204]; however, there exist some controversy in the literature about tissue contribution to 3-MH excretion. Previous studies stated that the skeletal muscle contributes only 50 % to the urinary 3-MH amount and that the gastrointestinal tract has a great impact on urinary 3-MH in female rats, and thus, 3-MH is not suitable as biomarker [205, 206]. In contrast, similar 3-MH and 3-MH/Crea in urine between healthy individuals and individuals with short bowels (28 % remaining gastrointestinal tract) were observed, and it was suggested that gastrointestinal contribution can be neglected and concluded that 3-MH is representative for skeletal muscle breakdown [207].

It was shown that 3-MH also might depend on muscle mass of individuals. Therefore, MH concentrations were normalized to creatinine levels to minimize the influence of muscle mass since creatinine was suggested as useful markers for muscle mass [120, 121, 123, 125, 194, 195]. Urinary creatinine was significantly positively correlated with skeletal muscle mass (r = 0.79) [123], which is in accordance to **Publication 1** showing that plasma creatinine positively correlated with skeletal muscle mass (r = 0.577) and grip strength (r = 0.482) of omnivores and with skeletal muscle mass (r = 0.607) of vegetarians. Unfortunately, no data on muscle mass or grip strength were available to evaluate their associations with creatinine and 3-MH in **Publication 2**.

Urinary creatinine excretion correlated significantly with urinary 3-MH [117, 123], which is similar to a positive correlation between plasma creatinine and 3-MH in omnivores and vegetarians in **Publication 1**. Urinary 3-MH and 3-MH/Crea excretion were shown to be positively correlated with fat-free body mass [208] as was the 3-MH excretion with skeletal muscle mass in 14 healthy men aged 20-30 years [123] and in 10 men aged 20-47 years [209]. Similarly, plasma 3-MH correlated positively with skeletal muscle mass and grip strength in vegetarians showing associations of 3-MH with muscle status in **Publication 1**.

Plasma 3-MH concentrations are further influenced by kidney function. In individuals with chronic renal failure, urinary as well as plasma 3-MH concentrations needed around 12 days on a vegetarian diet to reach basal levels whereas in healthy individuals basal levels were reached after two to three days [133, 134]. Similar results

were shown in individuals (N = 11, age: 37-77 years) with reduced kidney function reaching steady-state 3-MH plasma concentrations and urinary excretions around day 14 on a vegetarian diet [132]. Kidney function was negatively associated with higher age as previously shown in two cross-sectional studies [130, 131], which is in accordance to findings from **Publication 2**. Therefore, the 3-MH/eGFR ratio was calculated to overcome this potential limitation for plasma 3-MH concentrations in **Publication 2**. That was the first time that the 3-MH/eGFR ratio was used in context of frailty and represents a novelty of **Publication 2**.

Another limiting factor for measuring plasma 3-MH concentrations in the context of muscle protein turnover and frailty might be the intake of medication of individuals. Urinary 3-MH excretion in healthy adults (N = 7) increased after a treatment with thyroid hormone or β-adrenoceptor blocking agents [210]. Furthermore, urinary 3-MH excretion was higher in asthmatic children (n = 69) treated with glucocorticoids and beta2 agonists compared with a placebo group (n = 48) [211]. In contrast, no changes of 3-MH excretion after a glucocorticoid treatment nor differences compared to a placebo treatment were observed in healthy individuals (N = 13, age: 19-34 years) [140]. Similarly, low-calorie diet and ephedrine treatment had no effect on urinary 3-MH in obese individuals (N = 10) [212]. Treatment with an anti-thyroid drug led to a decline of elevated urinary 3-MH/Crea levels in individuals with hyperthyroidism, whereas no decline was observed after treatment with a β-adrenoceptor blocking agent [210]. However, the impact of medication intake on plasma 3-MH is not known. In **Publication 2**, plasma 3-MH concentrations and both 3-MH/Crea and 3-MH/eGFR ratios were positively correlated with the number of self-reported medication intake. Therefore, adjustments for medication intake in the statistical analyses for 3-MH and its ratios were performed considering this possible confounder for plasma 3-MH. Since the observed multi-medication of individuals in **Publication 2** may reflect a general unhealthy status, multi-morbidity as possible confounder for plasma 3-MH was also included in the analyses.

Exercise and protein intake may have an additional impact on muscle metabolism [213, 214] that may alter the release of 3-MH from muscles and thus, may affect plasma and urinary 3-MH and 3-MH/Crea [141, 213, 215]. However, results are not consistent in the literature. A resistance exercise program (3 months) did not change urinary 3-MH/Crea ratios in frail individuals (n = 12, ≥ 76 years) but did enhance muscle protein synthesis;

however, no healthy individuals were included in this study [150]. Similarly, a 3 months resistance exercise training did not change 3-MH/Crea excretion in healthy advanced-aged (n = 9, 62-72 years) and young (n = 9, 22-31 years) individuals [216]. Additionally, no differences between both age groups were found. Subsequently, controlling for exercise would be a benefit in studying 3-MH, however, this was not possible in **Publications 2** and **3** due to missing data. This was also the case for protein intake since data on nutrient intake are missing for **Publications 2** and **3**.

Possible confounders for frailty analyses

In European countries of the SHARE project (Survey of Health, Aging and Retirement in Europe), the mean prevalence of pre-frailty and frailty in community-dwelling individuals (65+ years group) was found to be 42.3 % and 17.0 %, respectively [22]. Furthermore, frailty prevalence was 39.1 % pre-frail and 17.0 % frail among French individuals (N = 2286) [217]. These are in accordance to the study populations in **Publication 2** (pre-frailty: 43.1 % and frailty: 19.2 %) and **Publication 3** (pre-frailty: 41.7 % and frailty: 22.1 %). In contrast, in the Cardiovascular Health Study (CHS; N = 5317, age: 65-101 years), 7 % of the cohort were frail and 47 % were pre-frail [5]. In the Atherosclerosis Risk in Communities study (ARIC study; N = 4987), 6.8 % of individuals were classified as frail [37]. In the Progetto Veneto Anziani (N = 2925, ≥ 65 years) study, 7.6 % were frail and 49.3 % were pre-frail [25]. Considering the prevalence of single European countries, there is a north-to-south gradient with lower prevalence in northern countries [22]. Associations with frailty status were observed for the different cohorts in **Publication 2** (two French cohorts) and **Publication 3** (two French, one Spanish and one Italian cohort), and thus, subsequent statistical analyses where adjusted for cohort. Frailty prevalence and incidence might also differ because of varying frailty measurements and definitions, and therefore, comparisons between studies might be difficult. Additionally, different prevalence occurs because frailty status is associated with several socio-demographic factors (**Figure 9**), which should be considered when investigating this syndrome, and which will be discussed in the following.

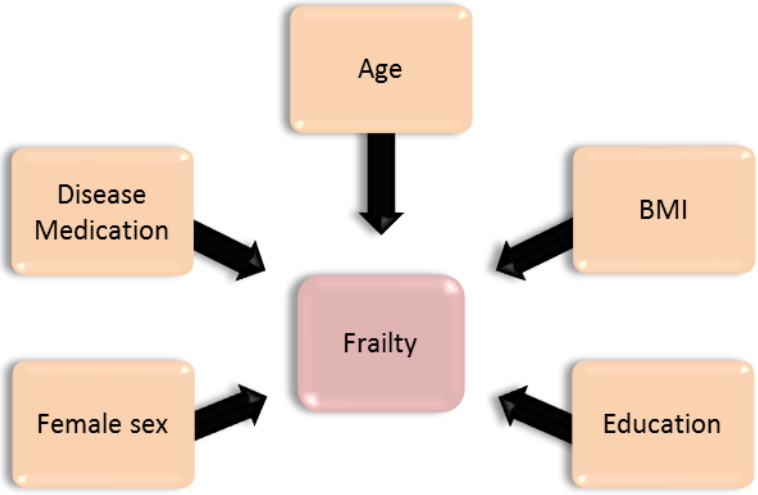

Figure 9. Possible confounders in the analyses of frailty. Orange boxes represent possible confounders for the frailty syndrome; red box represents the frailty syndrome. Abbreviation: BMI, body mass index.

Frailty prevalence was shown to increase with each 5-year age group in the CHS [5], and was higher in the 65+ years group than in the 50-64 years group in SHARE (N = 16,584) [22]. In the ARIC study, frail individuals were older (mean age: 78.0 years) than non-frail participants (75.4 years) [37]. Accordingly, frail individuals were significantly older than pre-frail and robust individuals in **Publications 2** and **3**. Therefore, the age of individuals was considered in statistical analyses in both publications, as was the sex of individuals since female sex was associated with a higher occurrence of frailty (**Publications 2** and **3**). Frailty prevalence was higher in women (7.3 %) than in men (4.9 %) in the CHS [5]. In SHARE, prevalence of frailty and pre-frailty was higher in women (5.2 % and 42.0 %, respectively) than in men (2.9 % and 32.7 %, respectively) in the 50-64 years group, as well as in the 65+ group, showing higher frailty and pre-frailty (21.0 % and 42.7 % respectively) prevalence women compared with men (11.9 % and 41.9 % respectively) [22]. In the ARIC study, frail individuals were more likely to be women than non-frail individuals [37], and the Progetto Veneto Anziani study found higher proportions of women in the frail and pre-frail groups [25].

Additionally, BMI of individuals might influence frailty prevalence and incidence. In **Publications 2** and **3**, BMI was similar between the three frailty groups. However, after categorizing individuals into normal weight (BMI < 25 kg/m^2), overweight (BMI: 25-29.9 kg/m^2) and obese (BMI ≥ 30 kg/m^2), frail individuals were highly prevalent in the obese group in **Publication 2**. Overweight individuals showed a lower prevalence of frailty

compared to normal weight and obese individuals in the English Longitudinal Study of Ageing (N = 3055, ≥ 65 years) [218]. In two Spanish population-based cohorts (Seniors-ENRICA: N = 1801, ≥ 60 years; Toledo Study for Healthy Ageing (TSHA), N = 1289, ≥ 65 years), normal weight compared with overweight was associated with an increased risk of frailty in the TSHA cohort after 3.5 years follow-up period; however, this was not the case in the Seniors-ENRICA cohort [75]. Obese individuals of these Spanish cohorts were at higher risk to become frail compared to overweight individuals during this follow-up period. Furthermore, overweight individuals and obese individuals which were non-frail at baseline, were at a higher risk of becoming pre-frail and frail during follow-up (mean 22 years) compared with normal weight individuals (N = 1119; Mini-Finland Health Examination Survey) [219]. In **Publication 3**, statistical models were adjusted for height and weight instead of BMI (in **Publication 2**), since BMI was not associated with frailty and both height and weight differed between the three frailty groups.

Frailty prevalence was positively associated with a lower educational level (and lower income) in the CHS [5] and in the ARIC [37] cohorts. Furthermore, frailty prevalence was found to be highest in individuals with lowest education (primary education or less) compared with individuals having secondary or tertiary education in independently living Dutch individuals (The Older Persons and Informal Caregivers Survey Minimum DataSet; ≥ 55 years) [24]. This was also true for frail individuals in **Publication 2** showing a higher prevalence of low education level (no schooling or primary education) than intermediate-to-high education level (secondary and/or vocational education and higher education). Therefore, statistical analyses were adjusted for education in **Publication 2**, but not in **Publication 3**.

Frailty was associated with poorer health, more co-morbid chronic diseases, lower cognitive capability and higher prevalence of depression in the CHS [5]. Multi-morbidity is defined by the presence of ≥ 2 long-term conditions that cannot be cured but can be controlled by medication or other treatment [220]. Most frail individuals are multi-morbid, but not vice versa. Multi-morbidity increases with age and there can be coexisting conditions like frailty and polypharmacy, and associations with frailty [220]. Frail individuals were more likely to have diabetes, CVD, anemia, and elevated levels of CRP [37]. Thus, statistical models were adjusted for multi-morbidity as well as for intake of medication in **Publication 2**. Here, frail individuals reported a higher intake of medication than robust and

pre-frail individuals. Accordingly, frail individuals had significantly higher medication intake than non-frail individuals and hyper-polypharmacy (≥ 10 drugs) was also more common in frail individuals [37]. In two French studies (N = 2286, ≥ 70 years; and N = 1890, ≥ 65 years), individuals taking polypharmacy (5-9 drugs) and hyper-polypharmacy were more likely to be pre-frail and frail compared to individuals taking ≤ 4 drugs and, polypharmacy was positively associated with the number of frailty criteria [217, 221]. However, no frailty classification was performed [221] and no objective measures of grip strength and walking speed were possible [217, 221]. In a German cohort (N = 3058, age: 57-84 years), polypharmacy and hyper-polypharmacy were positively associated with frailty prevalence at baseline as well as with frailty incidence after three years follow-up period [222].

Finally, in **Publications 2** and **3**, statistical analyses to investigate 3-MH and its ratios regarding their potential as biomarkers for frailty (risk), as well as associations of redox biomarkers and micronutrients with frailty where adjusted considering the aforementioned possible confounders.

3-MH, specific 3-MH ratios and redox status as potential biomarkers for frailty

Previously described biomarkers for the detection of muscle remodeling, muscle mass or muscle function/dysfunction were procollagen type III N-terminal peptide, CAF and myokines [9]; however, investigations on the associations of these biomarkers with the frailty syndrome in human studies are lacking. Several other biomarkers were described in the context of muscle mass, muscle function and the frailty syndrome (**Table 4**). These biomarkers were investigated mainly in cross-sectional and less in longitudinal studies and results were inconsistent. The inflammatory biomarkers CRP and TNF-α were not significantly associated with the frailty syndrome [223] and no association of advanced glycation end products with muscular strength occurred in hemodialysis patients [224]. Thus, further studies especially longitudinal and in frail cohorts are needed. Additionally, the oral administration of d_3-creatine and the resulting d_3-creatinine enrichment in urine was used as an estimation of muscle mass [225, 226]. However, due to its dependence on the availability of d_3-creatine, this method might be challenging and expensive for clinical routine; and validation and evaluation in the context of frailty is lacking.

Table 4. Potential biomarker for muscle mass, muscle function and frailty.

Category	Biomarker
Inflammatory cytokines	C-reactive protein [227-230]
	Interleukin-6 [223, 227-233]
	Interleukin-1-receptor antagonist [228]
	Tumor necrosis factor-α [230-232]
Clinical parameters	Hemoglobin [233, 234]
	Albumin [229, 230, 232, 235, 236]
	Creatinine [232]
Hormones	Dehydroepiandrosterone sulfate [237]
	Testosterone [238]
	Insulin-like growth factor-1 [233]
	Leptin and adiponectin [229, 233]
Oxidative damage markers	Advanced glycation end products [239]

Previously, urinary 3-MH and 3-MH/Crea was postulated as biomarker for muscle protein breakdown and both were shown to be elevated in muscle wasting disease. In two studies of Duchenne muscular dystrophy, higher 3-MH/Crea ratio was found in patients compared with controls [112, 114]. In rats, urinary and serum 3-MH were identified as biomarkers of drug-induced skeletal muscle necrosis [149]. Urinary 3-MH/Crea excretion detected an increased muscle protein turnover, which was associated with muscle weakness and inflammation in patients (N = 41) after cardiac surgery [240]. In the context of frailty diagnosis, higher urinary 3-MH/Crea excretion in frail women compared to healthy women was found suggesting an increased muscle protein breakdown [241].

In **Publication 1**, an association between plasma 3-MH and muscle status was shown in young vegetarians. Results from **Publication 2** showed that frail and pre-frail individuals had higher plasma 3-MH concentrations than robust individuals. Plasma 3-MH/Crea ratio and the newly introduced 3-MH/eGFR ratio were higher in frail individuals than in robust individuals in **Publication 2**. Since 3-MH concentrations and 3-MH/Crea ratios in plasma have rarely been investigated and the 3-MH/eGFR ratio was used for the first time regarding frailty diagnosis and risk assessment, no further comparison with existing literature is possible. Nevertheless, these biomarkers were evaluated in linear regression analyses, which showed positive associations between biomarker concentrations and frailty, even after adjustments to cohort, sex, age, BMI, education, multi-morbidity, and intake of medications, meat and fish (**Publication 2**).

Furthermore, 3-MH and 3-MH/eGFR were confirmed as potential biomarkers for frailty since frail individuals were more likely to be present in higher 3-MH and 3-MH/eGFR quintiles compared to robust individuals, in bivariate and multivariate logistic regression analyses (**Publication 2**).

Frailty might be influenced by micronutrient intake as well as micronutrient and OS status since it was previously described that micronutrients like VD, carotenoids and α-tocopherol, as well as redox biomarkers like PrCarb were linked to muscle wasting, impaired muscle function and sarcopenia [91].

Both pre-frail and frail participants showed lower β-cryptoxanthin, VD_3 and β-carotene concentrations than robust individuals and frail individuals had lower lycopene and lutein/zeaxanthin concentrations compared to robust and pre-frail individuals (**Publication 3**). Additionally, both pre-frail and frail individuals had higher PrCarb concentrations than robust individuals and frailty was positively associated with high PrCarb concentrations (**Publication 3**). Results from **Publications 2** and **3**, raise the assumption that there might be associations of OS biomarkers and micronutrients with 3-MH and its ratios subsequently possibly confirming the association of oxidative stress and an elevated muscle protein turnover; however, this has to be investigated in further studies.

Intake of fruits and vegetables was positively associated with low levels of OS biomarkers [242] and a low risk for frailty in individuals older than 60 years [75]. A higher likelihood to be frail than to be non-frail was shown for low VD and vitamin E intakes in individuals older than 65 years [78], and low VD was linked to a reduced muscle mass and physical performance in pre-frail and frail individuals (> 65 years) [85]. Inadequate micronutrient concentrations might be a result of a low fruit, vegetable, nut, seed and oil intake, particularly in advanced-aged individuals. Oral health status is one factor for dietary decision-making especially in higher-aged individuals, and a poor oral health status increases with age and was linked to frailty. Previously, cross-sectional positive associations between masticatory ability and oral pain with frailty criteria [243], as well as longitudinal associations between oral health and frailty [244] were described. Furthermore, impaired gastric emptying and digestive function might attenuate influences on plasma micronutrients as well as on 3-MH and its ratios in older individuals. Impairments in the gastrointestinal tract may result in reflux, heartburn or constipation, which might

subsequently lead to a reduced intake of fruits and vegetables. A decreased ability for older persons to go shopping or to prepare meals by themselves might be a further reason. However, data on the fruit and vegetable intake were not available for **Publication 3**, and thus, no adjustment for fruit and vegetable intake was possible. Inadequate exposure to sunlight, physical inactivity or frailty itself might result in insufficient VD levels. Fruit and vegetable intake as well as VD production in the skin by sunlight exposure underlie seasonal variations; therefore, statistical analyses were adjusted for season of blood sampling in **Publication 3**.

Similar to **Publication 3**, results from the Women's Health and Aging Study I and II (only women, age > 65 years) showed that frail women had lower plasma concentrations of VD, retinol, α-carotene, β-carotene, lycopene, lutein/zeaxanthin and β-cryptoxanthin compared to non-frail women [79]. In contrast, no associations between VD_3 and frailty status were observed in a study with individuals of the FRAILOMIC Initiative [77]. Different VD_3-cutoffs for tertiles and the additional pre-frail group used in **Publication 3** might explain these contradicting results. The lowest tertile of VD_3 (< 33.7 nmol/L) in **Publication 3** is in accordance to definitions for deficient or severely deficient VD_3 status, however, there exist different definitions for VD status [245]. Nevertheless, significant associations between frailty and micronutrient patterns low in vitamins A and E, and high in carotenoids were observed in individuals of the FRAILOMIC Initiative [77]. According to **Publication 3**, higher PrCarb concentrations in frail compared to non-frail individuals were previously shown in the TSHA cohort of FRAILOMIC [81]. Furthermore, higher OS levels were positively associated with frailty, unfortunately they did not measure PrCarb nor 3-NT as OS biomarkers [82] and thus, no further comparisons are possible. PrCarb concentrations correlated positively with age in robust participants, whereas no correlation of PrCarb with age was observed in frail individuals (**Publication 3**). Similarly, PrCarb was positively associated with age in advanced-aged individuals (61-85 years) compared with young individuals (21-40 years) [57] as well as in healthy persons (N = 80) between 18-85 years [58]. In contrast, PrCarb concentrations were not associated with age (65-95 years) in the TSHA cohort of FRAILOMIC [81].

Multivariable adjusted (cohort, season of blood sampling, gender, age, height, weight and smoking status) logistic regression analyses showed that pre-frailty and frailty were associated with low VD_3, low β-carotene and especially with low lutein/zeaxanthin

and β-cryptoxanthin concentrations, and that frailty was associated with low plasma α-tocopherol, α-carotene and lycopene concentrations (**Publication 3**). Frailty was significantly associated with lower total carotenoids, α-tocopherol and VD concentrations in logistic regression models, and the strongest association was found for β-carotene, lutein/zeaxanthin, and total carotenoids [79]. Results from **Publication 3** showed no associations of retinol and 3-NT with frailty status at all, which is in accordance with previous studies investigating individuals of FRAILOMIC and the InChianti cohort [77, 78]. Retinol is homeostatically regulated and therefore its circulating concentrations are considered a poor marker of vitamin A status [246].

Longitudinal data from the Women's Health and Aging Study I showed, that women in the lowest quartile of serum carotenoids were more likely to become frail during a three year follow-up period [80]. Furthermore, individuals (> 65 years) with insufficient VD levels (< 37.5 nmol/L) were at higher odds to become pre-frail as well as pre-frail/frail combined compared to individuals with adequate VD levels (≥ 75 nmol/L), during a follow-up period of three years [83]. No comparisons with these studies were possible, since longitudinal data were not available.

Possible confounding factors regarding 3-MH measures like muscle mass, meat/fish intake or renal function were considered by analyzing additional biomarkers like creatinine, 1-MH and eGFR, respectively, and calculating specific ratios (**Publication 1** and **2**; **Figure 10**). In **Publications 2** and **3**, a variety of possible confounders like demographic factors, health status as well as intake of meat, fish and medication, were included in the statistical analyses (**Figure 10**), and thus, strengthening the results. Furthermore, different lifestyles and a wide range of the society are represented in **Publications 1**, **2** and **3**, since cohorts from several European countries (Germany, France, Spain and Italy) including healthy as well as frail individuals and both young aged (20-30 years) and advanced-aged (≥ 65 years) individuals were investigated, and several redox biomarkers, and potential nutritional and muscle protein turnover biomarkers were measured, which is a unique feature of this thesis. Additionally, several socio-demographic factors as well as dietary and health data were available and further used to adjust the statistical models for possible confounders of the biomarkers of interest in **Publications 2** and **3**, further strengthening the presented results of this thesis.

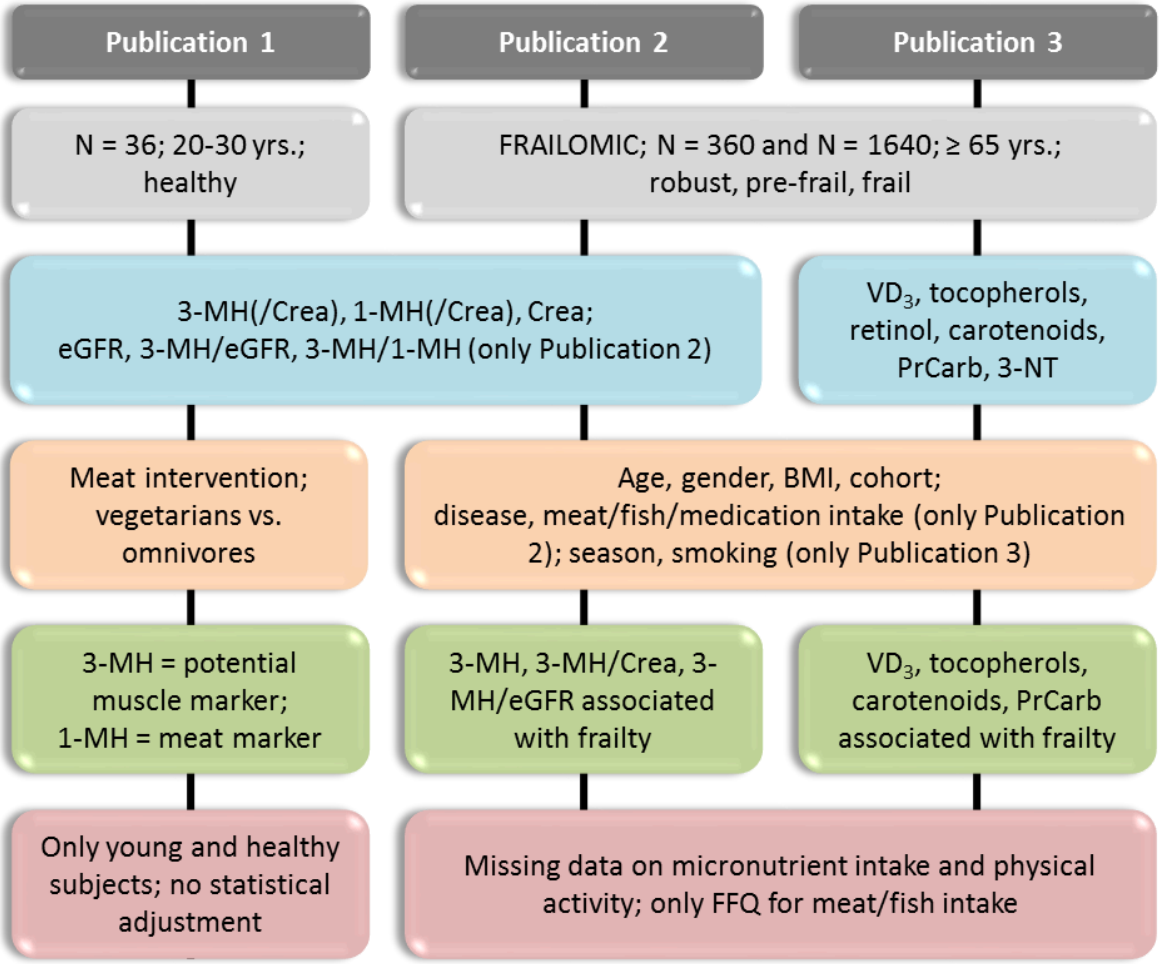

Figure 10. Biomarkers of muscle protein turnover and redox status as well as micronutrients in the context of frailty and its confounders. Grey boxes represent publications and cohorts used in this thesis; blue boxes represent biomarkers measured in plasma (eGFR was calculated using measured creatinine; 1-MH and 3-MH ratios were calculated); orange boxes represent possible confounders for either 3-MH or frailty syndrome or both and that were used in statistical models (except renal function and muscle mass) in Publications 2 and 3; green boxes represent results and conclusions of the publications; red boxes represent limitations of the publications. Abbreviations: 1-MH, 1-methylhistidine; 3-MH, 3-methhylhistidine; 3-NT, 3-nitrotyrosine; BMI, body mass index; Crea, creatinine; eGFR, estimated glomerular filtration rate; FFQ, Food Frequency Questionnaire; PrCarb, protein carbonyls; VD_3, vitamin D_3.

Outlook

The reliable analysis of 3-MH is of enormous importance to be used as a biomarker for frailty or elevated muscle protein turnover. Measurement reliability is dependent on a valid analytical technique and the plasma concentration is influenced by meat and fish intake, among others. The described UPLC-MS/MS method and results concerning the meat influence in **Publication 1** enables the further use of plasma 3-MH in clinical and research settings. However, the study population consisted only of young (20-30 years) healthy adults, and thus, the results on the impact of meat on plasma 3-MH should be evaluated in advanced-aged individuals, since their muscle metabolism and metabolism in general are different from young adults. Furthermore, adding additional metabolites like creatinine (as marker for muscle mass) or anserine (as additional biomarker for meat consumption) to the presented method would subsequently provide the opportunity for more informative analyses, e.g. meat and fish intake, or the nutritional impact on biomarkers.

In **Publication 2**, it was shown for the first time that higher 3-MH concentrations, and higher 3-MH/Crea and 3-MH/eGFR ratios in plasma were positively associated with frailty, and thus indicating that an elevated muscle protein turnover occurs in this condition. The newly introduced 3-MH/eGFR ratio opens new opportunities in frailty assessment. Additionally, these results strengthen the potential use of these biomarkers in the context of frailty or other muscle wasting diseases like sarcopenia. Future studies should evaluate the associations of these biomarkers with single frailty criteria. These biomarkers also need testing in combination with other possible biomarkers regarding muscle mass or muscle function since there is no single biomarker for frailty up to date [15]. Determining the associations between the 3-MH biomarkers and OS biomarkers as well as micronutrients could provide such a combinatory biomarker testing. Future studies should also include large-scale longitudinal cohorts to investigate how predictive 3-MH biomarkers are for frailty incidence since **Publication 2** provides only cross-sectional associations. This is also true for future studies regarding the investigated redox biomarkers in **Publication 3**. Plasma 3-MH concentrations as well as 3-MH/Crea and 3-MH/eGFR ratios need further investigations concerning their response to possible treatments of frailty like exercise and protein administration.

Results from **Publication 2** suggest that there might be threshold concentrations or ratios that can possibly diagnose frail individuals or identify individuals at a higher frailty risk. However, such possible thresholds need further evaluation in different cohorts in the future. Evaluating such thresholds or even finding "normal" values would be a major progress for clinical handling of frailty. Considering the possible influence of polypharmacy or specific drugs on frailty and plasma 3-MH, future investigations on the role of medication and types of drugs would be a great benefit. Including comorbidities or diseases into these determinations could provide additional knowledge on the multi-factorial pathogenesis of frailty. Future studies should consider physical activity and nutritional data of individuals to gain more information of their influences on frailty and potential biomarkers, as well as to be able to control for these parameters. Future studies might also consider measuring 3-MH-related biomarkers in plasma and in urine together to evaluate if possible confounders are similar for both matrices.

Conclusion

3-Methylhistidine was previously described as possible biomarker for muscle protein turnover when individuals stay on a meat-free diet for at least 2-3 days prior to blood samplings. However, this cannot be guaranteed especially in advanced-aged individuals or in large-scale research or clinical studies. Nevertheless, in **Publication 1**, a white meat intervention and an omnivorous eating behavior had only a weak influence on 3-MH and 3-MH/Crea in plasma. Therefore, it is suggested that 3-MH and 3-MH/Crea can provide reliable data and can function as biomarkers for muscle protein turnover when subjects do not consume meat 24 hours before blood sampling. Additionally, a white meat intervention and an omnivorous diet had a strong impact on plasma 1-MH and 1-MH/Crea in **Publication 1**. This confirms their potential use as biomarkers for meat consumption by signaling short-term meat consumption and displaying exogenous 3-MH in plasma.

Publication 2 presents novel data concerning 3-MH, 3-MH/Crea and 3-MH/eGFR in plasma, and their potential use as biomarkers for frailty. This was the first publication to use the 3-MH/eGFR ratio and to assess associations of plasma 3-MH and its ratios in individuals classified into robust, pre-frail and frail. Results showed that these biomarkers differ between robust, pre-frail and frail participants leading to the assumption of an elevated muscle protein breakdown in frail individuals. These biomarkers were also positively associated with the frailty status of individuals. Additionally, individuals were more likely to be frail than robust with every increase in 3-MH and 3-MH/eGFR quintile. In conclusion, plasma 3-MH, 3-MH/Crea and 3-MH/eGFR might be potential biomarkers to identify frail individuals or individuals with higher odds to be frail than robust. Additionally, there might be 3-MH and 3-MH/eGFR threshold concentrations and ratios that can identify frail individuals or those individuals that are more likely to be frail.

Results from **Publication 3** show that low plasma VD_3, β-carotene, lutein/zeaxanthin and β-cryptoxanthin concentrations and high plasma PrCarb concentrations are positively associated with pre-frailty and frailty. Thus, it is concluded that an impaired redox status is associated with frailty and that a diet rich in micronutrients, which might improve redox status could be supportive in frailty prevention.

Regarding several criteria that should be fulfilled for biomarkers (**Table 3**), data of **Publications 1** and **2** showed promising results for plasma 3-MH and its ratios as well as for

1-MH. **Publication 1** described an accurate, specific, sensitive and reproducible method for the detection and quantification of 3-MH and 1-MH that included a simple and fast sample preparation which enabled a high sample throughput. **Publication 2** showed that diseased (frail) individuals can be discriminated from healthy (robust) individuals based on 3-MH concentrations, furthermore, 3-MH was also cross-sectionally associated with frailty. Additionally, 3-MH is only weakly biased by meat intake as possible confounder as described in **Publication 1** after a white meat intervention. In **Publication 2**, associations of plasma 3-MH and 3-MH/eGFR with frailty did not change statistical adjustments for several confounders, suggesting no bias of these confounders, however, this has to be confirmed in future studies. Thus, plasma 3-MH and its ratios fulfill several required criteria to function as a biomarker.

In conclusion, these novel and promising results provide a solid basis for further studies evaluating plasma 3-MH, 3-MH/Crea and 3-MH/eGFR as biomarkers for frailty, frailty risk and an elevated muscle protein turnover.

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Lebenslauf

Die Seite 97 (Lebenslauf) enthält persönliche Daten. Sie ist deshalb nicht Bestandteil der Online-Veröffentlichung.

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

Nuthetal, den _____

Bastian Max Kochlik