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NUTRIENTS AND NUTRITION IN OLDER ADULTS: SEVERAL OPEN QUESTIONS AND SOME ANSWERS

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To my Family,

my greatest strength

INDEX

Page

1. INTRODUCTION

Nutrition is the set of integrated processes by which cells, tissues, organs and the whole body acquire the energy and nutrients for physiological structure and function, which is achieved at body level through dietary supply, and transformation of substrates and cofactors necessary for metabolism. An appropriate nutrition starting early in the life course has a significant impact on health as it could determine the future resilience of the individual to stresses and susceptibility to disease. Not only the quantity, but also the quality of the diet is important to ensure healthy growth and the appropriate proportions of lean and fat mass. Furthermore, nutrition is not simply a matter of diet, but should also take into account physical activity level, stressors and underlying conditions (physical, behavioral, social), which modulate nutrient availability and handling.

There is a clear evidence of the important role of nutrition in primary and secondary prevention for several diseases. With respect to primary prevention, this role is related both to specific dietary patterns, such as the Mediterranean diet, but also to single micronutrients, such as poli-unsaturated fatty acids (PUFAs). Besides Mediterranean diet, other nutritional patterns, such as some of spices used in in Asian cultures that have beneficial effects on human health and are commonly used in the Ayurveda, the Indian traditional medicine. One of these spices, curcumin, has also been studied in the occidental traditional medicine for its anti-inflammatory, anti-oxidant and anti-cancer properties, and new compounds derived from curcumin have been studied for the treatment of cancer and other diseases. On the other hand, also micronutrients usually introduced with the diet, such as vitamin D, or nutritional status *per-se*, have an important role in secondary prevention. Furthermore, nutrition can have a role in the context of other diseases, such as anemia in influencing biomarkers concentration in heart failure.

While the role of nutrition seems to be well established, some questions remain open, especially in older adults, that are a population usually less studied respect to younger adults. In fact, if we consider the "classical" associations, such as sodium intake and cardiovascular diseases, most of the studies did not include older adults, a population in which the coexistence of multiple diseases,

polytherapy, frailty, high prevalence and risk of disability, might influence these associations. Therefore, an association evident in young adults might lack or being more evident in older adults, thus changing dietetic indications in this specific population.

The aim of this thesis was to study the role of nutrition in influencing clinical outcomes in older adults, paying particular attention to:

- The role of micronutrients in primary prevention; with respect to this, we studied the association between sodium and PUFA intake and mortality in older adults
- The role of micronutrients and nutritional status in influencing outcomes; with respect to this, we studied the association between vitamin D and nutritional status in functional improvement in older adults admitted to rehabilitation settings and the association between nutritional status and functional status and outcomes in older adults affected by chronic heart failure
- The role of nutrition and nutrients in the context of other diseases; with respect to this, we studied the role of curcumin in lung diseases and melanoma and the role of anemia in influencing concentration of natriuretic peptides in older adults.

In the following sections will be reported all the studies developed during the PhD training on the previously reported main topics.

2. THE ROLE OF MICRONUTRIENTS IN PRIMARY PREVENTION

a. ASSOCIATION BETWEEN SODIUM EXCRETION AND CARDIOVASCULAR DISEASE AND MORTALITY IN THE ELDERLY: A COHORT STUDY

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Original Study

Association Between Sodium Excretion and Cardiovascular Disease and Mortality in the Elderly: A Cohort Study

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ABSTRACT

Objective: High dietary sodium intake is a risk factor for cardiovascular events and death. Recently, a J-shaped correlation between sodium intake and adverse outcomes has been shown. The evidence on the association between sodium intake and cardiovascular outcomes in the elderly is scant. The objective of this study was to evaluate the correlation between sodium intake and cardiovascular events and mortality in an elderly population, taking into account frailty status.
Design: Cohort study of community dwelling older people enrolled in the InCHIANTI (Invecchiare in Chianti - Aging in the Chianti) study from 1998 to 2000 and followed-up for 9 years.
Setting: Two communities in Tuscany, Italy.
Participants: A total of 920 participants 65 years of age and older, with 24-hour urinary sodium excretion data.
Measurements: Nine-year mortality and incident cardiovascular events were analyzed using Cox and nonlinear log-binomial models, stratified by frailty status. Sensitivity analysis in participants without hypertension and cardiovascular diseases was performed.
Results: Mean age of the population was 74.5 years (standard deviation 6.99); 55.4% were women. There was a bi-modal association between sodium excretion and mortality, with risk increasing only below sodium excretion of 6.25 g/d [hazard ratio (HR) 1.29, 95% confidence interval (CI) 1.20-1.38], confirmed in the adjusted model (HR 1.12, 95% CI 1.04-1.22). These results were confirmed in participants without cardiovascular diseases. After stratification for frailty phenotype, the association was stronger in frail participants (adjusted HR 1.23, 95% CI 1.02-1.50 vs HR 1.11, 95% CI 1.01-1.22 in robust participants). There was no association between 24-hour sodium excretion and 9-year incidence of cardiovascular diseases (adjusted risk ratio 0.96, 95% CI 0.90-1.02).
Conclusions: Reduced sodium excretion is associated with increased mortality in a sample of community-dwelling older people, especially among the frail participants. High levels of sodium excretion are not associated with adverse outcomes in this population; therefore, sodium restriction might not be beneficial in older people.

INTRODUCTION

Excess dietary sodium intake is considered an important cause of hypertension,[1] and very recently an urinary sodium excretion > 3.7 g/day has been associated with subclinical cardiovascular disease.[2] Several studies have shown a reduced risk of cardiovascular events in

people with lower sodium intake,[3] and reducing sodium intake is recommended for cardiovascular prevention.[4] There is evidence, however, that a J-shaped or U-shaped relationship may exist between sodium intake and adverse outcomes, with risk increasing when sodium excretion is below ~ 3 g/day or above ~ 5 g/day. [5,6] The increased risk associated with may be related to increased activity of renin-angiotensin-aldosterone system and sympathetic nerve activation.[7]

To our knowledge, only one study evaluated this association in an elderly population, finding no relationship between sodium intake and adverse outcomes.[8] In this study, sodium intake was evaluated using a food frequency questionnaire rather than 24-h urinary sodium excretion, that is considered the most accurate method to estimate sodium intake.[9] Thus, the evidence on this issue in the elderly are still not clear. In this population comorbidities, poly-pharmacotherapy, and frailty may act as an effect modifier, in the same way as it happens for hypertension, that does not seem to be associated with mortality in people with reduced gait speed.[10] The objective of our study was to evaluate the correlation between sodium intake, estimated using sodium urinary excretion, mortality and cardiovascular events in an elderly population. The association was analyzed separately in people with and without frailty.

METHODS

Data source and study design

We analyzed data from the longitudinal InCHIANTI study.[11] The baseline study was supported by the Italian Ministry of Health and also partly supported by the US National Institute on Aging. After obtaining informed consent, participants were randomly selected from the populations of two town areas; data collection started in September 1998 and was completed in March 2000. The eligible participants were interviewed at home by trained study researchers, the interview was followed by a physical examination at the study clinic and laboratory analysis. Follow-up visit were scheduled at 3, 6, and 9 years. The Italian National Institute of Research and Care on Aging Ethical Committee

approved the study protocol.

Sample selection

From 1170 participants with available 24-h urinary sodium excretion data, we excluded participants younger than 65 years (N: 250). Follow up data at 9 years to were available for 920 participants. For the analysis on incident cardiovascular events we selected participants without history of cardiovascular disease at baseline (N: 514).

Definition of exposure and outcome

Sodium intake was estimated using the 24-h sodium urinary excretion. On the day of the study visit, participants were provided with a plastic bottle containing 1 g of boric acid as preservative, and instructed to collect all the urine produced in the following 24 hours, making the maximum effort to avoid dispersing urine during the collection period.

We considered mortality for all causes and incident cardiovascular events (angina pectoris, myocardial infarction, heart failure, and stroke) as outcome measures. Cardiovascular events were ascertained using a questionnaire and reviewing clinical documentation in the follow-up visits. Data on mortality were collected from mortality registers.

Analytic approach

We reported the characteristics of the study sample using descriptive statistics (mean and standard deviation for continuous variables, proportion for categorical variables), according to quartiles of 24-h urinary sodium excretion. We took into account associated diseases (e.g. hypertension, diabetes), cigarette smoke (pack-years), blood pressure, total cholesterol, and estimated creatinine clearance (CKD-EPI). Total caloric intake, macronutrients and micronutrients intake, and alcohol intake were evaluated using the European Prospective Investigation Into Cancer and nutrition (EPIC) Questionnaire, a food frequency intake questionnaire validated in a Italian elderly population[13]. Finally, we took into account years of education and physical activity.

Mortality risk across quartiles of sodium excretion was calculated using the Kaplan-Meier method. We used Cox regressions to evaluate the relationship between sodium excretion and mortality. Both

linear and polynomial (restricted cubic splines) models were fitted, the goodness of fit of these models was evaluated using the log-likelihood test and the best fitting unadjusted model was then adjusted for potential confounders, selected on the base of the clinical significance, prior knowledge, and results of the univariate analysis. To explore the different role of demographic and clinical variables, we first adjusted for age and sex, and then for the other potential confounders (education, CKD-EPI, pack/year, hypertension, diabetes, BMI, caloric intake/body weight, anti-hypertensive drugs and diuretics). Finally, we repeated the analysis in participants with and without frailty, defined according to Fried's frail phenotype as the presence of three or more of the following criteria: unintentional weight loss (10 lbs in past year), self-reported exhaustion, reduced grip strength, slow walking speed, and low physical activity.[14] Since cardiovascular diseases (angina pectoris, myocardial infarction, heart failure, and stroke) and hypertension may significantly influence the relationship between sodium excretion and mortality, we planned a sensitivity analysis that excluded participants with these conditions at baseline (N: 312).

The relationship between sodium excretion and incident cardiovascular disease was analyzed in the subgroup of participants without prevalent cardiovascular disease (angina pectoris, myocardial infarction, heart failure, and stroke), ascertained by investigating the clinical history and documentation (N: 514). This relationship was evaluated by fitting linear log-binomial regression models in order to directly estimate relative risks. To allow for a non-linear relationship between exposure and outcome, we compared the goodness-of-fit of quadratic log-binomial and linear models using the Akaike Information Criteria (AIC). All analyses were performed using R version 3.3.3 (R Foundation for Statistical Computing, Wien, Austria).

RESULTS

The mean age of the sample was 74.5 years (SD 6.99); 55.4% were women. Cut-off for sodium excretion quartiles were 3.70 g/day, 5.44 g/day, and 7.04 g/day. Participants in the first (i.e., lower) quartile of sodium excretion were older (mean age 77.4, SD 7.6 vs 72.5, SD 6 in the fourth quartile,

P<0.001), more frequently women (68% vs 38% in the fourth quartile, P<0.001), and more sedentary (74% vs 52% in the fourth quartile, P<0.001). They also had lower estimated GFR and body mass index (BMI), and lower prevalence of diabetes, and higher blood systolic blood pressure and prevalence of dementia. Participants in the fourth quartile of sodium excretion had higher intake of energy (2093.5 kcal/day compared 1784.2 kcal/day, P<0.001), micronutrients, including sodium, potassium, and vitamins, and also had higher intake of alcohol (12.3 g/day in the first quartile vs 18 g/day in the fourth, P=0.001) (table 1).

Table 1
General Characteristics of the Population Distributed by Quartiles of 24-Hour Sodium Excretion

	I Quartile (n = 230)	II Quartile (n = 230)	III Quartile (n = 230)	IV Quartile (n = 230)	*P*
Age, mean (SD), years	77.4 (7.6)	74.9 (6.9)	73.2 (6.4)	72.5 (6)	<.001
Female sex, No. (%)	156 (68)	136 (60)	125 (55)	88 (38)	<.001
Education, mean (SD), years	5.3 (3.6)	4.9 (2.9)	5.5 (3.4)	5.6 (3)	.094
Sedentary, No. (%)	170 (74)	149 (65)	132 (58)	120 (52)	<.001
BMI, mean (SD), kg/m^2	26.6 (4)	27.4 (4.4)	27.5 (3.9)	28.1 (3.6)	.002
CKD-EPI, mean (SD), mL/min/1.73 m^2	68.4 (15.6)	71.1 (14.2)	72.5 (12.8)	72.5 (13)	.005
Total cholesterol, mean (SD), mg/dL	215.6 (36.8)	217.5 (40.5)	218.4 (40)	218.8 (38.1)	.819
Systolic blood pressure, mean (SD), mmHg	155.6 (20.4)	148.9 (17.8)	148.6 (18.6)	149.5 (20.3)	<.001
Diastolic blood pressure, mean (SD), mmHg	85.6 (8.2)	83 (7.8)	83.9 (9)	83.7 (8.6)	.01
Smoke, mean (SD), pack/year	11.2 (21)	10.4 (19.5)	13.6 (21.8)	16 (22.3)	.021
Hypertension, No. (%)	145 (63)	149 (65)	133 (58)	141 (61)	.462
Diabetes, No. (%)	26 (11)	27 (12)	21 (9)	42 (18)	.021
Peripheral Arteriopathy, No. (%)	36 (16)	29 (13)	21 (9)	23 (10)	.128
COPD, No. (%)	19 (8)	17 (7)	14 (6)	21 (9)	.652
Metabolic syndrome, No. (%)	48 (21)	59 (26)	53 (23)	57 (25)	.635
Dementia, No. (%)	18 (8)	12 (5)	4 (2)	4 (2)	.002

COPD, chronic obstructive pulmonary disease.

The median follow-up time was 9 years, with a cumulative follow-up time of 7009 years. Over this time, 281 participants died, with an incidence rate of 4.01/100 person-year (95% CI 3.57-4.49) and a cumulative risk of 31% (95% CI 28%-34%). There was no difference in the mortality risk between the third and the fourth quartile (22.7% and 20.2%, respectively), while participants in the first two quartiles had a clearly increased risk (46.2% and 34.7%, respectively) (figure 1).

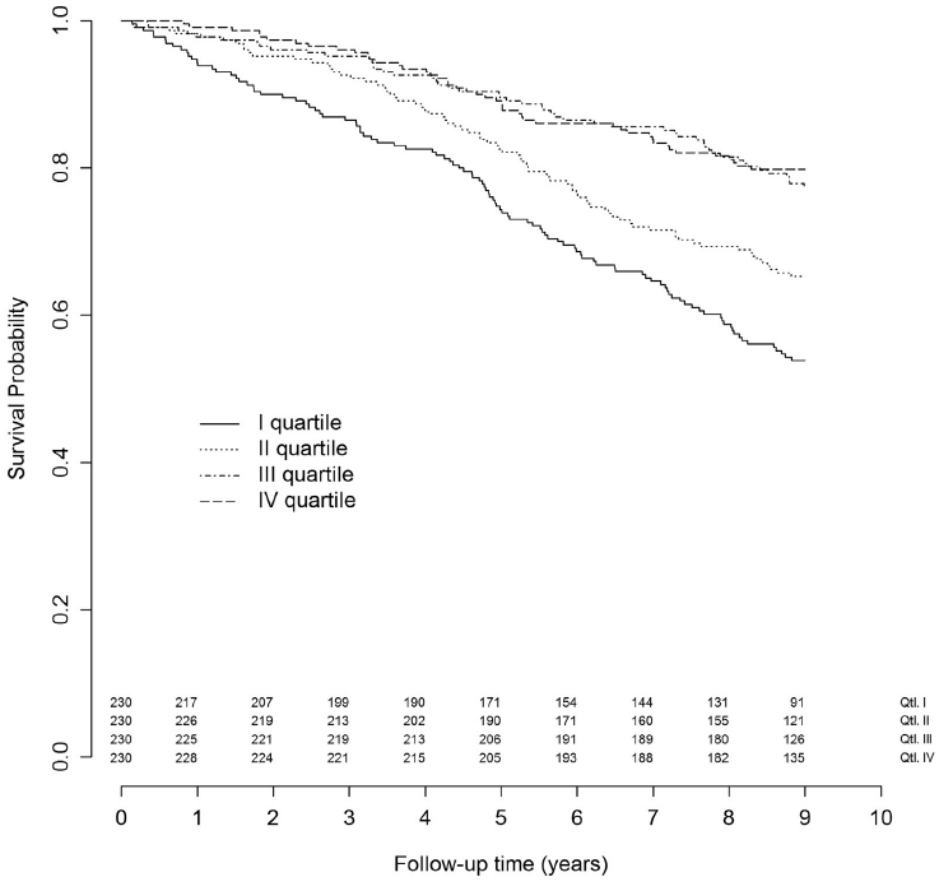

Fig. 1. Six-year all-cause mortality according to 24-hour sodium excretion quartiles.

The analysis of the relationship between sodium excretion and risk of mortality using a non-linear Cox model (restricted cubic spline with 3 knots) showed that a linear model was not appropriate to describe the data, while a linear spline allowing an increase in risk when sodium excretion is below 6.25 g/day seemed to provide an adequate fit (figure 2).

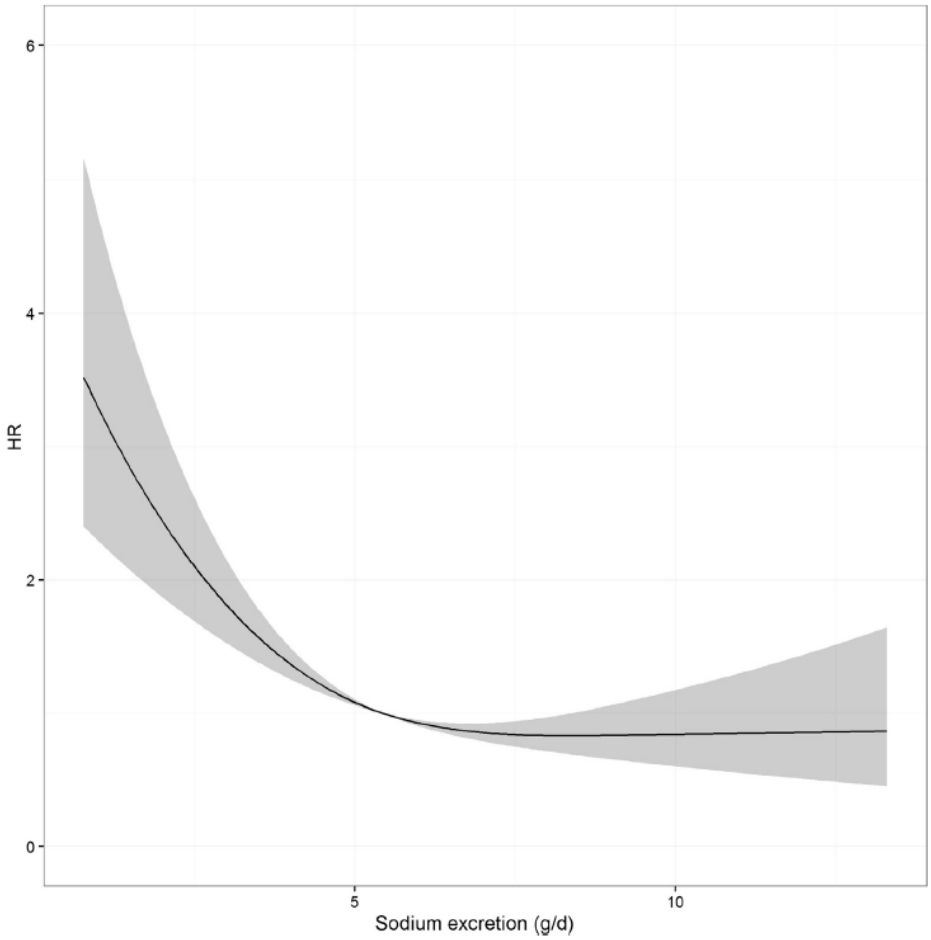

Fig. 2. Twenty-four-hour sodium excretion and HR for all-cause death

The likelihood ratio obtained with a linear, restricted cubic spline, or linear spline models were 36.7, 48.1, and 49.4, respectively. Accordingly, we chose to use the linear spline model with one knot at 6.25 g/day because it allows an easy interpretation of the coefficient, that express the increase of the risk for 1-unit decrease of sodium excretion below 6.25 g/day.

We found an association between decreasing sodium excretion and mortality (HR: 1.29; 95% CI 1.20 to 1.38), that was reduced but still significant after adjustment for age and sex only (HR: 1.15; 95% CI: 1.07 to 1.24), and after further adjustment for education, estimated GFR, systolic blood pressure, smoking history, hypertension, diabetes, BMI, caloric intake/body weight ratio, and anti-hypertensive drug and diuretics (HR: 1.12; 95% CI: 1.04 to 1.22). Compared to robust participants, frail participants were older (mean age 80.9 years, SD: 6.9 vs 73.7 years, SD: 6.5), more frequently women (64% vs 54%), and more frequently had a low level of physical activity (95% vs 58%), and

comorbidities, such as diabetes, peripheral arteriopathy, dementia; there was no difference in total energy intake/kg of body weight (28.9 kcal/kg, SD: 8.4 vs 26.6 kcal/kg, SD: 7.5 in frail vs robust group). After stratification for frailty phenotype, we found that the crude association between decreasing daily sodium excretion and mortality was more evident in robust (HR: 1.25, 95% CI: 1.15 to 1.30) compared to frail participants (HR: 1.15; 95% CI: 1 to 1.33). After adjustment for potential confounders, however, among frail participants the risk associated with decreased sodium excretion was higher (HR: 1.23, 95% CI 1.02 to 1.05) compared to robust participants (HR: 1.11, 95% CI: 1.01 to 1.22) (table 2). In the subsample of participants without prevalent cardiovascular disease or hypertension at baseline we observed a stronger association between sodium excretion and mortality (crude HR: 1.69, 95% CI 1.18-1.56; adjusted HR: 1.32, 95% CI 1.12-1.56). A stratified analysis for frailty was not possible for the small number of frail people in this subsample (N: 24).

Table 2
Association Between Decreasing 24-Hour Sodium Excretion and Mortality

	Overall	Frail	Robust
N	920	87	812
N events	281	69	197
Person/y	7009	783	6430
Crude model HR (95% CI)	1.29 (1.20-1.38)	1.15 (1-1.33)	1.25 (1.15-1.30)
Adjusted model 1 HR (95% IC)	1.15 (1.07-1.24)	1.19 (1.02-1.39)	1.11 (1.02-1.22)
Adjusted model 2 HR (95% IC)	1.12 (1.04-1.22)	1.23 (1.02-1.50)	1.11 (1.01-1.22)

Model adjusted 1: adjusted for age and sex.
Model adjusted 2: Adjusted for age, sex, education, CKD-EPI, systolic blood pressure, pack/y, hypertension, diabetes, BMI, caloric intake/body weight, and antihypertensive drugs and diuretics.

Over the 9year follow-up, we observed 169 cardiovascular events, with an incidence rate of 3.75/100 person-year (95% CI: 3.23 to 4.33). Fitting log-binomial models of 24-h sodium excretion vs incident cardiovascular disease, we found no differences in the goodness of fit comparing a linear (AIC=651.3) and a quadratic (AIC=653.2) model, suggesting a linear relationship. There was

a weak inverse association between 24-h sodium excretion and 9-year incidence of cardiovascular diseases (RR: 0.95; 95% CI: 0.90 to 1), that was not confirmed after adjustment of the model for possible confounders (age, sex, education, CKD-EPI, systolic blood pressure, pack/year, hypertension, diabetes, BMI, caloric intake/body weight, anti-hypertensive drugs and diuretics), with a RR of 0.96 (95% CI: 0.90 to 1.02).

DISCUSSION

We found that risk for mortality does not increase for sodium excretion of 6.25 g/day or more, and a linear increase in risk was evident for sodium excretion below 6.25 g/day. After stratification for frailty phenotype, this association was somewhat stronger in frail participants. We did not find an association between sodium excretion and cardiovascular events in a sample of older community dwelling people. To our knowledge, only one other study investigated this issue focusing on older people.[8] However, in that study sodium intake was assessed by a food questionnaire, that is not the gold standard,[9] and people with difficulties with walking, stair climbing or activities of daily living, or with cognitive impairment, that are at higher risk of negative outcomes, were excluded. The non-linear association between sodium excretion and mortality found in our sample is in line with the evidence obtained in younger people, in which different studies found an association between mortality and low sodium excretion, while an association with high sodium excretion was observed only in the hypertensive subgroup.[6,15] These findings were confirmed by a meta-analysis showing a 9% reduction of the risk of all-cause mortality in the usual vs. low sodium intake group.[16] In contrast with other studies,[5,17] we did not find an association between higher sodium excretion and mortality.[16] The discrepancy between these findings and our results may be due the difference in the sample characteristics, as the mean age in our study was considerably higher and also other selection criteria (e.g., baseline cardiovascular risk) differed.

To our knowledge, this is the first study comparing the effects of sodium intake in frail and robust people. Frailty is associated with reduced physical activity, that in the elderly is associated with a

reduced activation of sympathetic nervous system (SNS), but also of renin-angiotensin-aldosterone system (RAAS), although for the last one evidences are more contrasting;[18] therefore frail subjects may have an over-activation of these systems, which in turn may lead to increased risk for cardiovascular events and mortality.[19,20] This activation of SNS and RAAS may be exacerbated by low sodium intake,[7,21] explaining the stronger association with mortality compared to robust participants. Another mechanism underlying the stronger association between low sodium intake and mortality highlighted in frail people may be related to the lower caloric intake that we found to be associated with low sodium excretion. Frailty and malnutrition are closely related,[22,23] and frail people may have a reduced energy reserve, that would make them more susceptible to low caloric intake, with consequent high risk of macronutrients and micronutrients deficits. These nutrients have a primary role in cellular metabolism, but also in several biological processes,[24] and their deficit is associated with higher risk of mortality, especially in the elderly.[25]

The strength of this study is that it is the first study that evaluates the prognostic role of sodium intake in the elderly using the 24-h sodium excretion, the gold standard to estimate sodium intake. This is also the first study evaluating the role of frailty as an effect modifier of the relationship between sodium intake and clinical outcomes. Furthermore, our study represents a relative large sample of the real life community-dwelling elderly population, providing precious information on these persons, that are generally excluded in large clinical trials. A limitation of this study is the lack of information of sodium excretion at follow-up. Furthermore, we did not exclude people affected by hypertension to study the relationship between sodium excretion and cardiovascular diseases, and also those affected by cardiovascular diseases to study the relationship with mortality, with the risk of introducing a bias related to different dietary patterns due to medical prescriptions. However, we corrected our models for cardiovascular disease, hypertension and systolic blood pressure. Furthermore, the analysis performed on non-hypertensive participants confirmed our results in the total sample, despite a loss of statistical significance. Finally, our results may be partially related to the reverse causation. Elderly persons may have a reduction of total caloric intake, and thus sodium

intake, in their last years of life. Therefore, the association between reduction of sodium intake and mortality may be related to a physiological reduction of sodium intake in the last years of life of these subjects. However, the long follow-up time in frail persons may reduce this effect.

CONCLUSION

Reduced sodium excretion is associated with increased mortality in a sample of older community-dwelling people, especially among frail and those without hypertension or history cardiovascular disease. High levels of sodium excretion are not associated with adverse outcomes in this population; therefore, a sodium restriction might not be appropriated for older people, especially in those without cardiovascular disease.

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b. ASSOCIATION BETWEEN PUFA INTAKE AND SERUM CONCENTRATION AND MORTALITY IN OLDER ADULTS: A COHORT STUDY

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ABSTRACT

Background & aims: PUFA intake is associated with reduced cardiovascular and all-cause mortality in the general population. It is generally believed that PUFA dietary intake is associated with PUFA serum concentration and, therefore, changing PUFA content in the diet affect serum concentration; however, evidence about this association in older adults is controversial. The objective of the study was to evaluate the relationship between PUFA intake and serum concentration, and their association with all-cause and cardiovascular mortality.

Methods: in this cohort study, 927 community dwelling adults aged ≥65 years were enrolled in the InCHIANTI study from 1998 to 2000 and followed-up for 9 years. The association between PUFA intake and serum concentration was evaluated through scatterplot and Pearson correlation test; 9-years all-cause and cardiovascular mortality was analyzed using the Kaplan-Meier method and Cox regressions adjusted for potential confounders.

Results: mean age of the population was 75 years (SD 7.3), 55% were women. There was no association between intake of overall PUFAs, linolenic and linoleic acid and their serum

concentration. There was no association between quartiles (Q) of PUFA intake and all-cause mortality: compared to Q1 of PUFA intake, the adjusted HR (95% CI) for overall mortality were: 1.05 (0.74-1.50) in Q2, 1.10 (0.76-1.58) in Q3, and 0.98 (0.68-1.41) in Q4; this lack of association was confirmed for cardiovascular mortality. Considering PUFA serum concentration, compared to Q1, Q4 participants had lower risk of all-cause mortality (adjusted HR [95%CI]: Q2 1.10 [0.79-1.53], Q3 0.84 [0.60-1.19], Q4 0.66 [0.44-0.995]), no association was found for cardiovascular mortality.

Conclusions: PUFA intake is not associated with serum concentration in a sample of community-dwelling older adults. Interventions to modulate PUFA concentration based on dietary intake may not be effective in preventing mortality in this population.

INTRODUCTION

Both PUFA intake and PUFA serum concentration may have a role in the prevention of cardiovascular disease (CVD) and death (1). PUFA intake may influence health outcomes by influencing PUFA serum concentration. Indeed, observational studies found an inverse association between PUFA intake and CVD mortality and all-cause mortality (2–5). Assessing PUFA dietary intake has two major drawbacks: it is inherently imprecise, also in relation with the questionnaire used, and it is only a hypothetical proxy of the PUFA serum concentration. However, it has the advantage of being less expensive and more easily available; furthermore, it is a modifiable factor (6). However, while many observational studies on adult populations showed an inverse relationship between PUFA serum concentration and CVD and all-cause mortality (2,3), clinical trials on PUFA supplementation showed discordant results (4).

Few and discordant evidences are available on the correlation between PUFA and mortality in older adults. In a relatively small population, Solfrizzi et al. did not find a relationship between dietary PUFA intake and mortality (7), while Mozaffarian et al. showed a statistically significant inverse correlation between serum ω-3 PUFA serum concentration and all-cause and CVD mortality (8);

similar results were found by Wu et al for ω-6 PUFA concentration (9). However, Mozaffarian et al and Wu et al evaluated the association between PUFA intake and serum concentration using food-frequency questionnaires performed three years before the blood sampling, thus being not representative of the PUFA intake at the time of PUFA serum concentration assessment. Thus, it is not clear if the discordant results reported in the literature may be due to a weak relationship between PUFA intake and PUFA serum concentration or to biases in the available studies.
We speculated that the lack of association between PUFA intake and mortality in older adults might be underpinned by a weak relationship between PUFA intake and PUFA serum concentration in this population.
The objective of this study is firstly to evaluate the correlation between dietary intake of PUFA and PUFA serum concentration, and then to evaluate whether PUFA intake, serum concentration or both are associated with all-cause and cardiovascular mortality in a sample of community-dwelling older adults.

MATERIALS AND METHODS

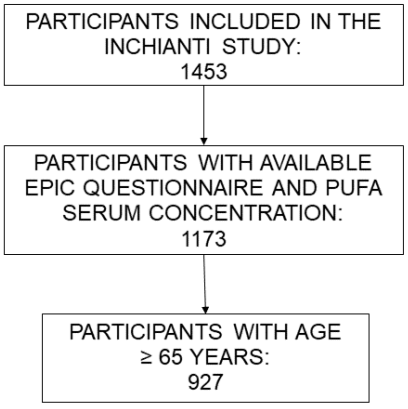
Data source and study design

We analyzed data from the longitudinal InCHIANTI study (10). The baseline study was supported by the Italian Ministry of Health and partly supported by the US National Institute on Aging. After obtaining informed consent, participants were randomly selected from the populations of two town areas in Tuscany, Italy; baseline data collection started in September 1998 and was completed in March 2000. The interviews of the eligible participants were performed at home by trained study researchers and were followed by a physical examination at the study clinic and a laboratory analysis. Follow-up visit were scheduled at 3, 6, and 9 years. The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 1983.The Italian National Institute of Research and Care on Aging Ethical Committee approved the study protocol.

Sample selection

From 1453 participants included in the InCHIANTI study, 1173 had available EPIC questionnaire and PUFA serum concentration; we excluded participants younger than 65 years (N: 246), obtaining a final sample of 927 participants (Supplementary figure 1).

Supplementary figure 1. Flow diagram of the study population selection.



Definition of exposure and outcome

PUFA dietary intake was estimated using the EPIC questionnaire (11). This questionnaire investigates intake frequency over the previous year of 236 specific foods, along with the average size of the servings, selected from a range as shown in photographs. The information derived from the questionnaire was automatically converted into data on energy, micro- and macronutrient intake by a specifically designed software. The EPIC nutritional assessment has been successfully validated in an older adult population, by comparing the dietary intake estimated by this method with the dietary intake estimated by a direct method of measuring, weighing and recording of seven

day-food consumptions (12). For the present analyses we used total PUFA intake, and linoleic and linolenic acid (g/day) (the essential and most represented ω-6 and ω-3 in the diet).

Blood samples for PUFA serum concentration were collected in the morning after the participants had been fasting for at least 8 h. Measurement of individual fatty acid was performed using gas-chromatography (Hewlett-Packard, Palo Alto, CA). Total PUFAs were calculated by summing C18:2 n-6 cis, C18:3 n-3 cis, C20:2 n-6 cis, C20:3 n-9 cis, C20:4 n-6 cis, C20:5 n-3 cis and C22:6 n-3 cis fatty acids and expressed in mg/l. The median time between food questionnaire and blood sample was 26 days.

We considered mortality for all causes and cardiovascular mortality (ICD-9 codes from 410 to 440.9 and from 444 to 444.91) as outcome measures; these data were collected from mortality registers and were available for all participants.

Analytic approach

We standardized PUFA for total lipid intake (PUFA intake) or total fatty acid weight (PUFA serum concentration). The characteristics of the study sample were reported using descriptive statistics (mean and standard deviation for continuous variables, proportion for categorical variables), according to quartiles of PUFA intake. We included associated diseases (e.g. hypertension, diabetes), cigarette smoke (pack-years), blood pressure, total cholesterol, estimated creatinine clearance (CKD-EPI), total caloric intake, and alcohol intake (EPIC Questionnaire). Finally, we considered years of education and physical activity.

The correlation between PUFA intake and PUFA serum correlation was evaluated using a scatterplot with LOESS interpolation. This analysis showed a linear association; therefore, we quantified the correlation using the Pearson coefficient. Mortality risk across quartiles of PUFA intake and serum concentration was calculated using the Kaplan-Meier method. The relationship between quartiles of PUFA intake and all-cause mortality and cardiovascular mortality was evaluated using Cox regressions. The models were then adjusted for potential confounders, selected on the base of the

clinical significance, prior knowledge, and results of the univariate analysis. To explore the different role of demographic and clinical variables, we first adjusted for age and sex, and then for the other potential confounders (education, CKD-EPI, pack/year, hypertension, diabetes, BMI, caloric intake/body weight, alcohol and oleic acid consumption). The same analysis was performed for PUFA serum concentration.

All analyses were performed using R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

General results

The mean age of the population was 75 years (SD 7.3), 55% were female. Cut-off values for quartiles of PUFA intake/lipid intake ratio (Q1-Q2-Q3-Q4) were 0.100, 0.107, and 0.117. Patients in Q3 were younger compared to the others (73.8 years vs. more than 75 years in the other quartiles). There were no differences across quartiles in sex, BMI, eGFR, and total cholesterol serum concentration. Total caloric intake, protein, lipids, and mono-unsaturated fatty acids intake decreased across quartiles, while there were no differences in total carbohydrates intake. The linoleic/linolenic acid ratio increased across quartiles (I quartile: 5.2, SD 0.7 to IV quartile: 6.7, SD 1.4, P<0.001). The prevalence of comorbidities did not change according with PUFA intake, with the exception of diabetes, that was more frequent in the IV quartile (19% vs. 7% in the I quartile, P=0.002) (Table 1).

Table 1. General characteristics of the population according with total PUFA/total lipid intake quartiles

Characteristics	I quartile (0.072-0.100) N=232	II quartile (0.101-0.107) N=232	III quartile (0.108-0.117) N=232	IV quartile (0.118-0.248) N=231	P
Age	75.4 (7.5)	75.1 (7.6)	73.8 (6.8)	75.7 (7.4)	0.030
Female sex	59	51	54	58	0.360
Education (years)	5.7 (3.7)	5.3 (3.1)	5.2 (3)	5.3 (3.5)	0.35
BMI (Kg/mq)	27.2 (3.7)	27.3 (3.7)	27.7 (3.9)	27.4 (4.7)	0.644

eGFR (CKD-EPI) (ml/min/1.73mq)	70.5 (13.8)	71.2 (14.2)	71.6 (14.7)	70.2 (14.8)	0.714
Total cholesterol (mg/dl)	217.1 (43.9)	215.2 (39.7)	217.4 (37.5)	214.8 (40.1)	0.864
Total Kcal intake	1997.8 (602.6)	1960.1 (563.1)	1937.4 (544.6)	1779.6 (519.6)	<0.001
Total Protein (g/day)	78 (22.2)	76.5 (19.9)	76.6 (20.6)	69.5 (18.1)	<0.001
Total lipids (g/day)	69.4 (21.3)	68 (19.7)	64 (19.3)	56.2 (17.5)	<0.001
Total glucids (g/day)	253.5 (86.5)	246.6 (77)	253 (78.5)	242.2 (78.2)	0.356
Linoleic/Linolenic acid ratio	5.2 (0.7)	5.7 (0.8)	5.9 (0.8)	6.7 (1.4)	<0.001
MUFA (g/day)	33.6 (11.5)	34.8 (10.5)	32.6 (11)	27.8 (9.2)	<0.001
Smoke (pack/year)	12.2 (20.7)	13.1 (22.8)	12 (19.8)	12.6 (19.8)	0.94
Reduced physical activity	66	63	64	66	0.892
Hypertension	59	56	64	65	0.190
Diabetes	7	13	13	19	0.002
COPD	6	12	8	10	0.125
Metabolic Syndrome	22	17	22	27	0.059
Gait speed (m/s)	1 (0.3)	1 (0.3)	1 (0.3)	1 (0.3)	0.167

Abbreviations: COPD: chronic obstructive pulmonary disease; eGFR: estimated glomerular filtration rate; MUFA: monounsaturated fatty acids.

The median follow-up time was 9 years, with a cumulative follow-up time of 6957 years. Over the follow-up time, 318 participants died, with an incidence rate of 4.57/100 person-year (95% CI 4.09-5.09) and a cumulative risk of 34.4% (95% CI 31.2-37.4); there were 114 cardiovascular deaths, with an incidence rate of 1.64/100 person-year (95% CI 1.36%-1.95%) and a cumulative risk of 16.9% (95% CI 11.5%-22%).

Association between PUFA intake and PUFA serum concentration

As shown in Figure 1, panel A, we found no association between PUFA intake and PUFA serum concentration (r=0.022, P=0.501). Results did not change after stratification for sex (male: r: 0.028, P=0.526; female: r: 0.005, P=0.924), or for age (age<80 years: r: -0.002, P=0.960; age≥80 years: r: 0.066, P=0.324) (data not shown). Similar results were found for the association between linolenic

and linoleic acid intake and serum concentration (r=-0.002, P=0.954 and r=-0.010, P=0.751) (Figure 1, panels B and C, respectively).

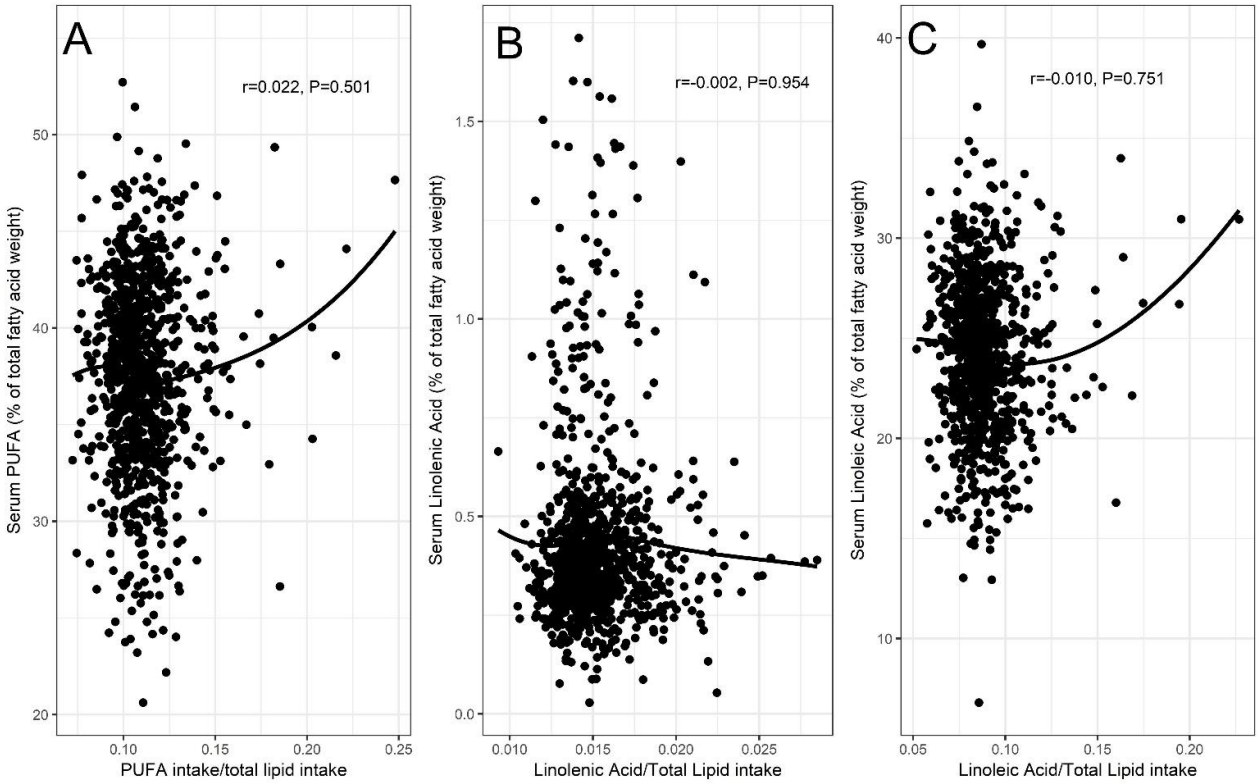


Figure 1. Correlation between intake and serum concentration of overall PUFAs (panel A), linolenic (panel B) and linoleic acid (panel C).

PUFA intake and mortality

Kaplan-Meier curves showed no differences in both all-cause and cardiovascular mortality across quartiles of PUFA intake/total lipid intake (Figure 2, panels A and B).

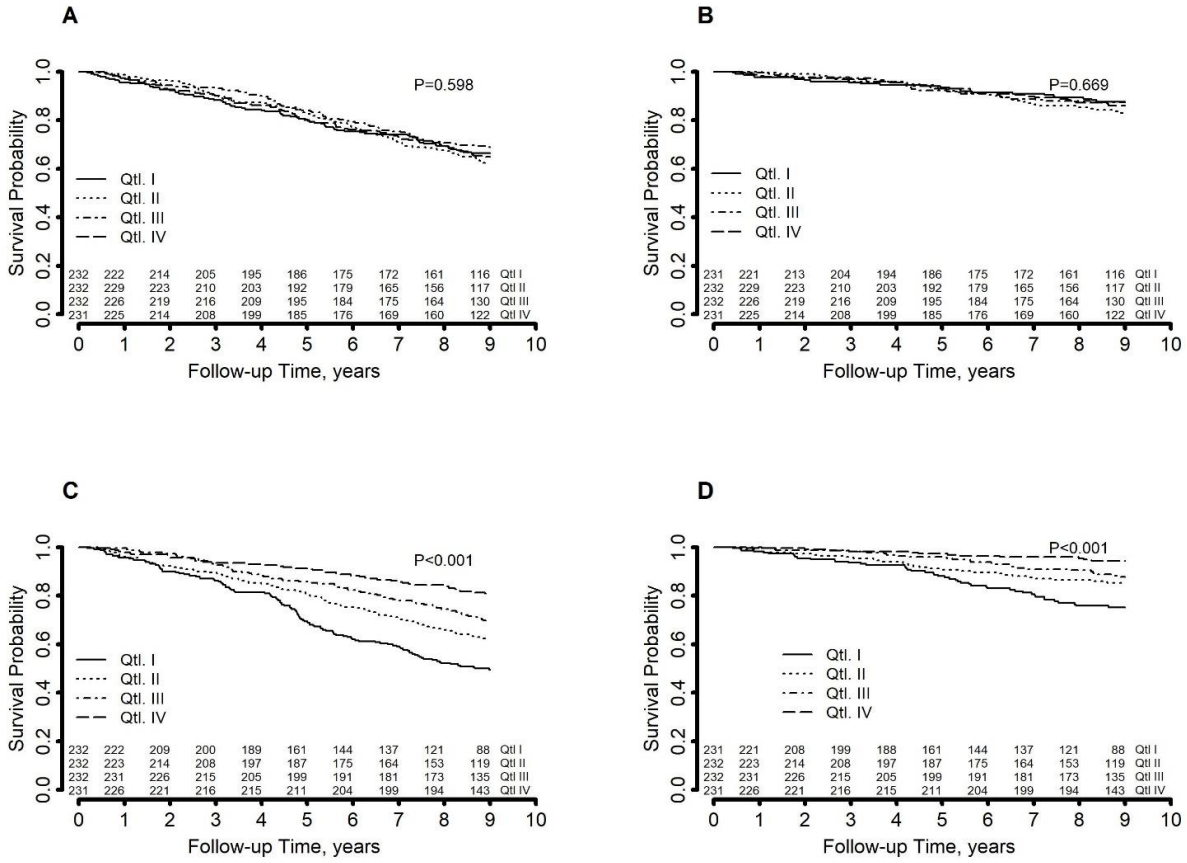
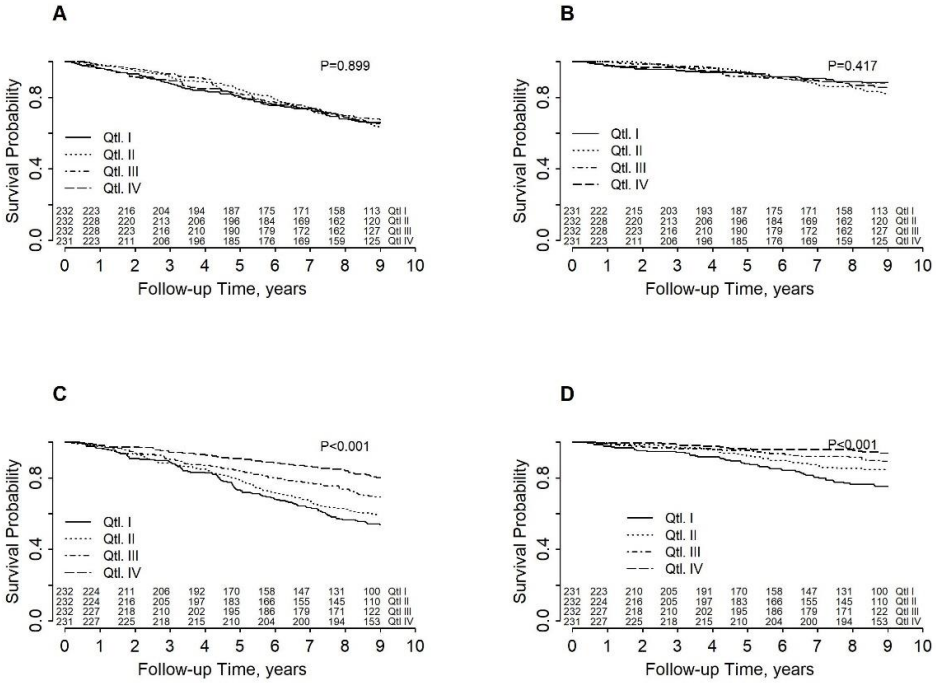
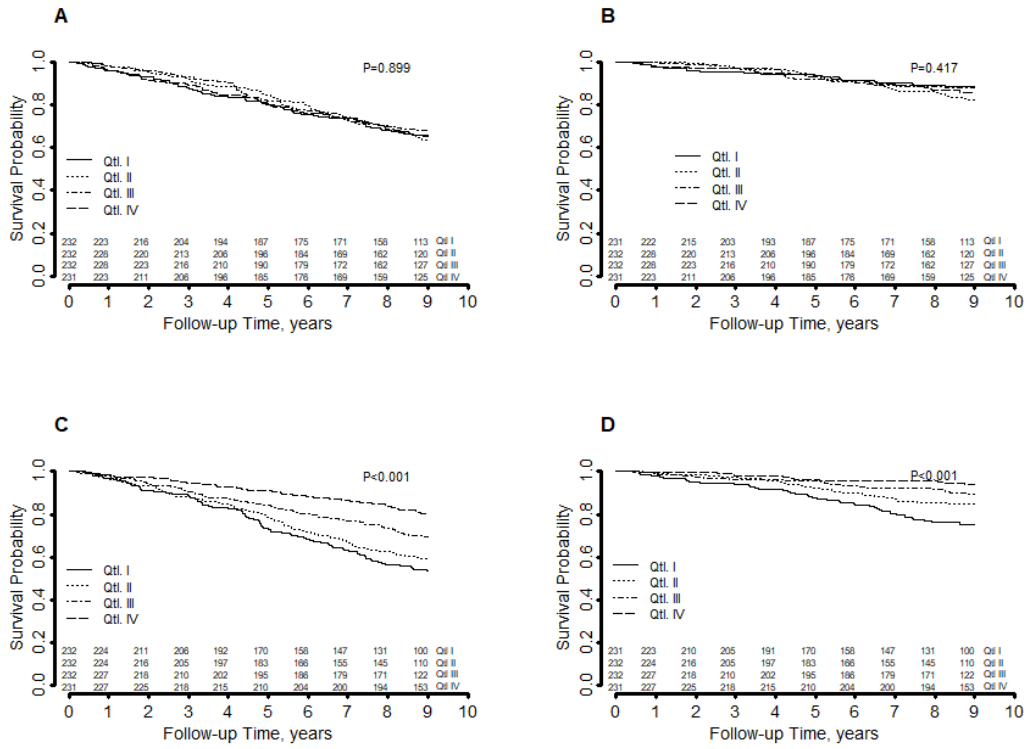


Figure 2. Kaplan-Meyer curves of overall and cardiovascular mortality by quartiles of PUFA intake (panels A and B, respectively) and quartiles of PUFA serum concentration (panels C and D, respectively).

Similar results were found for linoleic and linolenic intake (Supplementary figure 2 and 3).



Supplementary figure 2. Overall and cardiovascular mortality by quartiles of linolenic acid intake (panels A and B, respectively) and quartiles of linolenic acid serum concentration (panels C and D, respectively).



Supplementary figure 3. Overall and cardiovascular mortality by quartiles of linoleic acid intake (panels A and B, respectively) and quartiles of linoleic acid serum concentration (panels C and D, respectively).

The lack of association between PUFA intake quartiles and all-cause mortality was confirmed in Cox regression models adjusted for age, sex, education, CKD-EPI, pack/year, hypertension, diabetes, BMI, caloric intake/body weight, alcohol and oleic acid consumption with HR 1.05 (95% CI 0.74-1.50), 1.10 (0.76-1.58), and 0.98 (0.68-1.41) in Q2, Q3, and Q4, respectively. The corresponding adjusted HR (95% CI) for cardiovascular mortality were 1.22 (0.66-2.25), 1.40 (0.76-2.59), and 0.98 (0.51-1.86) (Table 2). The lack of association with all-cause and cardiovascular mortality was confirmed also for quartiles of linolenic and linoleic acid intake (Supplementary Table 1).

Table 2. HR for all-cause mortality and cardiovascular mortality according with PUFA/total lipid intake, using the I quartile as the reference one.

Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	1.10 (0.81-1.49)	1.05(0.74-1.50)	1.34(0.80-2.24)	1.22(0.66-2.25)
III quartile	0.89 (0.64-1.22)	1.10(0.76-1.58)	1.03(0.60-1.78)	1.40(0.76-2.59)
IV quartile	1.03 (0.76-1.41)	0.98(0.68-1.41)	1.11(0.65-1.90)	0.98(0.51-1.86)

[1]Models adjusted for age, sex, education, body mass index, estimated glomerular filtration rate (CKD-EPI), caloric intake/body weight, smoke, hypertension, diabetes, alcohol and oleic acid consumption.

Supplementary table 1. HR for all-cause mortality and cardiovascular mortality according with linolenic acid/total lipid intake and linoleic acid/total lipid intake, using the I quartile as the reference one.

Linolenic acid/total lipid intake				
Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	0.83(0.60-1.13)	0.83(0.58-1.18)	0.77(0.44-1.33)	0.77(0.41-1.45)
III quartile	1.04(0.77-1.41)	0.87(0.62-1.22)	1.24(0.76-2.01)	0.95(0.54-1.69)
IV quartile	0.89(0.65-1.21)	0.84(0.59-1.21)	0.86(0.51-1.46)	0.80(0.43-1.50)
Linoleic acid/total lipid intake				
Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	1.03(0.76-1.40)	1.07(0.75-1.53)	1.45(0.86-2.43)	1.52(0.81-2.85)
III quartile	0.92(0.67-1.26)	1.18(0.82-1.70)	1.00(0.57-1.76)	1.46(0.77-2.78)
IV quartile	1.01(0.74-1.38)	0.90(0.62-1.30)	1.20(0.70-2.06)	1.07(0.56-2.05)

[1]Models adjusted for age, sex, education, body mass index, estimated glomerular filtration rate (CKD-EPI), caloric intake/body weight, smoke, hypertension, diabetes, alcohol and oleic acid consumption.

PUFA serum concentration and mortality

Kaplan-Meier curves showed a significant decrease in both all-cause and cardiovascular mortality across increasing quartiles of PUFA serum concentration (Figure 2, panels C and D). However, in Cox-regression models adjusted for potential confounders, the association with all-cause mortality was evident only for Q4 (adjusted HR [95%CI]: Q2 1.10 [0.79-1.53], Q3 0.84 [0.60-1.19], Q4 0.66 [0.44-0.995], P for linear trend 0.028). The association was also evident for cardiovascular mortality (crude HR [95%CI]: Q2 0.57 [0.36-0.90], Q3 0.44 [0.27-0.72], Q4 0.20 [0.11-0.38]), but did not

reach statistical significance after adjustment for potential confounders (adjusted HR [95%CI]: Q2 1.14 [0.66-1.99], Q3 0.91 [0.52-1.59], Q4 0.62 [0.30-1.29], P for linear trend 0.213) (Table 3).

Table 3. HR for all-cause mortality and cardiovascular mortality according with PUFA serum concentration (% of total fatty acid weight), using the I quartile as the reference one.

Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	0.66(0.50-0.88)	1.10(0.79-1.53)	0.57(0.36-0.90)	1.14(0.66-1.99)
III quartile	0.50(0.37-0.67)	0.84(0.60-1.19)	0.44(0.27-0.72)	0.91(0.52-1.59)
IV quartile	0.30(0.21-0.42)	0.66(0.44-0.995)	0.20(0.11-0.38)	0.62(0.30-1.29)

[1]Models adjusted for age, sex, education, body mass index, estimated glomerular filtration rate (CKD-EPI), caloric intake/body weight, smoke, hypertension, diabetes, alcohol and oleic acid consumption.

Despite Kaplan-Meier curves documented a significant decrease in mortality across increasing quartiles of linolenic and linoleic serum concentration (Supplementary Figures 1 and 2), these results did not reach statistical significance after adjustment for potential confounders (Supplementary Table 2).

Supplementary Table 2. HR for all-cause mortality and cardiovascular mortality according with linolenic and linoleic acid serum concentration (% of total fatty acid weight), using the I quartile as the reference one.

Linolenic acid Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	0.63(0.47-0.84)	0.77(0.55-1.07)	0.47(0.29-0.76)	0.69(0.40-1.22)
III quartile	0.44(0.33-0.61)	0.74(0.52-1.05)	0.35(0.21-0.59)	0.72(0.40-1.30)
IV quartile	0.50(0.37-0.67)	0.75(0.53-1.07)	0.40(0.24-0.67)	0.69(0.39-1.23)
Linoleic acid Quartiles	All-cause mortality		Cardiovascular mortality	
	Crude HR (95% CI)	Adjusted HR[1] (95% CI)	Crude HR (95% CI)	Adjusted HR[1] (95% CI)
II quartile	0.84(0.64-1.11)	1.33(0.96-1.84)	0.59(0.37-0.93)	0.99(0.57-1.70)
III quartile	0.59(0.44-0.78)	1.08(0.76-1.53)	0.40(0.24-0.66)	0.85(0.48-1.50)
IV quartile	0.36(0.25-0.50)	0.92(0.62-1.38)	0.22(0.12-0.41)	0.72(0.36-1.46)

[1]Models adjusted for age, sex, education, body mass index, estimated glomerular filtration rate (CKD-EPI), caloric intake/body weight, smoke, hypertension, diabetes, alcohol and oleic acid consumption.

DISCUSSION

In a sample of community-dwelling older adults, we did not find an association between PUFA intake and PUFA serum concentration. There was no association between PUFA intake and mortality, while an inverse relationship was evident only between PUFA serum concentration and all-cause mortality, possibly with a threshold effect as indicated by the more evident reduction in risk in the highest quartile of PUFA serum concentration.

Few evidences are available about the association between PUFA intake and PUFA serum concentration in humans. To the best of our knowledge, this association was never reported in a cohort representative of the general population of older adults in which PUFA intake and serum concentration have been estimated within a narrow time window: Huang et al found an association between linoleic acid and docasahexaenoic acid serum concentration and intake, that was less evident for other PUFAs, but it was estimated in a sample of men with kidney failure (13). Studying a cohort of 60-year-old adults from Sweden, Laguzzi et al. found an association between fish intake and eicosapentaenoic and docosahexaenoic acid serum concentration, and between vegetable fat intake and linoleic acid and total PUFA serum concentration. However, the authors used a non-validated food questionnaire, that included only a few food items (14). This association was also studied in a sample of older adults free from cardiovascular disease in the Cardiovascular Health Study (CHS): Wu et al found a non-linear relationship between linolenic acid intake and serum concentration, that was more evident when intake of linolenic acid was <8% of total daily energy (9); in the same cohort, Mozaffarian et al. found an association between ω-3 PUFA intake and serum concentration, with a stronger association for ω-3 PUFA intake <400 mg/day (8). However, in the CHS the food questionnaire was administrated about three years before the serum assays, thus it might be not representative of the PUFA intake at the moment of the blood sampling, and the association found between PUFA intake and serum concentration might have been subject to bias.

In our sample, average PUFA dietary intake was similar to that reported by the above-mentioned

studies; however, we did not find an association with PUFA serum concentration. This discrepancy might be related to different sample characteristics (e.g. community dwelling older adults vs. older men with kidney failure).

The lack of association between PUFA intake and serum concentration in older adults might be related to the variability of the different processes involved in the absorption and metabolism of linoleic and linolenic acid, such as protein-mediated enterocyte PUFA uptake (15), or senescence-related epigenetic modifications of genes associated with lipid metabolism (16). These epigenetic modifications are expected to have less influence on the relationship between dietary intake and serum concentration of linoleic and linolenic acid, because they are at the beginning of the metabolic pathway of PUFA synthesis. However, our data indicate that no association is present even for these two PUFA precursors.

Regarding the association between PUFA intake and mortality, our results are in line and extend those previously reported by Solfrizzi et al in a sample of 278 older adults followed for 8.5 years, where no association was found between total PUFA intake and mortality for all causes (17). Our data confirm their results in a larger cohort, also for linoleic and linolenic acid intake, and not only for all-cause mortality, but also for cardiovascular mortality.

With respect to the association between PUFA serum concentration and mortality, in the CHS Wu et al in the found an inverse relationship between linoleic acid and all-cause and CVD mortality (9); similar results were found also for ω-3 PUFAs (8). Our results confirm the inverse association between overall PUFA serum concentration and all-cause, but not cardiovascular mortality, where only a trend in reduction of mortality was evident; furthermore, we did not find an association between mortality and linolenic and linoleic acid. These partially contrasting results might be related to the different characteristics of our population, since we did not exclude patients affected by cardiovascular diseases, and the sex distribution of our sample was more representative of the general population, and to the smaller sample size of our population with consequent fewer events.

Our study has many strengths: to the best of our knowledge, this is the first study on this topic estimating PUFA intake and serum concentration within a short period of time in a relatively large sample of community dwelling older adults. We used a food questionnaire for the estimation of the PUFA intake in the previous years validated in a similar population, thus providing reliable dietary information (11) and we did not use exclusion criteria, thus we provide data on a sample of "real life" older adults population. Finally, our study could help to clarify the discordant results of studies on PUFA dietary intake and serum concentration and mortality in older people.

On the other hand, we had a relatively small sample size, with consequent small number of events. We had information only on overall PUFA, linolenic and linoleic acid intake, therefore we could not evaluate study the association between dietary intake and serum concentration of all the individual fatty acids. However, linolenic and linoleic acid were the most represented ω-3 and ω-6 PUFA in the diet of our sample and have the advantage of being less influenced by lipid metabolism compared to other PUFA.

In conclusion, our results indicate that older people in the highest quartile of PUFA serum concentration have lower all-cause mortality risk. Nonetheless, we found no association between intake and serum concentration, suggesting that interventions to modulate PUFA concentration based on dietary intake may not be effective on mortality in this population.

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3. THE ROLE OF MICRONUTRIENTS AND NUTRITIONAL STATUS IN INFLUENCING OUTCOMES

a. 25(OH) VITAMIN D AND FUNCTIONAL OUTCOMES IN OLDER ADULTS ADMITTED TO REHABILITATION UNITS: THE SAFARI STUDY

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ORIGINAL ARTICLE

25(OH) vitamin D and functional outcomes in older adults admitted to rehabilitation units: the safari study

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Abstract

Summary Vitamin D (25(OH)D) deficiency is associated with poor physical performance; little is known about its impact on geriatric rehabilitation. We found a positive non-linear relationship between 25(OH)D and functional gain, stronger in levels < 16 ng/ml (below the cutoff for "deficiency"). An early 25(OH)D dosage may be advisable for this population.

Introduction Vitamin D (25(OH)D) deficiency is highly prevalent in older people, and it is associated with poor muscular strength and physical performance. Its impact on functional outcomes during geriatric rehabilitation has been poorly studied. We aim to analyze the association between 25(OH)D and functional recovery in geriatric rehabilitation units.

Methods We conducted a prospective multi-center cohort study including patients ≥ 65 years old admitted to 3 geriatric rehabilitation units in Italy and Spain, after orthopedic events or stroke. Outcomes were absolute functional gain (AFG, discharge-admission Barthel index) and ability to walk (AW) at 3 months after admission. The association between 25(OH)D quartiles (Q1-Q2-Q3-Q4) and outcomes was explored using linear or logistic regression models.

Results We included 420 patients (mean age = 81.2 years [SD = 7.7], 66.4% females, mean 25(OH)D concentration = 13.5 ng/ml [SD = 8.7]) (to convert to nmol/l multiply by 2.496). A non-linear relationship between 25(OH)D and AFG was found, with a stronger association for 25(OH)D levels < 16 ng/ml. Compared to Q1 (25(OH)D ≤ 6 ng/ml), participants in Q3 (25(OH)D 11.5–18.2 ng/ml) had the best AFG and AW (mean AFG [SD], Q1 = 28.9 [27.8], Q2 = 32.5 [23.5], Q3 = 43.1 [21.9], Q4 = 34.5 [29.3], R^2 = 7.3%; AW, Q1-Q2 = 80%, Q3 = 91%, Q4 = 86%). Regression models adjusted for potential confounders confirmed these results (AGF Q2, β = 2.614, p = 0.49; Q3, β = 9.723, p < 0.01; Q4, β = 4.406, p = 0.22; AW Q2, OR [95% CI] = 1.84 [0.67–5.33]; Q3, OR [95% CI] = 4.01 [1.35–13.48]; Q4, OR [95% CI] = 2.18 [0.81–6.21]).

Conclusions In our study, 25(OH)D concentration showed a positive association with functional outcomes at 3 months. The association is stronger below the usual cutoff for "deficiency." Dosage of 25(OH)D concentration may help identify geriatric rehabilitation patients at risk for a worse functional recovery.

INTRODUCTION

Vitamin D insufficiency (usually defined as 25(OH)D serum concentration between 21 and 29 ng/ml) is a highly prevalent condition worldwide, regardless of age and latitude. Prevalence has

been reported at around 40% of healthy adults, increasing up to 70-100% when hospitalized [1]. Vitamin D deficiency (usually defined as 25(OH)D serum concentration ≤20 ng/ml) is also very common, with a prevalence of around 35% in hospitalized patients [1]. Older adults are at a higher risk of vitamin D deficiency and insufficiency due to dietary deficiencies, lower sun exposure and a physiological age-related decrease of vitamin D3 cutaneous synthesis [2]. In community-dwelling older adults, vitamin D deficiency and insufficiency have a prevalence of about 30% and 75%, respectively [3, 4], while in rehabilitation settings insufficiency may result in more than 90% of patients [5].

In addition to the well-known role of vitamin D in improving bone mineral density [6], recent evidence documented that vitamin D contributes both to muscular health [7] and strength [8–10]. Furthermore, low 25(OH)D levels are associated with higher risk of incident disability, likely through the association with poor physical performance [11, 12] and neuromuscular disorders [13] [14]. However, a recent meta-analysis has raised controversy regarding the impact of vitamin D supplementation combined with resistance exercise training on musculoskeletal health, although confirming the association with muscular strength. This meta-analysis pointed out that few studies are available on this topic in older adults, and that further evidence is required to draw any final conclusions of the role of vitamin D on muscular health [15].

Due to the close relationship between vitamin D deficiency and physical performance, it is hypothesized that vitamin D deficiency might affect rehabilitation outcomes and physical function in older adults. However, limited and discordant evidence is available about the association between 25(OH)D and rehabilitation outcomes in this population: most of the studies examining this topic have a relatively small sample size and are not focused on older adults [5, 16, 17], a population in which the consequence of vitamin D deficiency may be even more evident, due to age-related body and muscle composition change [18].

Our hypothesis is that there is a positive association between 25(OH)D serum concentration and functional recovery. The objective of our study was to analyze the association between 25(OH)D

levels and functional recovery in an older population admitted to geriatric rehabilitation units for orthopedic conditions or stroke, the most prevalent conditions in this setting.

METHODS

Study design and setting

We analyzed data from the Sarcopenia And Function in Aging Rehabilitation (SAFARI) study, a multi-center international collaboration project on the identification of the frailty-related factors associated with functional improvement in older patients admitted to geriatric rehabilitation units.
This was a prospective multi-center cohort study conducted on patients aged 65 years or older and admitted after an orthopedic surgery (hip fracture and hip or knee replacement) or stroke in the rehabilitation departments of Gemelli Hospital, Rome (Italy), Ancelle Hospital, Cremona (Italy), and Parc Sanitari Pere Virgili, Barcelona (Spain) between December 2014 and May 2016.
Exclusion criteria included unstable medical conditions that may influence the rehabilitative program, terminal diseases, previous severe dementia (Global Deterioration Scale 6-7), and severe functional impairment before the event (i.e. Total Barthel index $\leq$40).
At admission, patients underwent a multidimensional geriatric assessment to develop an individualized treatment plan that included a rehabilitation program aimed to improve functional outcomes. The rehabilitation units were staffed by full-time geriatricians, nurses, and nursing assistants, and also by physical, speech and occupational therapists.
The study was approved by the Animal and Human Ethics Committee from each Institution.
Informed consent was obtained from all individual participants included in the study.

Measurements and outcomes

Baseline evaluation was performed within 72 hours of admission by geriatricians and trained nurses or physiotherapists. It included demographic characteristics, main diagnosis at admission (hip fracture, orthopedic elective surgery or stroke), body mass index (BMI), comorbidities (Charlson

index), cognitive function (Mini Mental State Examination [MMSE]), and nutritional status (Mini Nutritional Assessment-Short Form [MNA-SF]). Muscle strength was assessed by measuring the grip strength in the dominant hand (or in the preserved hand in post-stroke patients) with a Jamar® hydraulic dynamometer, and functional status was evaluated with the Barthel Index (BI, which rates 0-100, from complete dependence to independence in the basic activities of daily living), collected before the acute event (self-reported by the patient or the caregiver at admission), at admission, at 30 days and at 3 months after admission (via telephone interviews). BI has been validated for use with patients or proxies, and as well as for telephone interviews [19]. Length of stay (LOS) was also recorded.

25(OH)D concentration was determined at the time of admission to the rehabilitation units with the same technique, chemiluminescent immunoassay (CLIA), at each of the three local laboratories, with normal reference values ≥30 ng/ml. All centers participated in the DEQAS. The mean precision and accuracy of the centers were 8% and 4%, respectively. In all centers, vitamin D supplementation was administrated according to local protocols. This information, however, was collected by one center only.

The functional outcomes evaluated were the ability to walk and the Absolute Functional Gain (AFG), both at 30 days and at 3 months. The ability to walk was a dichotomous variable, defined by a cut-off of ten from the walking BI item (15-point item): patients with a walking-BI ≥10 (independent for indoor walking) were defined as able to walk. The AFG was defined as the difference between the total BI at 30 days or 3 months minus the total BI at admission.

Statistical analysis

We explored the relationship between 25(OH)D and AFG using graphic methods (scatterplot with spline interpolation). This exploratory analysis revealed a non-linear relationship between the two variables, with a positive association for 25(OH)D concentration below 16 ng/ml, and an inversion of the trend for concentrations above this value. Therefore, we decided to analyze data according to

25(OH)D quartiles (Q) (Q1: 3-6 ng/ml, Q2: 6.1-11.4 ng/ml, Q3: 11.5-18.2 ng/ml, Q4: 18.3-50.5 ng/ml). Supplementary analysis using the cut-offs of severe vitamin D deficiency (25(OH)D <12 ng/ml) [20], deficiency (25(OH)D 12-20 ng/ml), insufficiency (25(OH)D 20.1-29.9 ng/ml) and normal 25(OH)D concentration (≥30 ng/ml) [21]

We analyzed baseline characteristics of the study sample using descriptive statistics (mean and standard deviation for continuous variables, proportion for categorical variables), according to quartiles of 25(OH)D serum concentration. We evaluated the association between 25(OH)D and AFG and the ability to walk using linear or logistic regression models, as appropriate, crude and adjusted for potential confounders, using the first quartile as a reference. The relationship between 25(OH)D concentration and AFG was further explored with linear regression models performed separately for 25(OH)D values below and above 16 ng/ml, both crude and adjusted for potential confounders. Finally, the association between 25(OH)D and functional gain was studied through a linear mixed model, using BI as outcome over the time, both crude and adjusted for potential confounders, using the first quartile as a reference.

We selected the potential confounders included in the analyses on the basis of clinical significance, prior knowledge, and results of the univariate analysis. To explore the different roles of demographic and clinical variables, we first adjusted for age and sex, and then for the other potential confounders (i.e. Charlson index, MMSE, length of stay, BI at admission, hand grip strength at admission, eGFR, BMI at admission, study site, diagnosis at admission). All analyses were performed using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Mean age of the 420 study participants was 81.2 years (SD=7.7), 66.4% were female. Diagnosis at admission was hip fracture for 175 patients, elective orthopedic surgery for 136, and stroke for 138. Mean 25(OH)D concentration was 13.5 ng/ml (SD=8.7), with no difference in the periods of April-September (13.1 ng/ml) and October-March (13.8 ng/ml); 81.2% of the samples had 25(OH)D

concentration <20 ng/ml and 93.8% 25(OH)D <30 ng/ml (to convert ng/ml in nmol/l multiply values by 2.496). The mean length of stay was 29 days (SD=9.7).

Patients in the first quartile of 25(OH)D serum concentration were older (mean age [SD] 84.2 [7.3] years, 81.7 [7.2], 79.9 [7.8], 79.7 [7.6] across increasing quartiles, p for trend<0.01). There was no difference across quartiles in sex, diagnosis at admission, Charlson index, MMSE, BMI, eGFR estimated using the CKD-EPI formula, and albumin. Muscle strength was lower in the first 25(OH)D quartile (mean hand grip strength [SD] Q1: 12.3 [8.3] kg, Q2: 15.2 [10], Q3: 15.8 [9.2], Q4: 14.1 [8.4], p for trend=0.03). BI pre-event and at admission did not change throughout quartiles, while both BI at 30 days and 3 months increased across quartiles (Table 1).

Table 1 General characteristics of the population distributed by quartiles of 25(OH)D concentration

Characteristics	I quartile (3–6 ng/ml) N105	II quartile (6.1–11.4 ng/ml) N105	III quartile (11.5–18.2 ng/ml) N105	IV quartile (18.3–50.5 ng/ml) N105	*p*
Age (years), mean (SD)	84.2 (7.3)	81.7 (7.2)	79.9 (7.8)	79.7 (7.6)	< 0.01
Female sex, (%)	71	67	63	65	0.59
Hip fracture, (%)	30	25	23	22	0.12
Elective orthopedic surgery, (%)	19	23	33	26	
Stroke, (%)	25	28	20	28	
BMI (kg/m^2), mean (SD)	25.1 (5.3)	25.6 (4.3)	25.9 (4.2)	25.7 (4.1)	0.69
eGFR (ml/min1.73m^2), mean (SD)	67.1 (22.5)	64.6 (22)	69.3 (21)	71.8 (20.3)	0.20
Charlson Index, mean (SD)	3.3 (2.6)	3.6 (2.5)	4.1 (2.6)	3.7 (2.7)	0.22
MMSE, mean (SD)	22.7 (5.6)	22 (6.1)	23.8 (5.1)	22.9 (5.6)	0.14
Handgrip strength at admission (kg), mean (SD)	12.3 (8.3)	15.2 (10)	15.8 (9.2)	14.1 (8.4)	0.03
Length of stay, mean (SD)	29.6 (9.8)	29.5 (9.6)	27.4 (9)	29.4 (10.3)	0.30
BI before event, mean (SD)	87.9 (16.6)	88.9 (16.7)	92.3 (13.7)	90.7 (16.4)	0.19
BI at admission, mean (SD)	43.4 (19.1)	41.3 (22.5)	43.8 (22.3)	41.9 (20.1)	0.79
BI at 30 days, mean (SD)	67.7 (21.4)	63.5 (28)	73.2 (21.4)	70.4 (24.6)	0.03
BI at 3 months, mean (SD)	71.1 (29.3)	72.3 (28.3)	86.4 (19)	76.4 (29.5)	< 0.01
AFG at 30 days, mean (SD)	23.8 (18.7)	22.1 (19)	29.2 (17.1)	28.1 (18.2)	0.01
AFG at 3 months, mean (SD)	28.9 (27.8)	32.5 (23.5)	43.1 (21.9)	34.5 (29.3)	< 0.01
AW at 30 days (%)	71	66	68	72	0.80
AW at 3 months (%)	80	80	91	86	0.10

BMI, body mass index; *MMSE*, mini-mental state examination; *eGFR*, estimated glomerular filtration rate; *BI*, Barthel index; *AW*, ability to walk

Information on vitamin D supplementation was available for one center only (200 participants) and only 29 participants received supplementation. In this subgroup, supplementation did not significantly affect AFG at 30 days (21.2 vs 16.9, P=0.29), AFG at 3 months (22.1 vs 26.6, P=0.50), the ability to walk at 30 days (90% vs 78%, P=0.22), and the ability to walk at 3 months (88% vs 84%, P=0.74).

Figure 1 shows the relationship between 25(OH)D concentration and AFG at 30 days after admission. There was a non-linear relationship, with a positive association for 25(OH)D concentration values up to 16 ng/ml, however not confirmed by a linear regression model adjusted for the potential confounders (β 0.136, p=0.71), and no association when values where higher than 16 ng/ml. Splitting up the curve according to 25(OH)D quartiles, a positive association between 25(OH)D and AFG at 30 days was evident within the first three quartiles, while within the fourth quartile there was no further improvement in AFG (Figure 1).

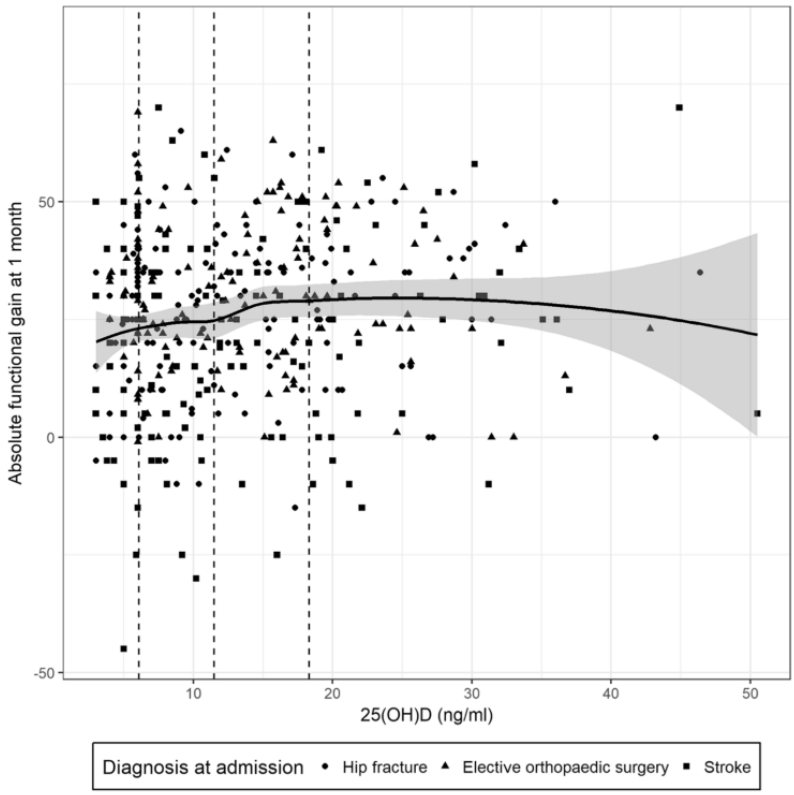

Fig. 1 Association between 25(OH)D and absolute functional gain at 30 days from admission. There is a non-linear relationship between 25(OH)D and absolute functional gain, with a weak positive association for 25(OH)D concentration values up to 16 ng/ml, and no association when values where higher than 16 ng/ml. Patients with different diagnosis at admission are equally distributed in the graph

The mean AFG at 30 days (SD) according to 25(OH)D quartiles was Q1: 23.8 (18.7), Q2: 22.1 (19), Q3: 29.2 (17.1) and Q4: 28.1 (18.2) (Table 1). Linear regression models, both crude and adjusted for potential confounders, did not show a statistically significant association between 25(OH)D quartiles and AFG at 30 days (Table 2).

Table 2 Linear regression models of the association between 25(OH)D quartiles and absolute functional gain

Quartiles	30 days		3 months	
	β crude (P)	β adjusted*(P)	β crude (P)	β adjusted* (P)
I quartile	Reference	Reference	Reference	Reference
II quartile	− 1.712 (0.51)	− 0.536 (0.85)	3.63 (0.36)	2.614 (0.49)
III quartile	5.383 (0.04)	3.025 (0.28)	14.205 (< 0.01)	9.723 (< 0.01)
IV quartile	4.337 (0.09)	3.802 (0.18)	5.661 (0.14)	4.406 (0.22)

*Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission, eGFR (CKD-EPI), BMI at admission, handgrip strength at admission, study site, diagnosis at admission

Regarding the relationship between 25(OH)D concentration and AFG at three months (Figure 2), we found a positive relationship for values below 16 ng/ml, that was confirmed by a linear regression model, both crude (β 1.89, P<0.01) and adjusted for the complete set of confounders (β 1.20, P<0.01). Instead, a weak inverse relationship was evident for 25(OH)D values over 16 ng/ml, that was not statistically significant in linear regression analyses (crude β -0.58, P=0.13; adjusted β -0.22, P=0.55, respectively). Dividing the curve according to 25(OH)D quartiles, a positive association between 25(OH)D and AFG at 3 months was evident within the first three quartiles, while within the fourth quartile there was no further improvement of AFG when increasing values of 25(OH)D (Figure 2).

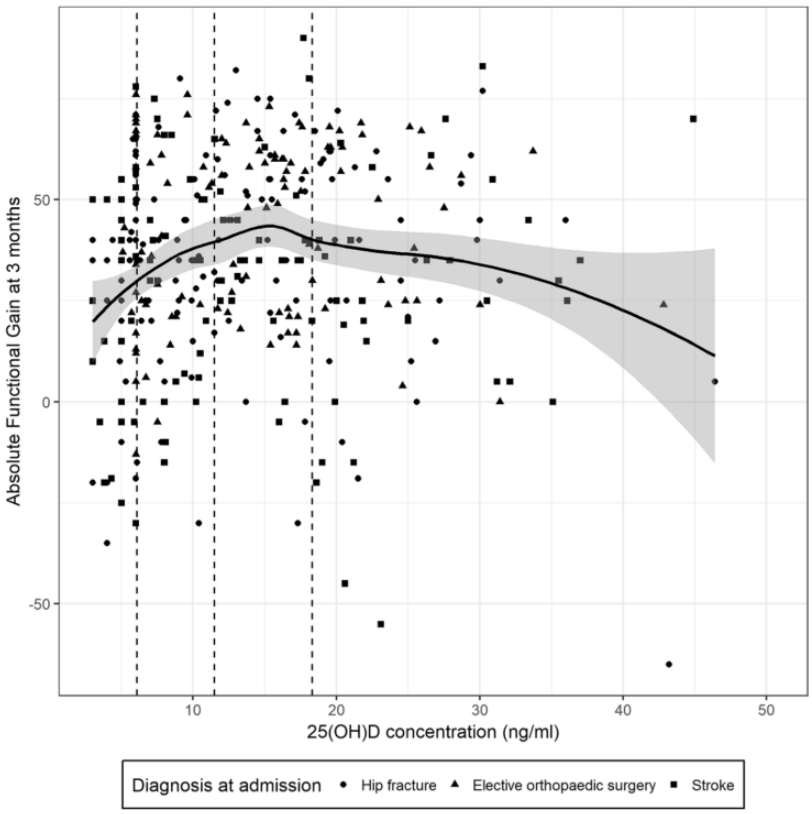

Fig. 2 Association between 25(OH)D and absolute functional gain at 3 months from admission. A non-linear relationship between 25(OH)D and absolute functional gain is evident for 25(OH)D values up to 16 ng/ml, and a weak inverse association for values higher than 16 ng/ml. Patients with different diagnosis at admission are equally distributed in the graph

There was an improvement in mean AFG across 25(OH)D quartiles, less evident in the fourth quartile (Mean [SD] Q1: 28.9 [27.8], Q2: 32.5 [23.5], Q3: 43.1 [21.9] and Q4: 34.5 [29.3]; $p<0.01$, R-squared=7.3%) (Table 1). This data was confirmed in a crude and adjusted linear regression model, where improvement was statistically significant in the third 25(OH)D quartile, compared to the first one (Crude model: Q2: β 3.63, p=0.36; Q3: β 14.21, $p<0.01$; Q4 β 5.66, p=0.14; Adjusted model: Q2: β 2.61, p=0.49; Q3: β 9.72, $p<0.01$; Q4: β 4.41, p=0.22) (Table 2).

In relation to the ability to walk, there was no difference across 25(OH)D quartiles in the proportion of patients able to ambulate at 30 days after admission (Table 1). These results were confirmed both in a crude logistic regression model (OR [95% CI] Q2 0.79 [0.44-1.44]; Q3 0.88 [0.48-1.60] and Q4 1.04 [0.57-1.92]), and after adjustment for potential confounders (OR [95% CI] Q2 0.86 [0.35-2.10], Q3 0.89 [0.37-2.13] and Q4 0.90 [0.38-2.13]) (Table 3).

At three months, there was an increased proportion of patients able to walk across 25(OH)D quartiles, improvement, again, less evident in the fourth quartile: Q1: 80%, Q2: 80%, Q3: 91% and

Q4: 86% (p for trend=0.095) (Table 1). In logistic regression models, compared to Q1, patients in Q3 had a significant improvement of the outcome, both in the crude (OR [95% CI] Q2: 1.00 [0.47-2.11]; Q3: 2.56 [1.11-6.30]; Q4: 1.58 [0.73-3.51]) and in the adjusted model (OR [95% CI] Q2: 1.84 [0.67-5.33]; Q3: 4.01 [1.35-13.48]; Q4 2.18 [0.81-6.21]) (Table 3).

Table 3 OR for ability to walk according to 25(OH)D quartiles

Quartiles	30 days		3 months	
	Crude (95% CI)	Adjusted* (95% CI)	Crude (95% CI)	Adjusted* (95% CI)
I quartile	Reference	Reference	Reference	Reference
II quartile	0.79 (0.44–1.44)	0.86 (0.35–2.10)	1.00 (0.47–2.11)	1.84 (0.67–5.33)
III quartile	0.88 (0.48–1.60)	0.89 (0.37–2.13)	2.56 (1.11–6.30)	4.01 (1.35–13.48)
IV quartile	1.04 (0.57–1.92)	0.90 (0.38–2.13)	1.58 (0.73–3.51)	2.18 (0.81–6.21)

*Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission, eGFR (CKD-EPI), BMI at admission, handgrip strength at admission, study site, diagnosis at admission

Similar results were found when 25(OH)D was categorized according to the clinical 25(OH)D cut-offs (Supplementary Tables 1 and 2).

Supplementary Table 1. Linear regression models of the association between 25(OH)D groups and absolute functional gain

25(OH)D groups	30 days		3 months	
	β Crude (P)	β Adjusted*(P)	β Crude (P)	β Adjusted* (P)
<12 ng/ml	Reference	Reference	Reference	Reference
12-20 ng/ml	5.41 (<0.01)	2.04 (0.38)	10.95 (<0.01)	6.91 (0.02)
21-29 ng/ml	7.18 (0.01)	5.29 (0.10)	3.70 (0.37)	2.29 (0.57)
≥30 ng/ml	3.25 (0.401)	7.50 (0.08)	-1.46 (0.81)	2.96 (0.60)

* Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission, eGFR (CKD-EPI), BMI at admission, hand grip strength at admission, study site, diagnosis at admission.

Supplementary Table 2. OR for ability to walk according to 25(OH)D groups

25(OH)D groups	30 days		3 months	
	Crude (95% CI)	Adjusted*(95% CI)	Crude (95% CI))	Adjusted* (95% CI)
<12 ng/ml	Reference	Reference	Reference	Reference
12-20 ng/ml	0.95 (0.59-1.55)	0.97 (0.48-1.98)	2.17 (1.08-4.67)	2.73 (1.09-7.72)
21-30 ng/ml	1.38 (0.70-2.84)	0.94 (0.37-2.40)	1.49 (0.65-3.86)	1.23 (0.40-4.37)
>30 ng/ml	1.18 (0.49-3.16)	1.26 (0.30-6.29)	0.87 (0.35-3.66)	1.06 (0.25-5.73)

* Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission, eGFR (CKD-EPI), BMI at admission, hand grip strength at admission, study site, diagnosis at admission.

Figure 3 shows BI changes over time across 25(OH)D quartiles: participants in quartile III resulted in better improvement in BI over time in respect to the other quartiles. Adjusted linear mixed models confirmed these results: participants in the III 25(OH)D quartile have a 7.52-point improvement in BI respect to I quartile at 30 days (P=0.03) and of 16.98 points at 3 months (P<0.01). An improvement in the Barthel index was also observed for IV quartile but reached the statistical significance only at the 3-months follow-up (Beta: 8.63, P=0.02) (Supplementary table 3).

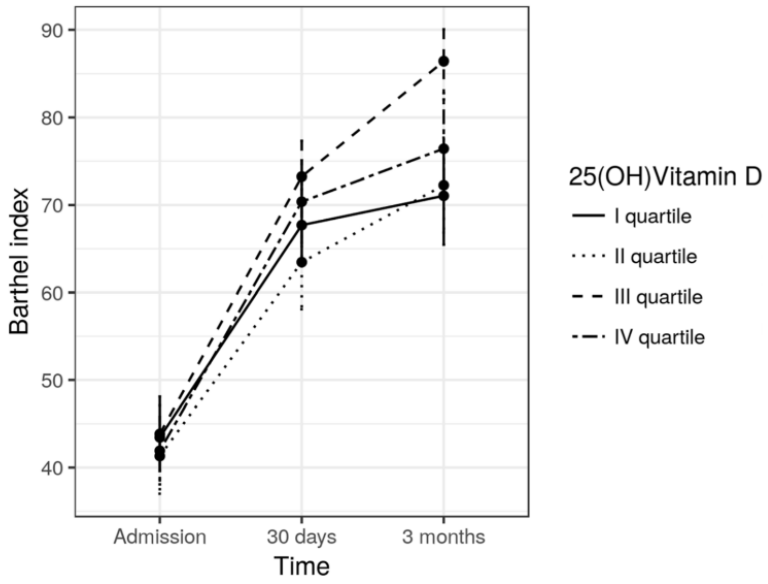

Fig. 3 Changes in the Barthel Index over time. Participants in the III 25(OH)D quartile had a better improvement in the Barthel index in respect to the other quartiles

Supplementary table 3. Linear mixed models of the association between 25(OH)D quartiles and the Barthel Index over time.

	Beta	p-value
I quartile	Reference	-
II quartile	-0.33	0.91
III quartile	-3.98	0.14
IV quartile	-2.35	0.39
30 days-time*II quartile	-0.37	0.92
3 months-time*II quartile	3.80	0.31
30 days-time*III quartile	7.52	0.03
3 months-time*III quartile	16.98	<0.01
30 days-time*IV quartile	6.32	0.07
3 months-time*IV quartile	8.63	0.02

Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission, eGFR (CKD-EPI), BMI at admission, hand grip strength at admission, study site, diagnosis at admission.

DISCUSSION

Our study documented a high prevalence of vitamin D insufficiency (81.2%) and deficiency (94.3%) in an older population admitted to the rehabilitation units. Despite our hypothesis, we did not find a statistically significant association with outcomes measured at 30 days from admission, with the exception of changes in Barthel index over the time, for which a non-linear relationship and a statistically significant improvement in the third 25(OH)D quartile respect to the first one was evident already at 30 days. At three months from admission we found a non-linear association between 25(OH)D quartiles and functional outcomes, with an association that was weaker in the fourth quartile.

Our results at 30 days from admission confirmed the precedent findings of Pellicane et al., that in a sample of patients with a mean age of 71 years admitted to an inpatient rehabilitation unit did not find differences in functional independence between those with 25(OH)D levels below or above 30 ng/ml [16]. Similar results were documented by Kiebzak et al. comparing patients with 25(OH)D levels lower and higher than the median 25(OH)D value (16.6 ng/ml) in a sample with a mean age

of 70 year-olds admitted to an ambulatory rehabilitation [5]. However, these two studies were not focused on older people, and had a relatively small sample size (about 100 patients). Another study on 456 older patients admitted after a hip fracture found a positive association between 25(OH)D concentration and BI at discharge [17]. However, this study was not comparable to the others, because it took into account only the BI at discharge (mean LOS about 37 days) and not the functional improvement after rehabilitation. Few and contrasting data is available for a longer and more thorough follow-up: in a post-stroke population of 50 patients with a mean age of 72 years, 25(OH)D concentration was not associated with BI and modified Rankin Scale (mRS) both at three and six months [22]. On the other hand, poor functional outcomes (evaluated with mRS) were observed in 266 non diabetic-patients with vitamin D insufficiency one year after stroke [23], and at 3 months after thrombolysis due to an ischemic stroke [24]. However, none of these studies were focused on older adults, nor took into account the modification of functional outcomes over time, or if the patients performed rehabilitation shortly after the acute event and for how long. To our knowledge, only one study analyzed improvement in functional outcomes both at discharge and after discharge from a rehabilitation setting: in a sample of 171 older patients surgically treated for inter-trochanteric hip fracture, Seng et al. did not find differences in the improvement of modified BI either at discharge, or at 6 and 12 months, between patients with 25(OH)D below or above 20 ng/ml [25].

The lack of association between 25(OH)D and functional outcomes documented in many studies might be explained by the non-linear relationship between these two variables that we proved in our study: a dichotomization of 25(OH)D or its analysis using linear models as a continuous variable may therefore not be representative of this relationship. This non-linear association was already documented in community dwelling older people: in a cohort of 4100 persons aged ≥60 years there was a more evident improvement in walking time and time to stand for 25(OH)D concentration above 16 ng/ml [26], and of physical performance for 25(OH)D concentration <20 ng/ml [12].

Instead, the lack of association between 25(OH)D and functional outcomes at 30 days, with the

exception of an improvement in Barthel index over the time, observed in our study might be explained by a longer recovery time after an acute event needed for older adults [27].

The association between 25(OH)D levels and muscular strength [15], which might partly justify the relationship between 25(OH)D and functional outcomes, may be explained by the important role played by vitamin D in the skeletal muscle. In this tissue, vitamin D receptors (VDR) are well represented, and vitamin D contributed to the regulation of ATP-dependent calcium uptake by sarcoplasmic reticulum and in the production of actin and myosin [28]. Furthermore, vitamin D supplementation increases VDR intra-myonuclear concentration in type II fibers, type II muscle diameter and representation in rats [29]. VDR activation may be significantly reduced for 25(OH)D serum concentration below a critical value, thus explaining why in our sample the association between 25(OH)D and functional outcomes is evident for 25(OH)D concentration values below about 16 ng/ml. After this threshold 25(OH)D concentration may not significantly influence VDR activation, thus explaining the lack of further improvement of functional outcomes for 25(OH)D values above 16 ng/ml. This value is included in the third 25(OH)D quartile; it might explain the lack of further improvement in the fourth quartile documented in our population.

The reference value for 25(OH)D insufficiency of 20 ng/ml was established by assessing the point where serum parathyroid hormone starts to rise [30]; however, according to our results, and to the previously mentioned studies, this cut-off could not be representative of vitamin D effects on clinical and functional outcomes, especially in older adults. Considering the non-linear relationship between 25(OH)D and functional outcomes found in our study and previously reported by other authors, further studies in larger cohorts could work to propose new 25(OH)D cut-offs for older adults.

Among the strengths of our study, we include the relatively large sample size compared to the majority of previous studies in the same setting. Second, it placed focus on older adults, a population in which vitamin D deficiency may be more evident also because of age-related modification of the body composition [31]. Thanks to its longitudinal design, this study provides

information on functional outcomes both during rehabilitation and after discharge in the same sample, thus giving precious information on different steps after an acute event or an elective orthopedic surgery. We also acknowledge different limitations in our study: despite its longitudinal design, it had a relatively short follow-up, thus it may not accurately capture long-time effects of 25(OH)D. Data on vitamin D supplementation was available for one center only and we cannot exclude that data from the other two centers might influence our results. However, our analyses in this subsample suggests that supplementation did not significantly affect our outcomes. Finally, 25(OH)D dosage might be influenced by the different study site; in order to reduce this bias, we adjusted the models for the study site.

In conclusion, 25(OH)D concentration is positively associated with functional outcomes in older patients admitted to a rehabilitation setting. Testing for vitamin D insufficiency (perhaps using a lower cut-off compared to the one currently suggested) might contribute in estimating t he chance of functional recovery after an acute event or an elective orthopedic surgery for knee or hip replacement. Further research should confirm these results, and possible intervention studies should assess the impact of interventions directed to increasing 25(OH)D levels in case of insufficient values in this population.

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b. NUTRITION AND FUNCTIONAL OUTCOMES IN OLDER ADULTS ADMITTED TO REHABILITATION UNITS: THE SAFARI STUDY

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Nutritional Status and Functional Outcomes in Older Adults Admitted to Geriatric Rehabilitations: The SAFARI Study

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ABSTRACT

Objective: Evidence about the role of nutritional status (NS) on functional outcomes (FO) after rehabilitation in older adults is scarce. Our aim was to analyze the association between NS and FO in older adults admitted to geriatric rehabilitation units.

Methods: The Sarcopenia And Function in Aging Rehabilitation (SAFARI) multicenter study enrolled patients aged ≥65 years admitted to geriatric rehabilitation units in Italy and Spain. FO were absolute and relative functional gain (AFG-RFG) in Barthel Index (BI) at 1 and 3 months after admission. The association between NS (Mini Nutritional Assessment–Short Form) and FO was explored using linear regression and mixed models, adjusted for potential confounders. Analyses were then stratified for diagnosis at admission.

Results: We included 415 patients (mean age 81.4 years [SD: 7.7]; 67% female; 9.4% malnourished [MN], 42.7% at risk of malnutrition [RM], and 48% well nourished [WN]). Admission diagnoses were hip fracture (39.5%), elective orthopedic surgery (EOS) (29.5%), and stroke (31%). In an adjusted linear mixed model, MN and RM participants had lower BI compared to WN (MN: β: −8.47, $p = 0.023$; RM: β: −5.22, $p = 0.031$), and differences between groups remained stable over time. After stratification for admission diagnosis, only MN patients admitted after EOS had worse FO, both at 30 days (AFG: β adjusted: −13.54, $p < 0.001$; RFG: β: −32, $p < 0.001$) and 3 months (AFG: β adjusted: −17.79, $p < 0.001$; RFG: β: −26.77, $p = 0.002$).

Conclusions: In our sample, poor NS is associated with worse BI in older adults admitted to geriatric rehabilitation units; in patients undergoing EOS, MN is associated with worse FO. Our results documented for the first time the importance of assessing nutritional status before EOS.

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KEYWORDS
Older adults; malnutrition; rehabilitation; Barthel Index; MNA-SF

INTRODUCTION

Malnutrition is considered a geriatric syndrome characterized by involuntary weight loss and/or an acute or chronic discrepancy between nutritional needs and nutritional intake, and loss of function [1]. This condition is the result of interaction of multiple diseases and factors and its prevalence in community dwelling older people has been reported around 17% [2], increasing up to 30% in rehabilitation settings [3], and up to 20%-60% in the acute care settings [4,5].

The correlation between malnutrition and sarcopenia (i.e. the loss of skeletal muscle mass and function) has been widely described in the literature [4]. Previous cross-sectional and longitudinal

studies have reported an association between the coexistences of both malnutrition and sarcopenia and a poor physical performance [6,7]. Therefore, a poor nutritional status, through its influence on physical performance, may be associated with poor rehabilitation outcomes and the persistence over time of a worse physical function. Currently, only two studies, with a relatively small sample size (less than 200 participants), and performed on older adults admitted for different causes, documented a correlation between malnutrition and poor physical outcomes in older adults undergoing rehabilitation treatment [8,9].

Hip fracture and stroke are the most prevalent diagnoses in older adults admitted to geriatric rehabilitation units, but this notwithstanding, evidences regarding the association between malnutrition and functional outcomes in these populations are scarce. While results of previous studies on post-hip fracture patients are contrasting [10–12], only one study has reported the positive association between nutritional improvement and better functional outcomes on post-stroke patients [13]. Another frequent diagnosis of admission in geriatric rehabilitation wards is elective hip or knee replacement. To the best of our knowledge, no study has assessed the association between nutritional status and functional gain in older patients undergoing rehabilitation after elective orthopaedic surgery.

The objective of our study was to analyse the association between nutritional status and functional recovery in an older population admitted to geriatric rehabilitation units, analysing data from the Sarcopenia And Function in Aging Rehabilitation (SAFARI) study, a multi-centre international collaboration project on the identification of the frailty-related factors associated to functional improvement in older patients admitted to geriatric rehabilitation units.

MATERIALS AND METHODS

Study design and setting

The SAFARI study is a prospective multi-centre cohort study conducted in the geriatric rehabilitation departments of Gemelli Hospital, Rome (Italy), Ancelle Hospital, Cremona (Italy),

and Parc Sanitari Pere Virgili, Barcelona (Spain), between December 2014 and May 2016.

The inclusion criteria were age of 65 years or older, and admission diagnosis of orthopaedic surgery (i.e. hip fracture, and hip or knee replacement), or stroke. Patients with severe medical conditions that could influence the rehabilitative program, terminal diseases, a previous diagnosis of severe dementia (Global Deterioration Scale 6-7), or a severe functional impairment before the event (Total Barthel index pre-event ≤40) were excluded.

A multidimensional comprehensive geriatric assessment was performed at admission in order to establish an individualized rehabilitation plan, aimed to improve patients' functional outcomes. The rehabilitation units were staffed by full-time geriatricians, nurses, and nursing assistants, and also by physical, speech and occupational therapists.

The study was approved by the Animal and Human Ethics Committee of each institution.

Measurements and outcomes

The baseline evaluation was performed within 72 hours of admission by geriatricians and trained nurses or physiotherapists, and it included demographic characteristics, main diagnosis at admission (hip fracture, orthopaedic elective surgery, or stroke), body mass index (BMI), comorbidities (Charlson index [14]), cognitive function (Mini Mental State Examination [MMSE] [15]), and nutritional status (Mini Nutritional Assessment-Short Form [MNA-SF] [16]). A blood sample was collected within 72 hours of admission for complete blood count, creatinine, albumin, and cholesterol concentration dosage. The muscle strength was evaluated by grip strength in the dominant hand (or in the preserved hand in post-stroke patients) with a Jamar® hydraulic dynamometer.

The functional status was assessed by the Barthel Index (BI), a functional scale validated also for proxy and telephone interviews [17]. BI was collected before the acute event (self-reported by the patient or caregiver), at admission, at 30 days and at 3 months from admission (through telephonic interview). The length of stay (LOS) in days was systematically collected for all patients.

The functional outcomes evaluated were the Absolute Functional Gain (AFG), and the Relative

Functional Gain (RFG), both at 30 days and at 3 months. The AFG was defined as the difference between the total BI at 30 days or 3 months minus the BI at admission, while the RFG was defined as the AFG divided by the difference between BI pre-event minus BI at admission, thus taking into account the functional status before the event.

Analytic approach

The characteristics of the study sample were reported using descriptive statistics (mean and standard deviation for continuous variables, proportion for categorical variables) stratified according to MNA-SF classification (i.e. malnutrition [≤7 points], at risk of malnutrition [between 8-11 points], and well-nourished [≥12 points]). The relationship between nutritional status and outcomes was explored using linear regression models, using well-nourished (WN) participants as the reference group. The models were then adjusted for potential confounders, selected on the basis of the clinical significance, prior knowledge, and results of the univariate analysis. To explore the different role of demographic and clinical variables, we first adjusted for age and sex, and then for the other potential confounders (i.e. Charlson index, MMSE, length of stay, BI at admission, diagnosis at admission). RFG was not adjusted for BI at admission, in order to avoid over-adjustment. The association between nutritional status and functional outcomes was studied also through a mixed linear model, using BI as outcome over the time. The model was then adjusted for age, sex, Charlson index, MMSE, length of stay, and diagnosis at admission. Finally, analyses were stratified by diagnosis at admission (i.e. hip fracture, elective orthopaedic surgery, and stroke).
All analyses were performed using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The mean age of the 415 study participants was 81.4 years (SD 7.7); 67% were female. Thirty-nine participants (9.4%) were malnourished (MN), 177 (42.7%) at risk of malnutrition (RM), and 199 (48%) WN. Diagnosis at admission was hip fracture for 165 (39.5%) participants, elective

orthopaedic surgery for 123 (29.5%), and stroke for 127 (31%).

MN and RM patients were older (Mean [SD] MN: 82.3 [8.6] years, RM: 82.7 [7.3], WN 80.1 [7.6], p=0.004), and more frequently women (MN: 79%, RM: 72%, WN: 59%, p=0.008). MN patients had a lower BMI (mean[SD] MN: 23.7[4.4] kg/m^2, RM: 24.8[4.4], WN: 26.7[4.3], p<0.001) and a higher Charlson index (mean[SD] MN: 4.4[3.2], RM: 3[2.6], WN: 4[2.3], p<0.001), while there were no differences across groups in MMSE, eGFR, haemoglobin, cholesterol, or length of stay. Barthel index, both pre-event, at admission, at 30 days and 3 months after admission, increased across nutrition groups (Table 1).

Table 1. General characteristics of the population according with nutritional status

	Malnourished (MNA-SF ≤7)	At risk of malnutrition (MNA-SF ≥8 and <12)	Well-nourished (MNA-SF≥12)	P
	N=39	N=177	N=199	
Age (years), mean (SD)	82.3 (8.6)	82.7 (7.3)	80.1 (7.6)	0.004
Female sex, %	79	72	59	0.008
Diagnosis at admission				
Hip fracture, %	7	50	44	0.003
Elective orthopaedic surgery, %	11	28	60	
Stroke, %	11	47	42	
BMI (kg/m^2), mean (SD)	23.7 (4.4)	24.8 (4.4)	26.7 (4.3)	<0.001
Charlson Index, mean (SD)	4.4 (3.2)	3 (2.6)	4 (2.3)	<0.001
MMSE, mean (SD)	21.7 (5.7)	22.7 (5.5)	23.3 (5.7)	0.256
eGFR (ml/min/1.73m^2), mean (SD)	72.9 (18.7)	69.4 (21.4)	65 (22.5)	0.092
Haemoglobin (g/dl), mean (SD)	11.2 (1.8)	10.9 (1.8)	10.9 (2.2)	0.699
Cholesterol (mg/dl), mean (SD)	156.6 (32)	157.2 (39)	153.9 (33.8)	0.756
Albumin (g/dl), mean (SD)	3.5 (0.4)	3.5 (0.4)	3.3 (0.4)	0.007
BI before event, mean (SD)	83.4 (18.3)	87.6 (16.1)	93.2 (14.7)	<0.001

BI at admission, mean (SD)	30.9 (18.9)	36.7 (16.7)	50.6 (22)	<0.001
BI at discharge, mean (SD)	57.5 (25)	63.1 (21.2)	76.4 (23.8)	<0.001
BI after 3 months, mean (SD)	72.3 (32)	72.1 (27.5)	83.4 (24.1)	<0.001
Ability to walk at discharge, %	49	64	78	<0.001
Ability to walk at 3 months, %	75	83	88	0.095
Length of stay (days), mean (SD)	29.7 (8.5)	29.4 (6.8)	28.4 (11.7)	0.523

Abbreviations: BMI: body mass index; eGFR: estimated glomerular filtration rate; BI: Barthel Index.

In the whole sample, linear regression models did not find an association between nutritional status and AFG at 30 days after admission (MN: β 1.294, p=0.687; RM: β 1.123, p=0.558), not either after adjustment for potential confounders (MN: β -3.141, p=0.313; RM: β -1.586, p=0.407); the absence of association was confirmed also at 3 months after admission (Crude model: MN: β 8.279, p=0.086; RM: β 2.180, p=0.451; Adjusted model: MN: β -2.001, p=0.631; RM: β -2.746, p=0.290) (Table 2).

Table 2. Linear regression models, for MNA-SF classification, crude and adjusted for potential confounders, using well-nourished as the reference group.

	30 days		3 months	
Absolute functional gain				
	β Crude (P)	β Adjusted* (P)	β Crude (P)	β Adjusted* (P)
Malnourished	1.294 (0.687)	-3.141 (0.313)	8.279 (0.086)	-2.001 (0.631)
At risk of malnutrition	1.123 (0.558)	-1.586 (0.407)	2.180 (0.451)	-2.746 (0.290)
Relative functional gain				
	β Crude (P)	β Adjusted (P) #	β Crude (P)	β Adjusted# (P)
Malnourished	-4.587 (0.647)	-4.344 (0.665)	20.049 (0.227)	15.968 (0.329)
At risk of malnutrition	-8.973 (0.134)	-6.082 (0.315)	-0.969 (0.923)	4.375 (0.661)

* Models adjusted for age, sex, MMSE, Charlson index, length of stay, Barthel index at admission,

diagnosis at admission.
Models adjusted for age, sex, MMSE, Charlson index, length of stay, diagnosis at admission.

There was not association between nutritional status and RFG, both at 30 days (Crude model: MN: β -4.587, p=0.647; RM: β -8.973, p=0.134; Adjusted model: MN: β -4.344, p=0.665; RM: β -6.082, p=0.315) and 3 months after admission (Crude model: MN: β 20.049, p=0.227; RM: β -0.969, p=0.923; Adjusted model: MN: β 15.968, p=0.329; RM: β 4.375, p=0.661) (Table 2). Figure 1 shows BI changes over time across nutrition groups: the BI decreased at admission, and then gradually increased during the time, but at 3 months a complete recovery was not observed in any of the groups.

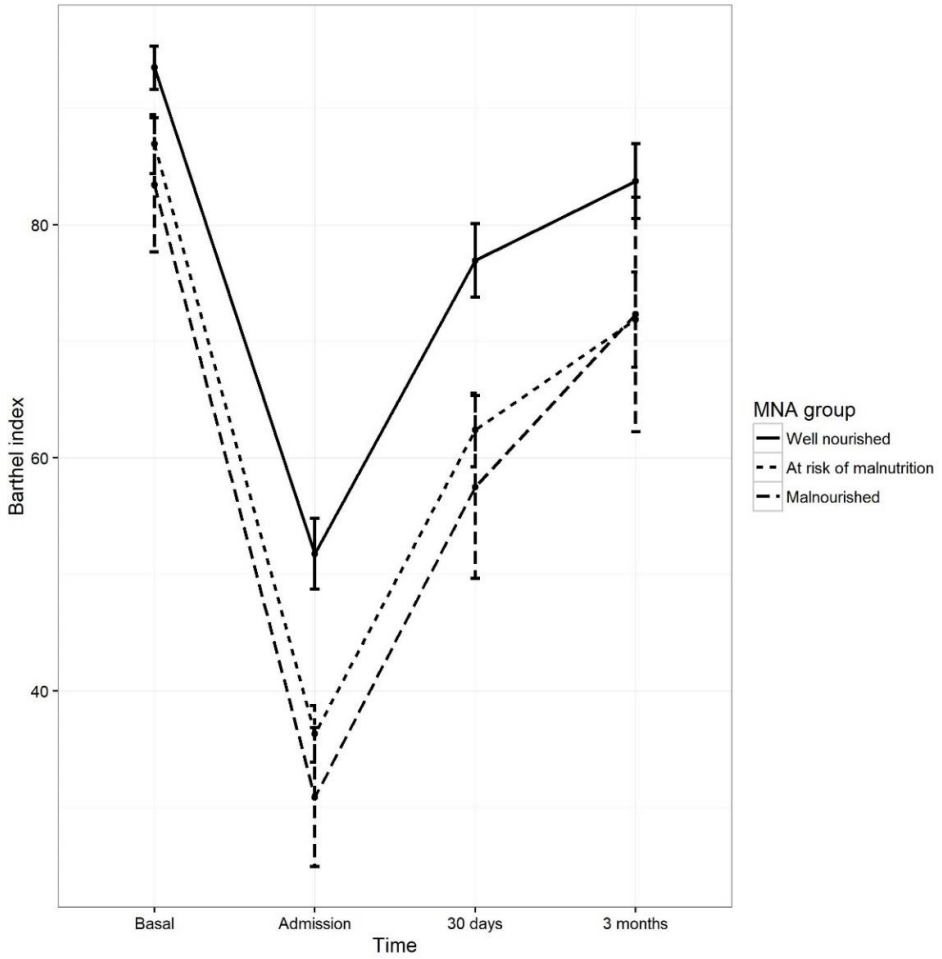

Figure 1. Changes in Barthel Index over the time according with nutritional status. Over the time, malnourished and at risk of malnutrition patients have a lower Barthel Index respect to well-nourished patients.

Adjusted linear mixed models showed that MN and RM participants had constantly a lower Barthel index over the time, respect to well nourished (mean BI difference: MN: -8.47, p=0.023 and RM: -5.22, p=0.031); furthermore, changes in BI over the time did not differ across the groups (Table 3).

Table 3. Linear mixed models of the association between nutritional status and Barthel Index over the time, adjusted for potential confounders, using well-nourished as the reference group

	Estimate	P-value
Malnourished (mean over the time)	-8.467	0.023
At risk of malnutrition (mean over the time)	-5.221	0.031
Admission time* Malnourished	-0.725	0.865
30 days-time* Malnourished	-0.182	0.966
3 months-time* Malnourished	3.432	0.432
Admission time* At risk	-1.523	0.588
30 days-time*At risk	-1.813	0.520
3 months-time*At risk	-3.787	0.188

Models adjusted for age, sex, Charlson index, MMSE, length of stay, diagnosis at admission.

When stratified for diagnosis at admission, linear regression models adjusted for potential confounders found no association between nutritional status and functional recovery in the hip fracture and stroke subgroups (data not showed), while in the subgroup of patients undergoing elective orthopaedic surgery, MN participants had an AFG significantly lower respect to well-nourished participants, both at 30 days and 3 months after admission, while in RM patients this association was confirmed only at 30 days (AFG at 30 days: MN: β -13.54 [p<0.001], RM: β -8.87 [p=0.002]; AFG at 3 months: MN: β -17.79 [p<0.001], RM: β -4.27 [p=0.243]). Similar results were found for the RFG (RFG at 30 days, Adjusted: MN: β -32.00 [p<0.001], RM: β -16.97 [p=0.016]; RFG at 3 months, Adjusted: MN: β -26.77 [p=0.002], RM: β 2.30 [p=0.721]).

DISCUSSION

Our study documented an association between nutritional status and physical function; however, the trajectory of functional recovery did not differ in patients with or without malnutrition. Stratifying by diagnosis at admission, we observed an association between nutritional status and functional outcomes only in patients admitted after an elective orthopaedic surgery.

The evidences on the association between nutritional status and functional outcomes in older adults admitted to rehabilitation units are scant, and this relationship has been previously investigated only cross-sectionally: in a population of 133 patients aged ≥65 years, admitted for different causes to a rehabilitation ward, Neumann et al. reported that patients who were malnourished or at risk of malnutrition had a worse function both at admission and after 90 days, but did not analyze the changes in physical function over the time [9]. Similarly, Chevalier et al. documented a positive relationship between nutritional status and gait speed in a sample of 182 older adults with disability (defined as impairment in activities of daily living) undergoing ambulatory rehabilitation [8]. The different approach to the functional outcomes might explain the discordance with our results: a cross sectional association between nutritional status and functional performance might not mirror the association with functional improvement over the time.

Few other studies analysed this association focusing on specific populations (post-stroke, hip fracture, and orthopaedic elective surgery for hip or knee replacement). With respect to stroke, to the best of our knowledge, only one study was performed, with a small sample size (N: 67), on older adults admitted to a rehabilitation setting, reporting a positive association between nutrition and physical function [13]. However, this study was focused on patients with a BMI <18 kg/m^2 or with a weight loss >2 kg after admission, therefore it is not comparable with the others previously mentioned; furthermore, the authors did not investigate the nutritional status *per se* but compared patients with and without nutritional improvement during hospital stay.

In a hip fracture population, the current evidence is contrasting, and none of the studies on this topic were performed in rehabilitation settings, but all in an orthopaedic ward; furthermore, in none of them there is a clear information about the actual rehabilitation after hip-fracture [10–12]. Li et al. documented in a population of 162 older patients admitted to an orthopaedic ward that malnourished patients had a worse functional recovery over 1 year of follow-up respect to well-nourished [10]. Otherwise, Goisser et al. in a population of 97 patients aged ≥75 years admitted to an orthopaedic ward after hip fracture, documented no correlation between nutritional status and functional gain over 6 month follow-up after hospital discharge, but observed a constant worse functional status in patients with a worse pre-fracture nutritional status [11]. Our findings are in line with these results, and extend them to a larger older adult population, admitted to rehabilitation not only after hip fracture, and confirmed these results both during rehabilitation and after discharge.

Surprisingly, in older patients admitted to rehabilitation after elective hip or knee surgery, nutritional status correlated with functional recovery, both at 1 and 3 months; furthermore, the functional status at three months remained worse respect to the pre-operative one. This discordance may be explained by the different characteristics of this sub-population: patients admitted after a hip fracture are frequently frail, older and with many comorbidities [18]; patients admitted after a stroke have also a slower recovery, that is significantly influenced by age [19], while patients undergoing and elective surgery are usually younger and have a better clinical and physical conditions [20], as in our sample. Therefore, patients admitted after an orthopaedic elective surgery may have less negative factors influencing the recovery process, and malnutrition might play a central role in defining the time and rate of the recovery. In the other subgroups, the effects of malnutrition might have been no detectable due to the presence of a stronger factor, the acute event.

This study had many strengths: it has a relatively large sample, and included patients admitted in rehabilitation for the most common described diagnoses in this setting. This is, to the best of our knowledge, the first study that investigates the association between nutrition and functional

recovery in older adults admitted to rehabilitation units after undergoing a hip or knee elective replacement surgery. This is a main strength, because a pre-operative nutritional assessment could detect patients with or at risk for malnutrition, in whom a tailored nutritional intervention before the surgery may improve functional recovery. Moreover, our study is focused on older adults, a population in which malnutrition strongly influence the risk of sarcopenia [21], physical function, and disability [6,22]. Finally, thanks to its longitudinal design, this study provides information on functional outcomes both during rehabilitation and after discharge, giving information about different steps after an acute event or orthopaedic elective surgery.

However, this study has many limitations: first of all, our study included only 39 malnourished patients (in line with other studies on this topic [11]); therefore, in order improve the accuracy of the statistical estimations, we decided to use the well-nourished group as the reference one. Furthermore, despite its longitudinal design, it has a relatively short follow-up. Finally, the BI pre-event of patients admitted after an elective orthopaedic surgery may be biased because influenced by the clinical condition that is treated by surgery. However, despite this limitation, these results are clinically significant and, if confirmed, may have an important impact on public health.

CONCLUSION

Poor nutritional status is associated with a worse functional status in older adults admitted to geriatric rehabilitation units; however, an association between nutritional status and functional improvement was observed only in patients undergoing an orthopaedic elective surgery. A screening for malnutrition in older adults in community could be helpful in preventing disability after an acute event. Furthermore, in consideration of the association between nutritional status and functional outcomes in patients admitted after elective orthopaedic surgery, a pre-operatory assessment of nutritional status should be taken into account in patients candidate to hip or knee

elective surgery, in order to reduce the risk of disability and/or lower functional recovery. Further studies are needed to substantiate these results.

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c. ASSOCIATION BETWEEN NUTRITIONAL STATUS AND OUTCOMES IN OLDER ADULTS AFFECTED BY CHRONIC HEART FAILURE

ABSTRACT

Introduction: Malnutrition is common in older adults affected by heart failure. It is related to poorer physical function and higher risk of hospitalizations, but evidence in older adults affected by chronic HF (CHF) is scant. No data are available in this population on the risk of acute heart failure not requiring hospitalization (AHFNH).

Objective: to evaluate whether nutritional status is associated to physical function (PF), hospitalizations and AHFNH in older adults affected by CHF. Secondary objective is to assess the role of energy intake and appendicular skeletal muscle mass index (ASMMI) in influencing the relationship between MNA and PF.

Methods: observational study including 101 older adults affected by CHF admitted to a cardiology outpatient clinic. Patients performed cardiological visit, echocardiogram, geriatric multidimensional evaluation, MNA, EPIC, bio-impedance analysis, gait speed and were followed-up for a mean of 519.9 days. MNA was not available for 2 patients; only one was categorized as malnourished and was excluded. MNA was analyzed both as continuous and categorical variable (at risk of malnutrition/malnourished). The association between nutritional status and PF was assessed using linear regression models, adjusted for potential confounders (age, sex, ejection fraction, energy intake and ASMMI). The association with hospitalization and AHFNH was assessed with adjusted Poisson regressions.

Results: mean age was 77.4 years, 74% were male. Patients at risk of malnutrition (RM) had a lower gait speed respect to well-nourished (WN) (0.8 vs 1m/s), data confirmed in linear models (RM β -0.167, P=0.007), but that did not reach the statistical significance after adjustment for

potential confounders (RM β -0.131, 95% CI 0.76-1.01). RM had a 303% higher risk of AHFNH (Adjusted IRR 3.03, 95%CI 1.04-8.63). No differences in risk of hospitalizations was found.

Conclusions: RM is associated with poor PF and higher risk of AHFNH respect to WN. Early assessment of nutritional status is recommended in older adults affected by CHF.

INTRODUCTION

Heart failure is a chronic progressive syndrome with a high prevalence worldwide, that increases with age (1), and with a natural history characterized by a progressive functional decline (2), hospitalizations, and death. This disease, especially in advanced stages, is characterized by a high prevalence of malnutrition, a syndrome characterized by involuntary weight loss and/or an acute or chronic discrepancy between nutritional needs and nutritional intake (3): malnutrition prevalence ranges from 16% to 62.4% in stable heart failure (4) and increases up to 80% in advanced HF (5) and to 75%-90% in patients hospitalized for acute heart failure (4). This large range of prevalence is related to different nutritional tools used and to different mean age of the studies: older adults affected by heart failure are at higher risk of malnutrition compared to younger people because of heart failure-related mechanisms, such as low nutritional intake due to intestinal edema and anorexia (6), liver disfunction (7), and cytokine-induced hyper-catabolism (8), but also because of age-related factors, such as comorbidities, polytherapy, and social factors. Malnutrition is associated with sarcopenia (9) and poor physical performance (10,11), but also to increased risk of hospitalizations and death (12). Therefore, in malnourished older adults with heart failure, the coexistence of two disease associated with negative outcomes in patients at higher risk respect to younger because of age-related risk factor, may cause a stronger association between nutritional status and outcomes, and thus the association might be evident also in patients at risk of malnutrition, and not only in the malnourished ones. Furthermore, in older adults affected by heart failure, the relationship between nutritional status and physical function might be not exclusively

related to caloric intake, muscle mass, and heart failure severity, but other factors, such as mood or social factors, may influence the relationship.

The role of nutritional status in influencing physical function in patients affected by heart failure has been poorly investigated: only few data are available on the association between malnutrition and physical function (13,14), and only one study was focused exclusively on older adults, documenting an association between malnutrition and a poorer physical function (15). However, this study included patients hospitalized for rehabilitation after heart failure and analysed physical function using the Barthel index at discharge. No data are available on older adults affected by chronic heart failure. Furthermore, to the best of our knowledge, no studies investigated the role of caloric intake and reduced appendicular skeletal muscle mass in influencing the association between malnutrition and physical function.

Despite recent evidences documented that malnutrition, evaluated through multidimensional tools, is also an important risk factor for hospitalizations and death in patients affected by heart failure (16–18), fewer evidences are available in older adults: Sargento et al. documented that malnutrition was associated with hospital admission in a population of older adults affected by chronic heart failure (19) and to mortality in a similar population, followed-up for 3 years (20). To the best of our knowledge, there are no evidences on the association between nutritional status and risk of acute heart failure not requiring hospitalization. The objective of this study was to analyse the association between nutritional status and physical function in older adults affected by chronic heart failure and to analyse the role of caloric intake and appendicular skeletal muscle mass index in influencing this relationship. Secondary objectives of the study were to analyse the association between nutritional status and acute heart failure not requiring hospitalization, heart failure-related and all-cause hospitalizations in this population.

METHODS

Study design and setting

In this prospective observational study, we enrolled patients aged 65 years or older affected by heart failure in NYHA class II-IV, attending an outpatient heart failure clinic at S. Camillo-Forlanini Hospital in Rome between January 2016 and September 2018. We excluded patients with acute cardiovascular events (history of myocardial infarction or acute heart failure in the previous month), severe dementia, visual or hearing impairment or severe functional limitation (not able to walk 4 meters). We also excluded participants affected by chronic kidney failure requiring emo-dialysis, active cancer, not compensated thyroid disease, terminal diseases (life expectancy < 1 year) or malabsorption diseases. The study was approved by the Ethic Committee of the S. Camillo-Forlanini Hospital (1578/CE Lazio 1).

Measurements and outcomes

At baseline all participants performed a cardiological visit, during which cardiologic history, comorbidities, and pharmacological therapy were collected. An echocardiogram was performed by a trained cardiologist with a Philips IE 33; left ventricular ejection fraction (4 chambers Simpson method) and filling pressures (mean E/e' ratio), tricuspid annular plane systolic excursion (TAPSE), chambers dimensions, evidences of valvular defects and inferior cava vein collapsibility were systematically evaluated. Blood examination including creatinine, electrolytes, complete blood count, and brain natriuretic peptide (BNP) was performed during the day of the first evaluation or in the two weeks before. Participants underwent to a geriatric multidimensional evaluation, including basic activities of daily living (ADL), instrumental activities of daily living (IADL), Montreal Cognitive Assessment (MOCA), geriatric depression scale (GDS), and euroQOL.

The nutritional assessment included the body mass index (BMI) and bio-humoral indicators (total cholesterol, total proteins, albumin). The risk of malnutrition was evaluated using the Mini Nutritional Assessment (MNA), a multidimensional evaluation tool approved by the American Society for Parenteral and Enteral Nutrition (ASPEN) and validated in older adults (21). The dietary

intake was evaluated using the EPIC questionnaire (22), that investigates intake frequency over the previous year of 236 specific foods, along with the average size of the servings, selected from a range as shown in photographs. The information derived from the questionnaire was automatically converted into data on energy, micro- and macronutrient intake by a specifically designed software. The EPIC nutritional assessment has been successfully validated in an older adult population (23). The body composition (free fat mass index, fat mass index, appendicular skeletal muscle mass index) was evaluated using bio-impedance analysis (BIA 101 New Edition, Akern).

Physical function was evaluated through the gait speed (4 meters at usual pace), performed the day of the first visit. It is a quick, reliable measure of functional capacity with well-documented predictive value for major health-related outcomes in older adults (24,25).

Participants were followed-up for 1 year. At follow-up participants performed a cardiological visit, an echocardiogram and a multidimensional evaluation, and data on relevant clinical events, were recorded. Acute heart failure not requiring hospitalization was defined as an acute worsening of clinical signs and/or symptoms of heart failure requiring an increase of diuretic therapy and/or a management of the heart failure therapy during the cardiologic outpatient visit.

Statistical analysis

The characteristics of the study sample were reported using descriptive statistics (mean and standard deviation or proportions, as appropriate), according to MNA classes (Malnourished: <17 points; At risk of malnutrition: 17 to 23.5 points; Well nourished: 24 to 30 points). The association between nutritional status and physical function (gait speed) was evaluated with the Pearson's correlation test. The results were then verified using linear regression models with MNA included both as continuous and categorical variable. Results were adjusted for potential confounders: age, sex, ejection fraction, appendicular skeletal muscle mass index (ASMMI), the daily caloric intake indexed for ideal body weight (calculated using the Lorenz formula), and the GDS.

The association between nutritional status and clinical outcomes was investigated using Poisson regressions and an incidence rate ratio was calculated, using well-nourished patients as the reference group. The models were then adjusted for age, sex, ejection fraction and geriatric depression scale.

RESULTS

The mean age of the 99 study participants at baseline was 77.4 years (SD 7.4); 74% were male. The mean ejection fraction was 40.4% (SD 10.1); 74.5% of the participants was in NYHA class II and 25.5% in NYHA class III; no participants were classified in NYHA class IV. Sixty-four participants were classified according to MNA as "well-nourished" (WN), 34 as "at risk of malnutrition" (RM) and only one as "malnourished", that was excluded. There were no differences in age and comorbidities between the groups, except for anaemia, that was more prevalent in the RM group (26% vs 9%, P=0.052), and COPD, that was more prevalent in the WN group (33% vs. 9%, P=0.017). RM participants were less frequently male (56% vs 84%, P=0.005). There were no differences between groups in BNP serum concentration (WN: 275.3, SD 353.7; RM: 270.8 pg/ml, SD 230.1; P=0.945). The daily energy and protein intake standardized for the ideal body weight (calculated using the Lorenz formula) did not differ between the two groups. The RM group had a lower BMI (24.8 kg/m^2, SD 3.3 vs 26.2 kg/m^2, SD 3.5, P=0.065), free fat mass index (18.9 kg/m^2, SD 2.6 vs 20.6 kg/m^2, SD 2.3; P=0.003) and appendicular skeletal muscle mass index (6.9 kg/m^2, SD 1.1 vs 8 kg/m^2, SD 2.8, P=0.007), while there were no differences in fat mass index (Table 1). There were no differences between groups in heart failure aetiology, but RM participants were more frequently in NYHA class III (38.2% vs 18.8%, P =0.062) (Table 1).

Table 1. General characteristics of the population at baseline

	Well-nourished	At risk of malnutrition	All	P-value

	N:64	N: 34	N: 98	
Age	77.4 (6.7)	77.5 (8.7)	77.4 (7.4)	0.949
Male sex	84	56	74	0.005
Hypertension	59	68	62	0.558
Type II diabetes	28	24	27	0.802
Thyroid disease	16	18	16	1
Anoemia	9	26	15	0.052
Atrial fibrillation	41	38	40	0.989
IHD	58	62	59	0.871
Dyslipidemia	42	33	39	0.570
Stroke	3	6	4	0.904
COPD	33	9	24	0.017
Creatinine (mg/dl)	1.3 (0.5)	1.2 (0.5)	1.3 (0.5)	0.141
eGFR (CKD-EPI, mL/min/1.73 mq)	54.7 (18)	61.8 (23.4)	57.2 (20.2)	0.136
Haemoglobin (g/dl)	13 (1.6)	12.5 (1.5)	12.8 (1.5)	0.096
BNP (pg/ml)	275.3 (353.7)	270.8 (230.1)	273.7 (314.5)	0.945
Total cholesterol (mg/dl)	151.6 (39.7)	160 (41.6)	154.5 (40.3)	0.355
Kcal/idea weight	29.3 (9.8)	27.5 (5.8)	28.7 (8.7)	0.249
Proteins/ideal weight	1.1 (0.3)	1.2 (0.3)	1.1 (0.3)	0.498
Fat mass index (kg/m2)	5.6 (2.5)	6.2 (2.6)	5.8 (2.5)	0.300
Free fat mass index (kg/m2)	20.6 (2.3)	18.9 (2.6)	20 (2.5)	0.003
Appendicular skeletal muscle mass index	8 (2.8)	6.9 (1.1)	7.7 (2.4)	0.007
BMI (kg/m2)	26.2 (3.5)	24.8 (3.3)	25.7 (3.5)	0.065
Uncertain	17.2	8.8	14.3	0.584
Ischemic cardiopathy	56.2	61.8	58.2	
Dilated cardiomyopathy	1.6	5.9	3.1	
Restrictive cardiomyopathy	1.6	0	1	
Valvular heart disease	17.2	14.7	16.3	
Hypertensive heart disease	4.7	2.9	4.1	
Degenerative heart disease	1.6	5.9	3.1	
NYHA class II	81.2	61.8	74.5	0.062
NYHA class III	18.8	38.2	25.5	

Abbreviations: IHD: ischemic heart disease; COPD: chronic obstructive pulmonary disease; BMI: body mass index; eGFR: estimated glomerular filtration rate; NYHA: New York Health Association.

RM group had a higher GDS score (5.7, SD 3.7 vs 3.5, SD 3.2, P=0.006. These patients had also more frequently an impairment in ADL (40.6% vs 15.3%, P=0.015) and urinary incontinence (31.2 vs 10.2%, P=0.025). There were no differences between groups in quality of life and cognitive function, while the number of years of education was lower in the RM group (6.9, SD 4.1 vs 9.6, SD 4.8, P=0.009) (Table 2).

Table 2. Multidimensional evaluation at baseline

	Well-nourished N: 64	At risk of malnutrition N: 34	All N: 98	P-value
MOCA	20.7 (4.6)	19 (5.6)	20.2 (4.9)	0.228
Geriatric Depression Scale	3.5 (3.2)	5.7 (3.7)	4.2 (3.5)	0.006
EuroQOL	65.3 (17.3)	65.2 (19.6)	65.2 (18)	0.984
Education	9.6 (4.8)	6.9 (4.1)	8.7 (4.8)	0.009
Gait speed (m/s)	1 (0.3)	0.8 (0.3)	0.9 (0.3)	0.006
ADL impairment	15.3	40.6	24.2	0.015
IADL impairment	40.7	53.3	44.9	0.363
Urinary incontinence	10.2	31.2	17.6	0.025
No caregiver for pharmacological therapy	98.3	78.1	91.2	0.004
Alone	8.5	13.3	10.1	0.757
Disabled Spouse	10.2	3.3	7.9	
No disabled spouse	55.9	53.3	55.1	
Son	18.6	23.3	20.2	
Caregiver	6.8	6.7	6.7	

Abbreviations: MOCA: Montreal Cognitive Assessment Test; GDS: geriatric depression scale; QOL: quality of life; ADL: activities of daily living; IADL: instrumental activities of daily living.

There were no differences between groups in ejection fraction (RM: 39.1%, SD 10.8; WM: 40.8, SD 9.8; P=0.443) nor in medium E/E' ratio (RM: 16.1, SD 9.1; WN: 13.8, SD 7.5; P=0.274) or in

severity of valvulopathies, with the exception of mitral regurgitation that was more frequent in the RM group (Table 3).

Table 3. Echocardiographic characteristics of the population

	Well-nourished N: 64	At risk of malnutrition N: 34	All N: 98	P-value
Ejection fraction (%)	40.8 (9.8)	39.1 (10.8)	40.2 (10.1)	0.443
Medium E/E' ratio	13.8 (7.5)	16.1 (9.1)	14.6 (8.1)	0.274
TAPSE (mm)	18.7 (3.6)	20.5 (5.1)	19.3 (4.2)	0.082
Left atrium area (cmq)	27.4 (6.3)	26.1 (6.7)	27 (6.5)	0.412
PAPS (mmHg)	37.3 (9.8)	38.7 (12.2)	37.8 (10.7)	0.562
Mild MR	52	31	45	0.019
Mild-moderate/moderate MR	31	59	41	
Moderate-severe/severe MR	0	3	1	
Mild AOS	2	0	1	0.542
Mild-moderate/moderate AOS	5	6	5	
Moderate-severe/severe AOS	2	6	3	
Mild AOR	25	41	30	0.245
Mild-moderate/moderate AOR	8	9	8	
Mild TR	59	56	58	0.629
Mild-moderate/moderate TR	12	9	11	
Moderate-severe/severe TR	2	6	3	
Inferior vena cava partially collapsible	23	30	25	0.597
Inferior vena cava not collapsible	10	15	11	

Abbreviations: TAPSE: tricuspid annular plane systolic excursion; sPAP: systolic pulmonary artery pressure; MR: mitral regurgitation; AOS: aortic stenosis; AOR: aortic regurgitation; TR: tricuspid regurgitation.

Respect to WN group, RM participants had a lower gait speed (0.8 vs 1 m/s, p=0.006) (Figure 1).

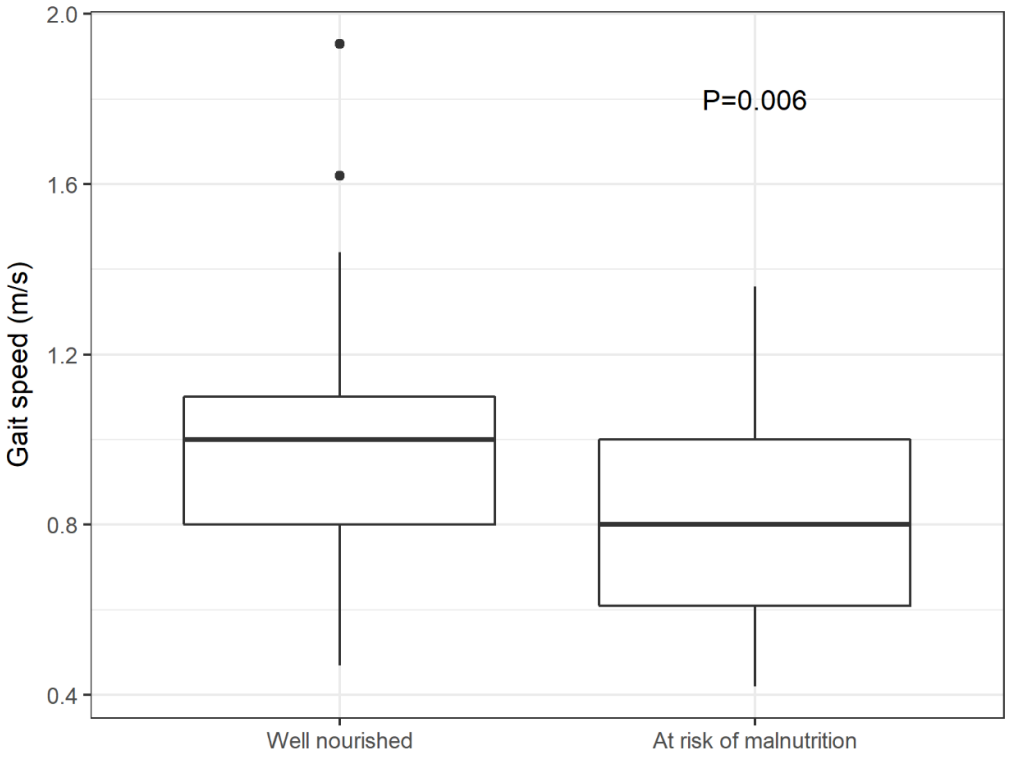

Figure 1. Differences in gait speed according to nutritional status.

These results were confirmed by a linear regression model, using WN as reference (β -0.167, P=0.006) The adjustment for potential confounder didn't cause a clinically significant change in the coefficient but the association didn't reach the statistical significance (β -0.131, P=0.07, 95% CI 0.76-1.01) (Table 4).

Table 4. Multivariable linear regression model of the association between nutritional status and gait speed

	β	P-value
Well-nourished	Reference	-
At risk of malnutrition	-0.131	0.069
Age	-0.005	0.230
Sex	0.112	0.179
Ejection fraction	-0.0006	0.848
GDS	-0.005	0.621
ASMMI	-0.013	0.260

Caloric intake/ideal body weight	0.0007	0.822

Abbreviations: GDS: geriatric depression scale; ASMMI: appendicular skeletal muscle mass index.

Analysing MNA as a continuous variable, it was positively associated with the gait speed (r: 0.290, P=0.007) (Figure 2). The association was confirmed by a linear regression model, both crude (β 0.027, P=0.007) and after adjustment for potential confounders: β 0.022, P=0.05) (Table 5).

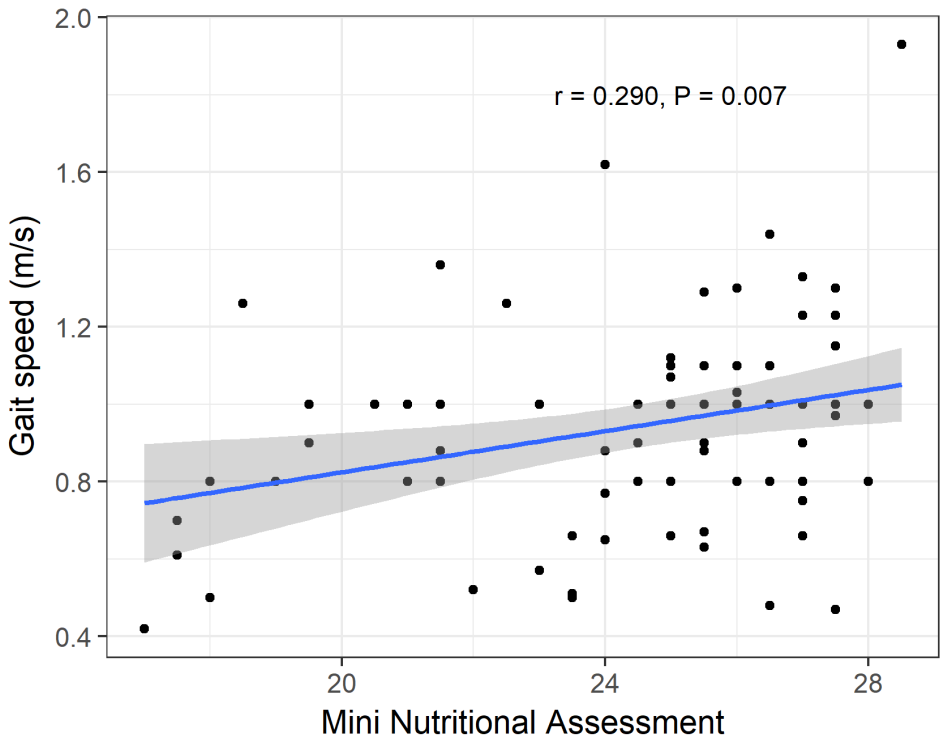

Figure 2. Association between Mini Nutritional Assessment and gait speed

Table 5. Multivariable linear regression model of the association between mini nutritional assessment and gait speed

	β	P-value
MNA	0.022	0.05
Age	-0.005	0.219
Sex	0.114	0.181
Ejection fraction	-0.001	0.828
GDS	-0.004	0.669
ASMMI	-0.012	0.294

Caloric intake/ideal body weight	0.0004	0.902

Abbreviations: GDS: geriatric depression scale; ASMMI: appendicular skeletal muscle mass index.

Of the 98 patients enrolled at baseline, 67 performed a clinical follow-up after a mean time of 519.9 days (SD 200.7). During the follow-up time, four participants died. Twenty-five participants had at least one hospitalization, 13 of which caused by exacerbation of heart failure. Finally, fifteen participants had at least one exacerbation of heart failure that didn't required hospitalization.

There were no differences between groups in incidence rate of hospitalizations, while participants at risk of malnutrition had a 303% higher risk of exacerbation of heart failure not requiring hospitalization respect to the well-nourished (Table 6).

Table 6. Incidence rate ratio of negative outcomes according to nutritional status, using well-nourished as the reference group.

	IRR crude (95% CI)	IRR adjusted* (95% CI)
All-cause hospitalization		
At risk of malnutrition	0.93 (0.44-1.85)	1.67 (0.71-3.70)
HF-related hospitalizations		
At risk of malnutrition	0.89 (0.28-2.39)	1.55 (0.41-5.16)
Exacerbated HF not requiring hospitalization		
At risk of malnutrition	1.42 (0.55-3.42)	3.03 (1.04-8.63)

* Models adjusted for age, sex, ejection fraction, geriatric depression scale.
Abbreviation: HF: heart failure.

DISCUSSION

Our sample of older adults affected by chronic heart failure was globally well-nourished using the WHO classification of the BMI (no participant was underweight, only 11.6% obese). This was confirmed by the Mini Nutritional Assessment, that classified only one participant as malnourished

(that was excluded from the study), 35% at risk of malnutrition and 65% well-nourished. Nonetheless, we documented that participants at risk of malnutrition were characterized by a poorer physical function and a higher risk of acute heart failure not requiring hospitalization with respect to well-nourished participant.

The association between nutritional status and physical function has been previously reported by other authors, but only in patients hospitalized for rehabilitation (14,15) or with a first diagnosis of heart failure with preserved ejection fraction (13): Katano et al. in their observational study including 145 participants aged ≥65 years old admitted to rehabilitation for heart failure documented that malnutrition, defined using the MNA short form (MNA-SF), was an independent predictor of functional dependence, evaluated using the Barthel index, at discharge (15). In a similar setting, in a sample of 105 patients with a mean age of about 73 years, Matsuo et al. documented that MNA-SF was linearly associated with the BI (14). Kinugasa et al documented that in a sample of patients with mean age 77 years affected by heart failure with preserved ejection fraction, moderate or severe nutritional risk, evaluated using the Geriatric Nutrition Risk Index (GNRI), was associated with a lower Barthel index at discharge (results not adjusted for potential confounders) (13). However, only the study of Katano et al was focused on older adults, and the association between nutritional status and physical function in chronic heart failure has not been previously studied. Furthermore, the impact of risk of malnutrition only has never been previously assessed. Our results are in line with the previous studies and extend their results: we confirmed the association between MNA and physical function, and we documented that in older adults not only malnutrition (as previously reported) but also the risk of malnutrition is associated with a poorer physical function respect to well-nutrition.

Our study analysed for the first time the role of energy intake and appendicular skeletal muscle mass index in influencing the association between nutritional status and physical function. Interestingly, differently from what would have been expected, nor energy intake, adjusted for ideal

body weight, nor appendicular skeletal muscle mass significantly influenced this association. The explanation of these results could lie in the fact that MNA is a multidimensional tool, that take into account not only dietary intake and the body mass index, but also other factors, such as polypharmacy, mood and cognitive impairment, muscle mass (21). This approach could be more effective in older adults, that are characterized by comorbidities, polypharmacy and higher risk of disability, and in whom the association between nutritional status and physical function may not be exclusively related to a reduced energy intake or reduced muscle mass but also to multiple other factors, such as social factors, poli-therapy, recent acute events, depression, that together contribute to the outcome.

To the best of our knowledge, only Sargento et al studied the association between nutritional status and hospitalization in older adults affected by heart failure (19). The authors documented that in a sample of 50 outpatients aged ≥65 years affected by heart failure with reduced ejection fraction, malnutrition was associated with a higher risk of hospitalizations at 12 months (19). The lack of association between nutritional status and hospital admissions found in our study could be explained by the absence of malnourished patients in our sample: in fact, the risk of malnutrition could be an intermediate condition, identifying patients at higher risk of negative outcomes respect to well-nourished patients, but with a lower risk respect to malnourished patients. For example, our group documented in a sample of older adults admitted to rehabilitation units that patients at risk of malnutrition had a lower Barthel index respect to well-nourished patients, but higher respect to malnourished patients (11). At further support of our hypothesis, we documented in our population that patients at risk of malnutrition were at higher risk of acute heart failure not requiring hospitalizations respect to well-nourished patients. This is a more frequent outcome respect to hospitalization; therefore, a statistically significant difference between the study groups could be identified during a shorter follow-up.

The present study has several limitations: first, the relatively small sample size that, however, it is similar to the other studies on this topic. Second, the relatively short follow-up time that, in patients with chronic heart failure could not be adequate in discriminating differences between groups. Third, malnourished patients were not represented in our population. However, the lack of this subgroup in our population allowed us to analyse separately from the other groups patients at risk of malnutrition, thus highlighting the association between this nutritional class and outcomes, never studied alone before. Fourth, physical function was evaluated exclusively with gait speed; nevertheless, it has the advance of being quick, executable also by patients with reduced physical function (it requires only a 4-meter walk) and predictive of health-related outcomes in older adults (24,25).

In conclusion, our results suggest that an early assessment of nutritional status using the Mini Nutritional Assessment should be performed in older adults affected by heart failure to identify patients at higher risk of negative outcomes. Further studies are needed to confirm these results and intervention trials including not only nutritional supplementation but a multidimensional approach for the treatment of malnutrition are desirable.

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4. THE ROLE OF NUTRITION AND NUTRIENTS IN THE CONTEXT OF OTHER DISEASES

a) CURCUMIN USE IN PULMONARY DISEASES: STATE OF THE ART AND FUTURE PERSPECTIVES

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Review

Curcumin use in pulmonary diseases: State of the art and future perspectives

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ABSTRACT

Curcumin (diferuloylmethane) is a yellow pigment present in the spice turmeric (*Curcuma longa*). It has been used for centuries in Ayurveda (Indian traditional medicine) for the treatment of several diseases. Over the last several decades, the therapeutic properties of curcumin have slowly been elucidated.

It has been shown that curcumin has pleiotropic effects, regulating transcription factors (e.g., NF-kB), cytokines (e.g., IL6, TNF-alpha), adhesion molecules (e.g., ICAM-1), and enzymes (e.g., MMPs) that play a major role in inflammation and cancerogenesis.

These effects may be relevant for several pulmonary diseases that are characterized by abnormal inflammatory responses, such as asthma or chronic obstructive pulmonary disease, acute respiratory distress syndrome, pulmonary fibrosis, and acute lung injury. Furthermore, some preliminary evidence suggests that curcumin may have a role in the treatment of lung cancer.

The evidence for the use of curcumin in pulmonary disease is still sparse and has mostly been obtained using either in vitro or animal models. The most important issue with the use of curcumin in humans is its poor bioavailability, which makes it necessary to use adjuvants or curcumin nanoparticles or liposomes.

The aim of this review is to summarize the available evidence on curcumin's effectiveness in pulmonary diseases, including lung cancer, and to provide our perspective on future research with curcumin so as to improve its pharmacological effects, as well as provide additional evidence of curcumin's efficacy in the treatment of pulmonary diseases.

INTRODUCTION

Curcumin is a component of turmeric, which is derived from Curcuma longa and used as a dietary spice and coloring agent. It has been used for centuries in Ayurveda (Indian traditional medicine) and in traditional Chinese medicine to treat several illnesses such as anorexia, hepatic disorders, and arthritis [1].

The biological effects of curcumin are mediated by modulation of several molecular targets through its action on multiple signalling pathways and by its regulation of the expression of several transcription factors, inflammatory cytokines, enzymes, growth factors, receptors, adhesion molecules, antiapoptotic proteins, and cell cycle proteins and their associated anti-inflammatory, antioxidant, and anticancer activity [2]. Furthermore, curcumin is well tolerated in humans [3].

In the last decades the role of curcumin in several diseases, such as inflammatory bowel disease, rheumatoid arthritis, psoriasis, and cancer, has been elucidated [4]. Curcumin may also have a role in respiratory diseases such as chronic obstructive pulmonary disease (COPD) [5], asthma [6], pulmonary fibrosis [7], and acute lung injury [8,9], which are characterized by either chronic inflammation or abnormal inflammatory responses. Additionally, curcumin has been studied in lung cancer.

The aim of this review is to summarize the available evidence on curcumin's effectiveness in pulmonary diseases (see table), including lung cancer, and to provide our perspective on future research on curcumin so as to improve its pharmacological effects, and to provide additional evidence of curcumin's efficacy in the treatment of pulmonary diseases.

BIOLOGICAL EFFECTS OF CURCUMIN

The most important biological effects of curcumin are anti-inflammatory, anti-oxidant, and anti-neoplastic, which are mediated by modulation of several molecular targets such as transcription factors, inflammatory cytokines, and proteins involved in cell replication and survival [2].

In vitro and *in vivo* models show that curcumin's anti-inflammatory effect is mediated by the modulation of several targets; one of the most important is nuclear factor kappa B (NF-κB), a transcription factor that regulates the expression of many genes that are involved in innate and adaptive immunity, and in inflammation [10]. The action on this pathway is of particular importance because NF-κB also regulates the expression of genes involved in cell survival and

proliferation, angiogenesis, and, consequently, invasion and metastasis, which play an important role in carcinogenesis [10] (Figure 1).

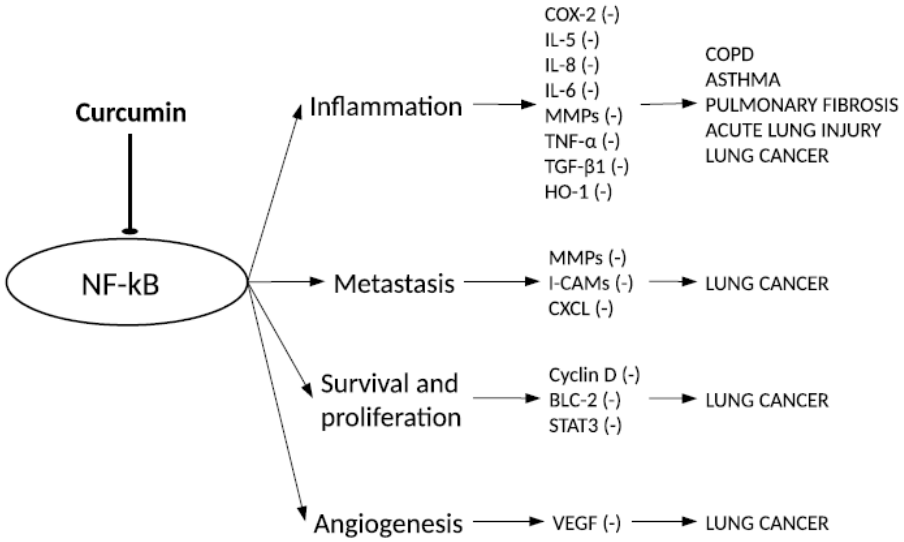

Fig. 1. Therapeutic effects of curcumin mediated by inhibition of NF-kB.
Curcumin inhibits NF-kB, a transcription factor implicated in inflammation, metastasis, cell survival and proliferation, and angiogenesis. This action is mediated by regulation of many genes and proteins, with consequent biological effects in many pulmonary diseases.
Abbreviations;: COX-2: cyclo-oxygenase 2; IL: interleukin; MMP: metalloproteinase; TNF: tumor necrosis factor; TGF: tissue growth factor; HO: heme oxygenase; I-CAM: intracellular adhesion molecule; CXCL: chemokine CXC motif ligand; BCL: B-cell lymphoma; STAT: signal transducer and activator of transcription; VEGF: vascular endothelial growth factor.

Downregulation of NF-κB by curcumin is mediated by inhibition of IκBα kinase and AKT, which are needed for NF-κB activation [11]. NF-κB downregulation leads to reduced levels of COX-2 and 5-LOX, which are implicated in prostaglandin synthesis from arachidonic acid (a key feature of inflammation) [12], and to downregulation of inflammatory cytokines such as IL-5 and IL-8 (produced by monocytes, macrophages and lymphatic cells) [13]. Furthermore, curcumin has effects both *in vitro* and in animal models many inflammatory cells; for example, it inhibits the proliferation of lymphocyte T helper (Th) 1 and Th2 cells, with a consequent reduction in IgG secretion [14]. Curcumin also inhibits mast cells, blocks histamine release [15], and acts on neutrophils by downregulating IL-8, which inhibits their migration due to a direct cytotoxic effect. Moreover, curcumin acts on macrophages, with subsequent downregulation of IL-1, IL-6, and TNF-α [4] and upregulation of IL-10 [14]. Finally, curcumin *in vitro* downregulates histone deacetylase 2 (HDAC2), a nuclear enzyme that plays a critical role in the expression of inflammatory genes by

reversing the hyperacetylation of core histones [16,17], with downregulation of anti-inflammatory genes.

The anti-oxidant properties of curcumin, described both *in vitro* and in animal models, are derived from its action as a scavenger of free radicals, such as superoxide anion and hydrogen peroxide [18]. Curcumin, both *in vitro* and *in vivo*, also reduces lipid peroxidation and maintains the activity status of various antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase by activation of NF-E2 related factor 2 (NRF-2), a key antioxidant transcriptional factor [17,19–22]. Additionally, curcumin inhibits inducible nitric oxide synthase (iNOS) and, consequently, the reduction of NO-mediated inflammation [12]. Furthermore, curcumin both *in vitro* and in animal models induces the expression of heme oxygenase-1 (HO-1), an enzyme metabolizing heme that accumulates in tissues and facilitates the production of metabolites that aid antioxidant defense, such as CO, which is involved in the downregulation of inflammatory cytokine IL-1β and TN F-α and upregulation of IL-10, an anti-inflammatory cytokine [22–24].

Beside its anti-inflammatory action, curcumin has an important anti-fibrotic effect, described both *in vitro* and *in vivo*, derived from NF-κB-mediated downregulation of metalloproteinase (MMP)-9, MMP-2, and upregulation of tissue inhibitor of matrix metalloproteinases (TIMPs), which are implicated in extracellular matrix (ECM) homeostasis [25]. An additional benefit of curcumin is the downregulation of transforming growth factor beta 1 (TGF-β1), which is a cytokine that mediates the activation of inflammatory cells and fibroblasts to elaborate ECM and fibroblast differentiation in myofibroblasts [26]. Finally, curcumin increases the levels of cathepsin K and L, collagenase, and elastase implicated in lung fibrosis [27].

Curcumin's anti-cancer activity is mediated by both direct and indirect action on many molecular targets, such as transcription factors, genes, enzymes, and adhesion molecules [28]. One of the most important of the above-mentioned effects is the downregulation of the transcription factor NF-κB, which is constitutively activated in cancer cells and which results in dowregulation of genes

regulated by NF-κB that are involved in cell survival (e.g., Blc-2), proliferation (e.g., cyclin D), invasion (e.g., MMP-9), adhesion (e.g., ICAM-1) and angiogenesis (e.g., VEGF) with consequent inhibition of proliferation of cancer cells and induction of apoptosis [28,29]. Curcumin also downregulates other transcription factors, such as activator protein-1 (AP-1), which is implicated in the transformation and proliferation of tumor cells [30], as well as Egr-1, which is implicated in angiogenesis [31]. Lastly, curcumin upregulates Nrf2, a transcription factor that regulates cytoprotective genes [22]. One of the most important genes downregulated by curcumin is p53, a very important survival gene in neoplastic cells. Importantly, downregulation of p53 by curcumin inhibits cell growth and induces apoptosis [32] (Figure 2). All these effects have been described both *in vitro* and *in vivo*.

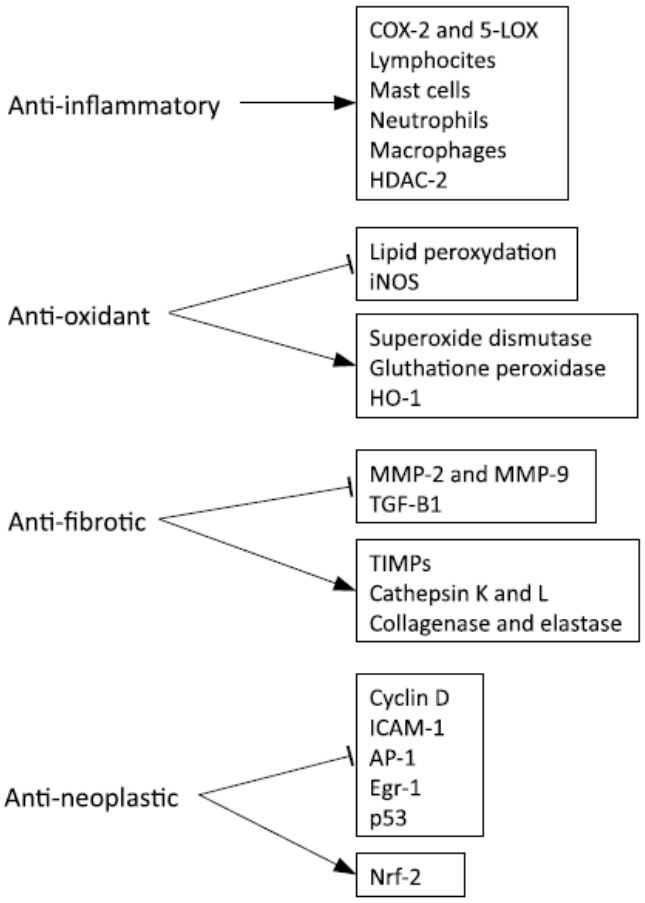

Fig. 2. Main biological effects of curcumin.
Curcumin has anti-inflammatory, anti-oxidant, anti-fibrotic, and anti-neoplastic effects, mediated by stimulation or inhibition of many genes, transcription factors, proteins, cells.
Abbreviations;: COX: cyclo-oxygenase 2; LOX: lipoxygenase; HDAC: histone deacetylase; iNOS: inducible nitric oxide synthase; HO: heme oxygenase; MMP: metalloproteinase; TGF: transforming growth factor; TIMPs: tissue inhibitor of matrix metalloproteinases; ICAM: intracellular adhesion molecule; AP: activator protein; Egr: early growth response protein; Nrf: nuclear factor erythroid related factor.

In this context, curcumin may have a role in the treatment of different pulmonary diseases, with COPD, asthma, pulmonary fibrosis, cystic fibrosis, acute lung injury, and lung cancer being the most important. While several pathways are involved, the effects of curcumin seem to be mediated by the modulation of the same mediators (such as NF-κB and AP-1) in the different diseases, leading to its anti-inflammatory, anti-oxidant, anti-fibrotic and anti-cancer effects. The only

exception is cystic fibrosis, in which curcumin modulates the expression and activity of cystic fibrosis transmembrane conductance regulator (CFTR).

The exact mechanisms by which curcumin exhibits these properties are still actively under investigation. The phenolic OH groups contained within curcumin's chemical structure are responsible for its reactive oxygen species (ROS) scavenging or anti-oxidant activities, ascribed either to hydrogen atom transfer, or sequential electron and proton transfer from the phenolic OH groups (see Figure 3 for chemical structure of curcumin and its derivatives).

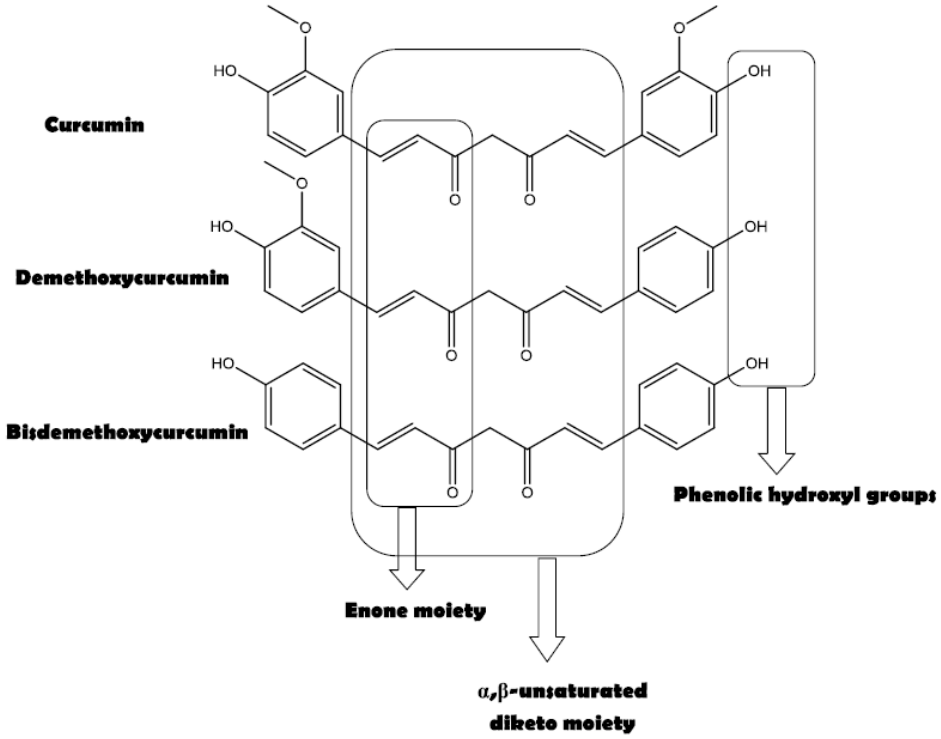

Fig. 3. Structure of curcumin and its derivatives. Structure of curcumin with highlighted its reactive groups mediating biological effects.

Curcumin also interacts with multiple target molecules within cells, which include proteins, DNA, lipids, metals, and metalloproteins [2,33–35]. The nature of the interactions of curcumin with these biomolecules is either covalent or non-covalent. The non-covalent interactions include hydrophobic interactions, $\pi-\pi$ interactions, and extensive hydrogen bonding, which gives curcumin many possible mechanisms to interact with target proteins. An example of a non-covalent interaction is with hydrophobic region of serum albumin, while covalent interactions are primarily due to reaction with

protein thiols and metals, for example curcumin-GSH conjugates or GSH adducts of feruloylmethylketone and feruloyl aldehyde [36]. Curcumin is a very good inhibitor of glutathione S-transferase (GST) activity and forms adducts with cysteine by covalently binding to the diene moiety [37]. It is for this reason that curcumin most likely undergoes rapid degradation in biological fluids and results in low plasma concentrations despite oral ingestion of gram quantities [34]. Similar covalent association has been proposed for curcumin with many other proteins involved in inflammation and cancer [38,39].

The α,β-unsaturated keto-enol structure of curcumin is associated with the anti-tumor activity of curcumin [33,40,41]. Curcumin can also bind directly to cellular DNA in cancer (as well as normal) cells. Specifically, curcumin binds with the minor groove in AT-rich regions of DNA, since it is not a compound that intercalates with the DNA structure [42–44], but binds with DNA through hydrophobic interactions or hydrogen bonding. Curcumin also binds with cell membranes at low concentrations through hydrogen bonding near the phosphate group of choline, which is similar to the way that cholesterol inserts itself into a cell membrane. The resulting structural change influences exocytosis and membrane fusion processes within cells [45,46].

The α–β-unsaturated β-diketone moiety of curcumin (enol form) is also an excellent chelating ligand and forms strong complexes with positively-charged metals and metal oxides [47]. Curcumin-metal complexes can exhibit therapeutic anti-tumor, and anti-oxidant properties. Additionally, curcumin-metal complexes can function as ROS scavengers and prevent free radical-induced damage to various biomolecules. In fact, a complex of Vanadyl-curcumin $(VO(Cur)_2)^{+2}$ was shown to be a more potent anti-oxidant than curcumin itself [48,49]. Lastly, it has been demonstrated that Cu^{+2} increases the cytotoxicity of curcumin to tumor cells by potentiating the curcumin-induced suppression of the NF-κB pathway [50]. Since metal complexes of curcumin can increase anti-tumor activity, there have been various attempts to modify the curcumin parent structure (*e.g.*, dimethoxy curcumin and diacetyl curcumin) to further increase anti-cancer activity.

THE ROLE OF CURCUMIN IN COPD

COPD PATHOGENESIS

COPD is a chronic disease characterized by bronchial obstruction that is not reversible after administration of bronchodilator drugs, deregulated chronic inflammation, and progressive destruction of the lungs. Inflammation-related contributors to lung tissue injury include cells and mediators of both innate and adaptive immunity, reactive oxygen species (ROS), and an imbalance of local proteolysis/antiproteolysis state [5].

After an epithelium and endothelium injury resulting from cigarette smoking and/or chemical fumes [51], pathogenic bacteria (e.g. *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*), or air pollutants [52], there is an enhanced production of free radicals, IL-8, chemokine CXC motif ligands 2 (CXCL2), and leukotrienes. All of these factors induce neutrophil migration in lung parenchyma with subsequent production of proteinases and elastases. These enzymes contribute to tissue breakdown and production of chemoactractive cytokines that are responsible for perpetuation of inflammation [53,54].

After a lung injury, there also is activation and recruitment of machrophages, activation of NF-κB [55] and AP-1 [56] pathways, and subsequent release of TNFα, IL-8, and CXC chemokines. These substances are chemotactive and serve to propagate inflammation and the release of elastolytic enzymes, including MMP-2, MMP-9, MMP-12 and cathepsins K, L and S; all of which are responsible for tissue breakdown [57,58]. Other cells involved in the pathogenesis of COPD are T lymphocytes; for example, CD4 Th1-type cytokines participate in perpetuating an autoimmune response by producing IFNγ, which then leads to an uncontrolled inflammatory response, tissue damage, and emphysema [59]. In fact, CD8 cytotoxic lymphocytes kill damaged cells and can be correlated with the severity of tissue destruction [60]. Inflammation is also mediated by reduced HDAC2 activity in peripheral lung tissue and in alveolar macrophages secondary to increased

oxidative and nitrative stress [61] and a reduction in the expression of HO-1 [23,24]. An increase in inflammatory cell activity and the activity of bacterial proteases progressively results in unbalanced proteolysis, which leads to proteolytic-mediated ECM degradation and the production of fragments that act as chemokines. This cascade of events promotes inflammation [62,63] and progression of the disease.

In addition to these factors, accelerated cell senescence with reduced repair and regeneration [64] and insufficient autophagy give rise to the accumulation of damaged cellular components [65], which has been implicated in the pathogenesis of COPD.

BIOLOGICAL EFFECTS OF CURCUMIN IN COPD

Some in vitro and in vivo evidence is available about curcumin's efficacy in COPD, but only one observational study [66] and one randomized controlled trial [67] are available in humans.

Curcumin's effectiveness in COPD originates from its anti-oxidant and anti-inflammatory properties and is mediated by different mechanisms (Figure 4).

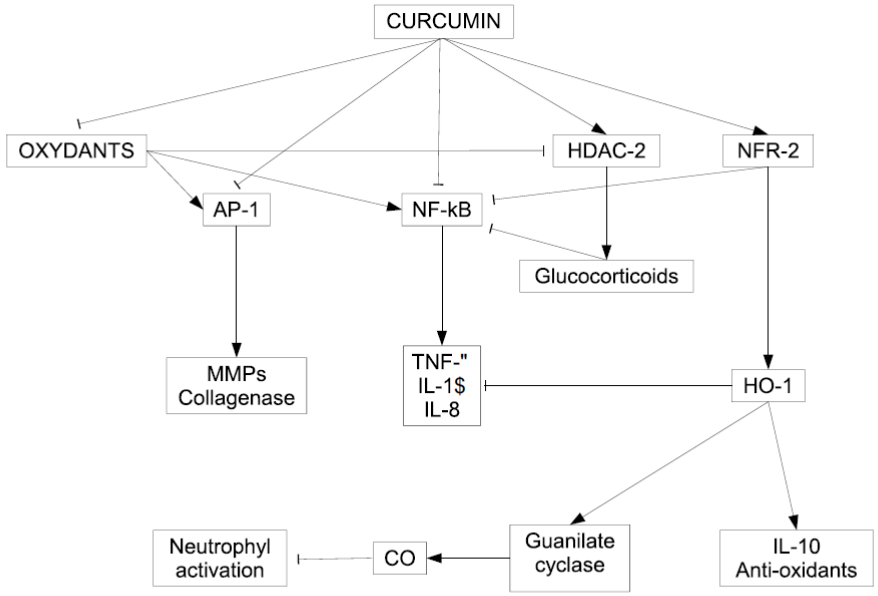

Fig. 4. Main biological effects of curcumin in COPD.
Biological effects of curcumin in COPD are mediated by modulation of several pathways.
Abbreviations;: AP-1: activator protein; HDAC2: histone deacetylase; HO-1: haeme oxygenase; NF-κB: nuclear factor kappa B; Nrf-2: nuclear factor erythroid 2-related factor 2; TNF: tumor necrosis factor.

In human cells treated with hydrogen peroxide, curcumin reduced the concentrations of oxygen radicals and IL-8, increased glutathione levels, and finally, modulated NF-κB and AP-1 activation [68]. In a murine model, intragastric administration of curcumin improved oxidative stress by modulating alkaline phosphatase, lactate dehydrogenase, lipid peroxidation and by augmentation of the antioxidant defense system (glutathione, glutathione peroxidase, superoxide dismutase and catalase) during nicotine-induced lung toxicity [69]. This action is also mediated by upregulation of nuclear factor erythroid 2–related factor 2 (NRF-2). Furthermore, in mice models of COPD it has been documented that the anti-inflammatory and anti-oxidant biological effect of curcumin, administrated orally, is also mediated by suppression of the increase in total cells, neutrophils, and macrophages in bronchoalveolar lavage fluid (BALF) [21]. The suppressive effect of curcumin on neutrophil recruitment was associated with a markedly reduced level of the neutrophil chemokine KC, a functional mouse homologue of human IL-8, in BAL, with consequent inhibition of neutrophil migration in the lungs [70].

HDAC2 dowregulation induced by oxidative stress and chronic inflammation is responsible for poor therapeutic benefits of chronic corticosteroid treatment in COPD patients. The mechanism involved includes the glucocorticoid receptor, which, following corticosteroid binding, translocates to the nucleus and must be deacetylated by HDAC2 to inhibit NF-κB [61]. Curcumin has been shown to restore, in a concentration-dependent manner, HDAC2 activity back to normal levels, and, consequently, restores corticosteroid-mediated suppression of pro-inflammatory cytokine release in vitro by monocytes after induction with hydrogen peroxid and cigarette smoke. This action is thought to be mediated by blockage in the degradation of HDAC2 induced by oxidative stress [17] and also by inhibition of histone acetyltransferase (HAT) [71], which is responsible for the acetylation of core histones and subsequent gene activation [61].

In humans, the effectiveness of dietary curcumin in ameliorating pulmonary function was evaluated in one observational study, in which a positive correlation was found between FEV1 and the

frequency of curry intake (evaluated using a food frequency questionnaire) in a Chinese community-dwelling population of 2478 patients. This correlation was stronger in current and past-smokers compared to non-smokers and in patients with history of asthma or COPD compared to the other participants [66]. Panahi et al. performed a randomized controlled trial to explore the therapeutic efficacy of 4-week supplementation with curcuminoids in subjects suffering from chronic COPD symptoms due to sulfur mustard exposure. This trial reported that short-term curcuminoids supplementation improves the severity and frequency of respiratory symptoms as well as health-related quality of life [72]. These improvements were accompanied by elevation of serum levels of reduced glutathione, while the levels of malondialdehyde and pro-inflammatory mediators (IL-6, IL-8, TNF-α, TGF-β, C-reactive protein, calcitonin gene-related peptide and macrophage chemotactic protein-1) were reduced [67,72]. With respect to spirometric parameters, curcuminoids did not affect FEV1, but improved FEV1 to FVC ratio [72].

THE ROLE OF CURCUMIN IN ASTHMA

ASTHMA PATHOGENESIS

Asthma is an inflammatory disease characterized by lung infiltration of eosinophils, lymphocytes, and neutrophils, as well as mucus hypersecretion and airway hyper-responsiveness (AHR) [73]. The most important cells in the pathogenesis of asthma are CD4 Th2 cells, which are required for antigen-induced allergic airway inflammation and AHR [74].

When exposed to allergens, airway epithelial cells produce IL-8, which stimulates neutrophil recruitment and TGF-β1. This, in turn, activates myofibroblasts [75]. This cascade continues with the production of GM-CF, which stimulates maturation of dendritic cells (DC) and subsequent migration and activation of CD4 Th2 cells that produce IL-4, IL-5, and IL-13 [76,77]. In turn, these cytokines activate eosinophils, NTK cells, mast cells, and basophils, all of which, produce IL-4, IL-5, and IL-13, with perpetuation of inflammation [6].

The main effector cells in asthma are eosinophils that secrete pro-inflammatory cytokines and peroxidases, cationic proteins, eotaxin, and MMP-9, which enhance inflammation and increase airway remodeling by stimulating subepithelial fibrosis [78]. MMP-9, produced by eosinophils, but also by the other cells mentioned above [79], activates TGF-β1 and mediates differentiation and activation of fibroblasts and myofibroblasts that determine remodeling of ECM and eventually airway fibrosis [26].

Recently, CD Th17 cells have been described to have a role in the pathogenesis of asthma. In inflammation, there is a dysregulation between regulatory T (Treg) cells that have an anti-inflammatory role, and Th17 cells, with subsequent production of TNF-α, IL-6, IL-17, and, ultimately, the development of inflammation and autoimmune tissue injuries [80].

BIOLOGICAL EFFECTS OF CURCUMIN IN ASTHMA

As in COPD, most data about curcumin's effectiveness in asthma have been acquired using in vitro or in vivo animal models, while only a few observational studies are available in humans, with discordant results.

In a mouse model of asthma, inhibition of NF-κB activity reduces AHR and inflammatory cell airway infiltration and determines the attenuation of IgE levels in BALF. It should be noted that oral administration of curcumin only produced minimal improvements compared with controls, while intraperitoneal administration was more effective [81]. Liu L et al. showed that curcumin, in addition to inhibiting NF-κB pathway, activates the Nrf2/HO-1 signaling pathway in a dose- and time-dependent manner, with consequent reduction of TNF-α, IL-1β, and IL-6 levels in vitro, decrease in eosinophil and WBC counts in BAL, and reduction of AHR in a murine model of asthma [82]. These effects are at least in part mediated by suppression of Th17 cells and stimulation Treg cells, with secondary reduction of IL-17A and an increase of IL-10 levels [83]. Furthermore, curcumin inhibits lymphocyte proliferation and the production of IL-2, IL-5, GM-CSF, and IL-4 in vitro [84], at least in part levels through inhibition of NO and inducible nitric oxide synthase

(iNOS) [85], that are also implicated in the recruitment of eosinophils and in the differentiation of Th2 cells [86]. Another signaling pathway involved in asthma is the Notch signaling pathway, which promotes peripheral T cell activation/proliferation and cytokine production [87], that is downregulated by curcumin [88].

Some studies have demonstrated that curcumin may reduce airway remodeling in asthma. Karaman et al. showed that intraperitoneal administration of curcumin reduces, in a dose-dependent manner, the thickness of epithelium and the subepithelial smooth muscle layer in a murine model, and that at the highest dose of curcumin, the effect on histopathological characteristics was comparable with that of dexamethasone [89]. These histological effects of curcumin were confirmed by Zeng et al., who also showed that intraperitoneal administration of curcumin reduces the degree of inflammatory cell infiltration in the lungs and inhibits airway smooth muscle cell proliferation in a dose-dependent manner by downregulating the ERK signaling pathway [90] (Figure 5).

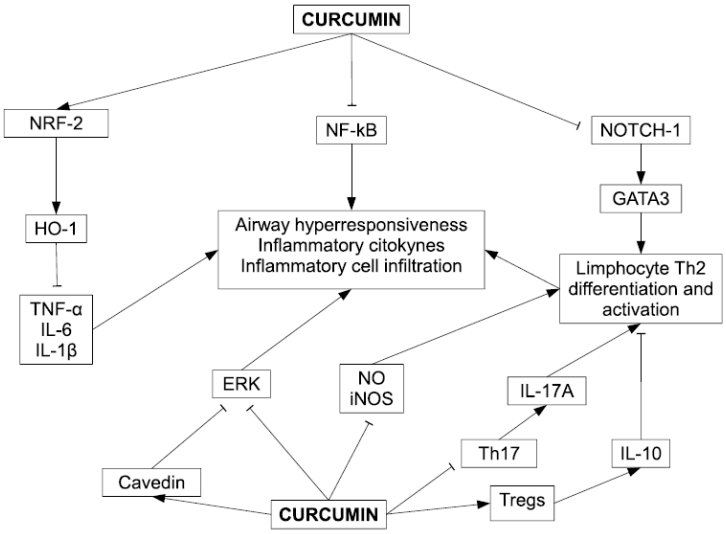

Fig. 5. Main biological effects of curcumin in asthma.
Biological effects of curcumin in asthma derive from modulation of several pathways, leading to reduction of Airway hyperresponsiveness, inflammatory citokynes and inflammatory cell infiltration.
Abbreviations;: AP-1: activator protein; ERK: Extracellular signal–regulated kinase; HO-1: haeme oxygenase; iNOS: inducible nitric oxide synthases; NF-κB: nuclear factor kappa B; Nrf-2: nuclear factor erythroid 2-related factor 2; TNF: tumor necrosis factor; Tregs: limphocyte T regulators.

In humans, Kim et al. performed a randomized, double blinded, controlled pilot study in 13 patients with stable atopic asthma; 6 assigned to placebo and 7 treated with 1000 mg bid of curcumin. After

50 days of treatment, there were no significant differences in FEV1, frequency of use of a rescue bronchodilator, dose of inhaled corticosteroid, blood IgE levels, or WBC counts between the groups [91]. In contrast, Abidì A et al., in a randomized study of 77 patients, demonstrated that curcumin (500 mg bid), relative to standard asthma therapy, improves FEV1. However, there were no differences between the two groups in the severity of symptoms [92]. The small sample of the studies and the availability of only two studies in humans make difficult to interpret these discordant results; therefore, more and larger trials are necessary to evaluate the effectiveness of curcumin in asthma.

THE ROLE OF CURCUMIN IN PULMONARY FIBROSIS

THE PATHOGENESIS OF PULMONARY FIBROSIS

Pulmonary fibrosis is the end stage of many pulmonary inflammatory diseases, and is characterized by an increase in collagen content, accumulation of ECM, and infiltration of inflammatory cells. This disease progressively leads to a decrease in gas exchange and pulmonary compliance and, consequently, the development of respiratory failure and death [7,93].

Injuries, such as inhalation of toxic environmental particulates, or infections, may cause damage of endothelial and epithelial cells, with subsequent release of inflammatory mediators and activation of the anti-fibrinolytic coagulation cascade [94]. This damage leads to vasodilation and increased vascular permeability, as well as production of MMPs (MMP-2 and MMP-9) that destroy basement membrane and results in the migration of inflammatory cells in the lung parenchyma [95,96]. Inflammatory cells and damaged endothelial and epithelial cells produce inflammatory cytokines and ROS, which causes a dysregulation in the equilibrium between oxidants and anti-oxidants. The most important cytokine involved in this process is TGF-β, which contributes to the activation of inflammatory cells and fibroblasts and maintenance of inflammation and ECM production, as well as fibroblast differentiation in myofibroblasts [26]. Other cytokines implicated in pulmonary

fibrosis are IL-4 [97], a profibrotic cytokine that promotes activation of macrophages and differentiation of Th2 cells with consequent production of TGF-β, IL-13, MMPs [98], and IL-13, which determines the differentiation of fibroblasts into myofibroblasts and stimulates production of TGF-β [99].

BIOLOGICAL EFFECTS OF CURCUMIN IN PULMONARY FIBROSIS

Currently, there are no human studies available that have assessed the efficacy of curcumin in pulmonary fibrosis. However, much evidences has been gathered using murine models, in which. fibrosis has been induced by bleomycin [100–102], cyclophosphamide [103], chemotherapeutic agents, radiation [104–106], or viruses [107]. All these models lead to similar inflammatory and oxydative responses and, consequently, histopathologic changes.

In all of the animal models evaluated, treatment with curcumin leads to a reduction of inflammatory cells and improvement in inflammation and collagen deposition [100–104,106,107]. Curcumin reduces, both in vitro, and in animal models, the levels of TGF-β1 [26,27,100,103,104,107]. Furthermore, it may improve oxidative stress in models of pulmonary fibrosis by reducing levels of iNOS [101,102], NOS [102], ROS, and by increasing levels of HO-1 [106]. These effects of curcumin seem to be comparable to oral administration of hydrocortisone [101]. Some evidence indicates that curcumin may improve survival in models of pulmonary fibrosis induced by bleomycin [100,102] or radiation [106].

The effects of curcumin on pulmonary fibrosis derive from its action on multiple pathways and through multiple mechanisms. As in asthma, the curcumin inhibition of NF-κB has a role in pulmonary fibrosis, by causing a reduction of TNF-α and cyclo-oxygenase 2 (COX-2) levels [104] and TGF-β1 levels [107]. The reduction in TGF-β1 levels results in anti-inflammatory and anti-fibrotic effects. Additionally, AP-1 is inhibited by curcumin, which results in inhibition of TGF-β1 production and myofibroblast differentiation [26]. Cathepsins K and L, collagenases, and elastases implicated in caspase-independent apoptosis pathways [108] and in the downregulation of TGF- β1

[109], are upregulated by curcumin, with consequent antifibrotic effect [27]. Reduction of TGF-β levels is also related to curcumin's direct inactivation of fibroblast TGF-β receptor through inhibition of its phosphorylation [100] (Figure 6).

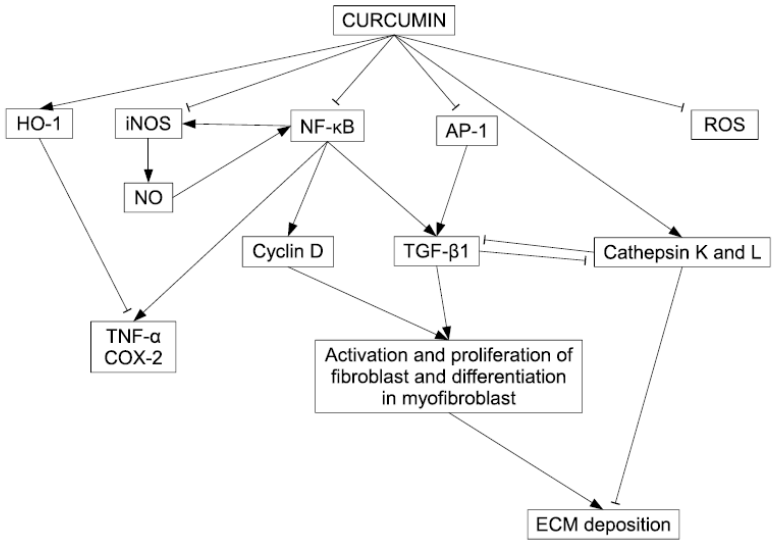

Fig. 6. Main biological effects of curcumin in pulmonary fibrosis.
Curcumin induces inhibition of ECM deposition and reduction of TNF-α and COX-2 through modulation of different pathways.
Abbreviations;: AP-1: activator protein; COX: Cyclooxygenase; ECM: extracellular matrix; HO-1: haeme oxygenase; iNOS: inducible nitric oxide synthases; NF-κB: nuclear factor kappa B; ROS: reactive oxygen species; TGF: transforming growth factor; TNF: tumor necrosis factor.

In contrast to studies showing that curcumin has biological effects regardless of the route of administration (oral, intra-gastric, intra-peritoneal), Smith et al reported a lack of efficacy of curcumin treatment administered orally, but a significant improvement following intraperitoneal administration [100]. The discrepancy between oral and intraperitoneal administration may be explained by the poor absorption of curcumin when administrated by the enteral route.

THE ROLE OF CURCUMIN IN CYSTIC FIBROSIS

PATHOGENESIS OF CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive disease caused by mutations of the cftr gene, which results in a reduction in the expression, or function, of cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a chloride ATP-dependent channel that mediates electrolyte transport across of a variety of epithelia, such as nasal and airway epithelia, and epithelium that lines the pancreatic

ducts [110]. Additionally, CFTR is composed of two transmembrane domains (TMD1 and TMD2), four intracellular loops (ICLs), two nucleotide binding domains (NBD1 and NBD2, which bind and hydrolyze ATP), and a regulatory domain (R domain) [111]. The R domain must be phosphorylated by phosphokinase A (PKA) to dimerize the two NBDs and consequently open and activate the channel [112]. The most common gene mutation is ΔF508 [113], which leads to a deficit in channel gating and cell surface residence. This, in turn, leads to the accumulation of immature protein in endoplasmic reticulum (ER) [114]. Another common mutation of the cftr gene is G551D, which determines an alteration of NBD1 structure. This makes it impossible for ATP to bind to NBD1 and consequently activate the channel [115]. W1282X and Δ1198 mutations determine a deletion of most all the NBD2, respectively [116]. Clinically, in the lungs, this disease is characterized by impaired mucus composition, which leads to progressive pulmonary damage, inflammation, and increased susceptibility to bacterial infections [117]. Recurrent respiratory infections lead to chronic inflammation, airway remodeling, and fibrosis [118], which results in high rates of mortality [119].

BIOLOGICAL EFFECTS OF CURCUMIN IN CYSTIC FIBROSIS

Given the high morbidity and mortality of patients affected by cystic fibrosis, and of the lack of effective treatments for these patients, many studies have recently investigated new compounds, including curcumin, with the intent of improving the natural history of this disease. Many studies have been performed in vitro, but, to date, only a few animal studies and no human studies have been performed. Curcumin's effectiveness in cystic fibrosis was mostly evaluated in ΔF508 mutation models, and the results are not unequivocal. Egan et al. documented that curcumin increases the surface delivery of ΔF508 CFTR in vitro, and, in vivo, demonstrated that oral administration of curcumin increases the nasal potential difference (i.e. the potential difference measured at the nasal mucosa) to levels comparable with those of wild type mice. The authors speculated that the effect may be a result of curcumin-mediated inhibition of the interaction between calnexin (a calcium binding protein) and CTFR, which reduces the calnexin-mediated

retention and degradation of CFTR in the ER [120,121]. Other studies, however, did not find a shift of ΔF508 CFTR to plasma membrane in vitro [122] or an increase in the membrane ΔF508 CFTR gating [123]. In vivo, Song et al. could not prove an increase of nasal potential difference in mice treated with oral administration of curcumin [123]. However, other evidence supports the hypothesis of curcumin's efficacy in cystic fibrosis. In vitro, curcumin determines the increase of the CFTR 'burst duration' in all of the most common CFTR mutations [124–126]. This action is likely due to an oxidation reaction [127] and appears to be dependent on PKA phosphorylation and independent of ATP [124,125], which is related to a curcumin cross-link with CFTR in an ATP-binding site [125]. In fact, the cross link is more efficient in immature CFTR forms (ΔF508 CFTR) compared to the mature forms [127]. In ΔF508 CFTR in vitro models, Lipecka et al. confirmed the effect of curcumin in determining the migration of ΔF508 CFTR from the ER, where it is retained, to the cytoplasm and the plasma membrane [128]. These authors speculated that the effect may be mediated by a reorganization of Keratin 18, a cytokeratin implicated in the inhibition of ΔF508-CFTR trafficking [129]. Finally, Wang G et al. demonstrated in vitro that curcumin is also an important chelator of Fe3+, a molecule that inhibits channel dimerization and opening despite phosphorylation of the R domain and ATP-dimerization of NBDs [130], and that it is a stabilizer of the R-ICL1/ICL4 interaction necessary for CFTR opening [131].

THE ROLE OF CURCUMIN IN ACUTE LUNG INJURY

PATHOGENESIS OF ACUTE LUNG INJURY

Acute lung injury (ALI) is a disease characterized by an acute inflammatory response that leads to endothelial and alveolar damage, neutrophil accumulation, protein-rich alveolar edema and hemorrhage [132]. It may be triggered by different causes, such as bacteria [133], intestinal ischemia/reperfusion [134], or sepsis [135]. Clinically, it is characterized by respiratory distress, refractory hypoxemia, and pulmonary edema, with a high rate of mortality [136].

Regardless of the cause, in ALI there is a NF-κB and AP-1 mediated release of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, that play a critical role in inflammation-induced lung injury [137], with consequent migration and activation of macrophages and neutrophils. These inflammatory cells are responsible of the oxidative damage that characterize ALI. With regard to oxidative changes, there is an increase in the levels of ROS and free radicals [138]. Additionally, acute inflammation leads to a reduction in endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase [139]. Moreover, inflammation and alveolar damage lead to decreased and impaired synthesis, secretion, function, and composition of surfactant protein D (SP-D) [140], a protein that plays an important role in innate lung host defense and in the regulation of surfactant homeostasis [141].

BIOLOGICAL EFFECTS OF CURCUMIN IN ACUTE LUNG INJURY

In recent years, many animal models of ALI have been developed, which have been induced by ischemia-reperfusion [142,143], paraquot [8], thermal inhalation [144], bacteria (such as *S. aureus and K. pneumoniae*) [9,145], lipopolysaccharide (LPS) [146,147], sepsis [142,148], gastrointestinal decontamination agents [149], and material aspiration [150] so as to assess the effectiveness of curcumin in this disease. All of these models induce similar inflammatory and oxidant responses and pathological alterations. To date, no trials on humans have been performed.

In the above mentioned models, curcumin is reported to decrease inflammation through reduction of neutrophils [8,145–148,151], macrophages [147], and lymphocytes [8,151] in BALF. Furthermore, curcumin facilitates a reduction of proinflammatory cytokine levels implicated in ALI, such as TNF-α [8,145–149,151], IL-1β [149], IL-8 [144,151], IL-6 [142,146,148], MIP-2 [146,148] and, finally, adhesion molecules [150]. As in other pulmonary diseases previously treated, in ALI curcumin has been shown to reduce oxidative stress [8,142,149] by reducing the levels of nitrites [145,147], as well as iNOS activity [143,150], and by increasing the levels of antioxidants [8,142,143,147,149–151]. Finally, curcumin causes an increase of SP-D levels in lung alveola

[143,150]. The reduction of inflammation and oxidative stress with curcumin treatment leads to an improvement in the histopathological alterations that characterize ALI. Some of these pathological features positively affected by curcumin treatment include a reduction of peribronchial and alveolar septal inflammatory cell infiltration, alveolar edema and exudate, interstitial fibrosis, necrosis and alveolar haemorrhage [8,9,142,148–151]. Overall survival in mouse models of ALI is improved with curcumin treatment [8,151].

Although many studies were performed to assess the effectiveness of curcumin in ALI, few studies concerning the molecular mechanism of action of curcumin have been performed. However, as in other pulmonary diseases, also in ALI the anti-inflammatory effects of curcumin are mediated, at least in part, by inhibition of NF-κB, with consequent reduction of IL-6 [142,146,148], TNF-α, MIP-2 [9,146], IL-1β, TGF-β [9], IL-8, COX-2, PGE2 [144], and I-CAM-1 [142], which, in turn, leads to a reduction in inflammation and oxidative stress. Dong ZW et al. demonstrated that curcumin directly downregulates TGF-β/SMAD3 pathway and, consequently, inhibits the increase in endothelial and epithelial permeability and inflammatory cell recruitment (Figure 7).

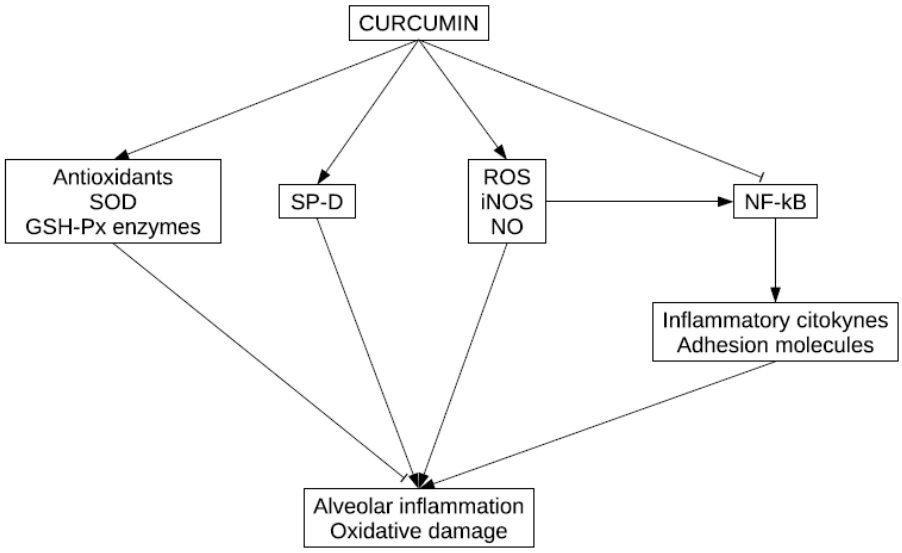

Fig. 7. Main biological effects of curcumin in acute lung injury.
Biological effects of curcumin in acute lung injury are mediated by different pathways.
Abbreviations;: iNOS: inducible nitric oxide synthase; NF-κB: nuclear factor kappa B; ROS: radical oxygen species;surfactant protein D.

THE ROLE OF CURCUMIN IN LUNG CANCER

PATHOGENESIS OF LUNG CANCER

Lung cancer is one of the most common malignancies worldwide [152]. It is classically subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which represents approximately 85% of lung cancers [153]. This disease is characterized by poor survival, which is related to the diagnosis being made in the advance stage of this disease and the lack of effective treatments [154].

Lung cancer has a complex pathogenesis that involves several pathways and is characterized by dysregulation of many genes, transcription factors, enzymes, growth factors, and cytokines, which ultimately lead to inhibition of apoptosis, uncontrolled replication, invasion, and migration. In this context, the role of epigenetic regulation of gene expression related to micro RNAs (miRNAs), small non-coding RNAs located in the intron segment of genes, is crucial. These miRNAs play an important role in the regulation and expression of genes implicated in cell proliferation and apoptosis [155,156]. Specific patterns of miRNA are typically dysregulated in lung cancer [157]. DNA methylation is another epigenetic mechanism implicated in gene expression [158]. Lung cancer cells characteristically present with hypermethylation and subsequent silencing of the promoter region of tumor suppressor genes, such as retinoic acid receptor β (RARβ) [159]. This leads to uncontrolled replication and inhibition of apoptosis [160].

Besides epigenetic mechanisms, other factors may act on apoptosis, proliferation, and/or migration of cancer cells. For example, the p53 gene physiologically induces cell cycle arrest and apoptosis, particularly in cells with DNA damage [161]. In neoplastic cells, mutant p53 loses its ability to induce cell cycle arrest and apoptosis, leading to uncontrolled replication [162]. Furthermore, many factors may influence the proliferation of cancer cells; one of the most important being the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, which regulates cell growth and proliferation, and that, when constitutively active, such as in cancer cells, facilitates

uncontrolled cell replication [163,164]. Also, NF-κB is implicated in proliferation of cancer cells, because it regulates the expression of many genes involved in this process [10]. Migration of cancer cells is a primary step for the development of metastasis in cancer and requires the detachment of cancer cells from epithelium due to a loss of adherence junctions [165]. This process is mediated by EGR-1 expression, which is involved in cell migration and inducing the transcription of MMP-9.MMP-9 plays a crucial role in degrading ECM and in cytoskeleton rearrangement [166]. Another pathway involved in the loss of adherence junctions is the Rac1/PAK1 pathway, which is a protein implicated in cytoskeleton rearrangement and cell adhesion via the activation of PAK1 and, consequently, the production of MMP-2 and MMP-9 [167].

BIOLOGICAL EFFECTS OF CURCUMIN IN LUNG CANCER

Although many drugs and radiation therapy have been developed to treat lung cancer, conventional treatments at the present time are poorly effective in changing the natural history of this disease. Patients are burdened by many adverse effects of these treatments, many of them permanent [168]. In this context, curcumin have recently been investigated with the intent of improving the quality of life and survival of patients affected by lung cancer. Many studies have been performed in vitro and in vivo subcutaneous xenograft mouse models, but, to date, no data are available on humans.

In in vitro models of NSCLC, curcumin induces apoptosis through modulation of the miRNA pathways with inhibition of caspase-3 [169], inhibition of the Pi3K/Akt pathway (implicated in growth factor-mediated cell survival) [170], and inhibition of X-linked inhibitor of apoptosis (XIAP) [171]. In contrast with its anti-oxidant properties documented in non-cancer cells, curcumin is described to have cytotoxic properties in NSCLC and SCLC cells in vitro, which are mediated by an increase of ROS and apoptosis [172,173].

The most important pathway by which curcumin inhibits lung cancer cell proliferation is the JAK/STAT3 pathway. It has been shown that curcumin blocks this pathway in vitro, with not only subsequent suppression of proliferation, but also of migration, invasion, and angiogenesis of SCLC

cells [174]. These data have been confirmed in vitro and in vivo for NSCLC cells [175]. Furthermore, curcumin inhibits the JAK2/STAT3 pathway in cancer stem cells in vitro [176], which are implicated in tumor recurrence and in drug resistance [177]. This inhibition leads to an inhibition of tumor growth in vivo. Another mechanism by which curcumin inhibits the proliferation of SCLC cells is the induction of forkhead box protein O1 (FOXO1), a transcription factor that regulates cell proliferation, differentiation, and DNA damage repair [178]. Curcumin's induction of FOXO1 upregulates p21 and p27 and downregulates cyclin D, inducing cycle arrest and apoptosis [179]. Inhibition of cell proliferation by curcumin also arises from epigenetic effects by reactivation of silenced tumor suppressor genes. In NSCLC cells, curcumin reduces RARβ promoter methylation, which induces the expression of RARβ in vitro and leads to the inhibition of tumor growth in vivo [180].

The antineoplastic action of curcumin is also mediated by the inhibition of cancer cell migration. In in vitro models of NSCLC, curcumin downregulates early growth response protein 1 (EGR-1) with enhancement of cell-cell adhesion [181]. Furthermore, curcumin inhibits the production and activity of MMPs by several mechanisms. In NSCLC, both in vitro and in vivo, this compound inhibits phosphokinase A, with consequent inhibition of NADPH oxidase-2 and a reduction in ROS production. ROS are required to activate transcription factor-2 (ATF-2), induces MMP-9 production [182]. Another suggested mechanism is the inhibition of the Rac1/PAK1 pathway, leading to a downregulation of MMP-2 and MMP-9 and inhibition of cell migration [183]. Finally, in both in vitro and in vivo NSCLC models, curcumin downregulates adiponectin, a cytokine produced by adipose tissue and implicated in lung cancer [184]. This downregulation leads to inhibition of NF-κB and, consequently, a reduction in the production of MMPs. This, in turn, causes a reduction in the migration and invasion capability of these cells [185] (Figure 8).

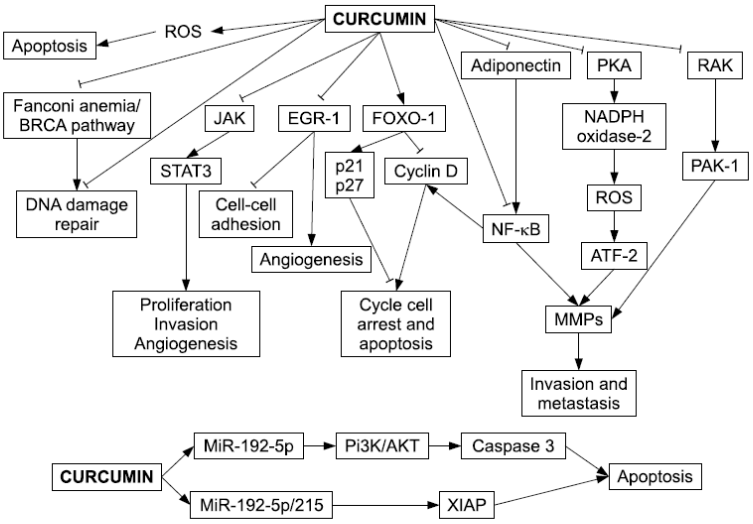

Fig. 8. Main biological effects of curcumin in lung cancer.
Biological effects of curcumin in COPD are mediated by modulation of several pathways.
Abbreviations;: ATF: activate transcription factor; BRCA: breast cancer; EGR-1: early growth protein 1; FOXO: forkhead box protein, JAK: janus kinase; MMPs: matrix metalloproteinases; NF-κB: nuclear factor kappa B; ROS: Reactive Oxygen Species; STAT: signal transducer and activator of transcription.

The most common chemotherapeutic agent used in NSLCL is cisplatin, and resistance to this drug is generally associated with cellular DNA repair [186]. Curcumin reverses cisplatin resistance in NSCLC cells in vitro and enhances the cisplatin-mediated inhibition of proliferation and induction of apoptosis. This action may potentially be mediated by inhibition of the Fanconi anemia/BRCA pathway, a DNA cross-link damage repair pathway, which is implicated in cellular resistance to DNA damage by anti-tumor agents [187]. Curcumin-induced reversal of cisplatin resistance, inhibition of proliferation, and induction of apoptosis in NSCLC cells, is also mediated by inhibition of Hypoxia-Inducible Factor (HIF)-1α, a transcription factor involved in angiogenesis, tumor growth, and chemoresistance [188]. Furthermore, curcumin increases cisplatin's efficacy toward cancer stem-like cells in vitro by inducing caspase-9 and p21. The effects of inducing caspase-9 and p21 is cell apoptosis and an inhibition of cell migration [189]. Finally, curcumin enhances the effectiveness of chemotherapy using erlotinib [190], docetaxel [191] and gefitinib [192] both in in vitro and in vivo NSCLC models. Finally, cancer cells have increased DNA-repair mechanisms, which confer chemoresistance properties to them. However, curcumin reduces in a dose-dependent manner the expression of DNA repair proteins and enhances p53 levels, inducing apoptosis in vitro [193].

LIMITATIONS OF CURCUMIN USE AND FUTURE PERSPECTIVES

The main drawback of curcumin as a pharmacological agent is its poor bioavailability after oral administration [34], due to low intestinal absorption, rapid hepatic and intestinal metabolism via glucuronidation and sulfation [194], and rapid elimination via the bile and excretion into the feces. In many animal studies it has been shown that intraperitoneal administration has higher bioavailability and effectiveness respect to oral administration, due to the by-pass of intestinal absorbption. [195]. Nonetheless, the rapid metabolism of curcumin by the liver causes a poor bioavailability of this substance even after parenteral administration [196].

In the last several years, curcumin adjuvants that reduce its metabolism, or novel delivery systems, have been evaluated in order to enhance curcumin's efficacy in vivo. In this context, piperine has been the most studied adjuvant. Piperin is a bioactive alkaloid that inhibits hepatic and intestinal glucoronidation. In animal models, piperine enhances curcumin's bioavailability by 154% relative to curcumin without piperine. In human volunteers, the increase in the bioavailability was approximately 2000% when compared to curcumin without piperine. Importantly, this increase in the bioavailability of curcumin when administered with piperine was not associated with adverse effects in the human volunteers [197]. Additional methods that have been studied to increase curcumin's bioavailability include curcumin nanoparticles, liposomes, micelles, phospholipid complexes, and structural analogues. All of these approaches are directed at achieving a longer half-life, increased concentrations in tissues, and resistance to metabolic processes [198]. However, whether these approaches to optimize the pharmacological action of curcumin are effective in humans remains to be explored. In rats, curcumin-phospholipid complex, compared to curcumin alone, yielded higher serum concentrations and increased the time period that the curcumin serum concentration exceeded the minimum effective concentration (MEC) [199]. Oral administration of curcumin formulated with phosphatidylcholine, compared with unformulated curcumin, resulted in

higher plasma and liver concentrations in rats [200]. Finally, using a mouse model of asthma, Wenrui Wang et al. has demonstrated the greatest enhancement in curcumin plasma and tissue concentrations by utilizing curcumin-solid lipid nanoparticles that were administered by intraperitoneal injection [201].

In conclusion, a large body of evidence is accumulating concerning the efficacy of curcumin in lung diseases. These data concerning curcumin's therapeutic benefits in treating numerous lung diseases is growing as the results of hundreds of in vitro and in vivo (animal models) studies are reported in the literature. However, unfortunately, there are few, and, at times, contrasting and equivocal results concerning curcumin's efficacy in humans for treating lung diseases. With regard to curcumin's bioavailability, adjuvants and many newly-created analogs of curcumin, as well as novel curcumin formulations and delivery approaches, are being evaluated with very promising results. Additional studies are needed to assess the clinical effectiveness of both curcumin and its analogs in human lung diseases.

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b) CURCUMIN AND LUNG CANCER: THE ROLE OF MICRO-RNAS

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REVIEW ARTICLE

BENTHAM SCIENCE

Curcumin and Lung Cancer: the Role of microRNAs

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Abstract: ***Background:*** Lung cancer is one of the most common types of cancer worldwide and is characterized by a poor prognosis, related to both late diagnosis and lack of effective treatments. In the last years, microRNAs (miRNAs) have been demonstrated to have an important role in tumor microenvironment and immune regulation. These RNAs can be categorized into tumor-suppressor genes, such as let-7 family and miR-34, and oncogenes such as miR-221 and miR-222. Curcumin is a bioactive polyphenol that is documented to have promising anti-cancer activity, and to be well tolerated in humans.

Methods: The present review aims to gather available evidence on the involvement of mRNAs in the therapeutic effects of curcumin against lung cancer.

Results: The anti-cancer properties of curcumin against lung cancer have been shown in both cellular and experimental models and are mediated by modulation of several molecular targets that regulate the expression of transcription factors, inflammatory cytokines, enzymes, growth factors, receptors, adhesion molecules, antiapoptotic proteins, and cell cycle proteins, leading to cell apoptosis, inhibition of cell proliferation and migration, and also chemo- and radio-sensitization of lung cancer cells. Recent studies have documented that pharmacological effects of curcumin in lung cancer are also mediated by modulation of several miRNAs, such as downregulation of oncogenic miR-21 and upregulation of oncosuppressive miR-192-5p and miR-215.

Conclusion: Further studies are necessary to explore this very promising field and the link between regulation of oncogenic and tumor-suppressive miRNAs and putative anti-cancer properties of curcumin.

Keywords: Curcumin, epigenetic, lung cancer, MicroRNA, RNA interference, tumor.

INTRODUCTION

Lung cancer is one of the most common types of cancer worldwide; it is classicaly differentiated in non-small cell lung cancer (NSCLC), that represents about 80% of all lung cancers, and small cell lung cancer (SCLC), that represents the remaining 20% [1]. This disease represents the first cause of death from cancer, accounting for more than 1,4 million deaths each year [2]. Despite improvements in diagnostic and therapeutic strategies, the overall 5-year survival from lung cancer remains only 10-20% [1] Poor prognosis is generally related to late diagnosis and lack of effective treatments [3].

Micro-RNAs (MiRNAs) are small single-strand non-coding RNAs, with a size range of 19-25 nucleotides that are implicated in post-transcriptional regulation of gene expression. MiRNAs are loaded into the RNA-induced silencing complex (RISC) that recognizes the complementary sequence of target mRNAs and induces their degradation or translational repression. MiRNAs can

silence multiple genes owing to their partial base complementarity with corresponding mRNAs [4,5]. Therefore, miRNAs can regulate multiple genes and have an important role in cell differentiation, proliferation, growth, mobility, and apoptosis. MiRNAs can be categorized into oncogenes and tumor-suppressor genes [6] and are implicated in tumor microenvironment regulation such as stimulation of angiogenesis, matrix degradation [7], and activation of cancer-associated fibroblasts that generate an environment promoting tumor growth and invasiveness [8]. MiRNAs can also regulate immune response through multiple mechanisms such as downregulation of MHC I [9], ICAM-1 [10], regulatory T lymphocytes (Tregs), CXCL12, and TGF-β production [11].

Curcumin is a component of turmeric, derived from Curcuma longa, and characterized by many biological and pharmacological effects, that are mediated by modulation of several molecular targets and signalling pathways. Therefore, curcumin regulates of the expression of several transcription factors, inflammatory cytokines, enzymes, growth factors, receptors, adhesion molecules, antiapoptotic proteins, and cell cycle proteins, with associated anti-inflammatory, antioxidant, and anticancer activity [12]. Furthermore, curcumin is well tolerated in humans [13]. Curcumin anti-cancer activity has been demonstrated in different types of cancer, such as melanoma [14], colon [15], pancreatic [16], and lung [17] cancer. These effects are, at least in part, mediated by modulation of miRNAs. For example, in human colon cancer cell lines, curcumin downregulates in a dose-dependent manner miR-21, an oncogenic miRNA, and induces the expression of the tumour suppressor Programmed Cell Death Protein 4 (PDCD4), which is a target of miR-21. These data are also confirmed in *in vivo* chicken-embryo-metastasis assay, where it is demonstrated to inhibit cancer cell metastasis [18]. MiRNA-21 is also implicated in pancreatic cancer, and it is a target of difluorinated curcumin (CDF), a curcumin analogue characterized by greater bioavailability [19]. In fact, in human pancreatic cell lines, CDF inhibits miR-21 and consequently induces tumor-suppressor phosphatase and tensin (PTEN), a target of miR21 [20]. Curcumin modulation of miRNAs is documented also in melanoma: Dahmke et al. described that dietary

intake of curcumin modulates the expression of 147 miRNAs in an engrafting mouse melanoma model, the most upregulated of which was mmu-miR-205-5p, with consequent downregulation of Bcl-2 and proliferating cell nuclear antigen (PCNA), that are involved in cancer-related pathways, including apoptosis and proliferation [21].

In this review we briefly describe the action of curcumin in regulating the miRNA involved in lung cancer

MICRORNAS AND LUNG CANCER

In the last years, microRNAs (miRNAs) have been demonstrated to have an important role in pathogenesis of lung cancer and development of drug-resistance.

Recent studies have reported an aberrant expression of miRNAs in lung tumor tissues compared with the corresponding normal lung tissues, suggesting the involvement of miRNAs in lung cancer pathogenesis [22].

In NSCLC, many miRNAs described to have a tumor-suppressor activity are down-regulated, while those inducing proliferation are up-regulated. The let-7 family of miRNA inhibits the expression of oncogenes (e.g. Ras, Myc and cyclin D) that are implicated in cell-cycle regulation and proliferation and thus inhibit tumor growth both *in vitro* and *in vivo* [23,24]. Another miRNA with tumor-suppressor activity is miR-34 that is transcriptionally activated by p53 in response to DNA damage. This miRNA regulates cell cycle, apoptosis and senescence by targeting *Bcl-2*, *Myc*, *MET* and *PDGFR* genes [25]. Both let-7 and miR-34 are downregulated in NSCLC cell lines [26]. MiR-29 family members may target DNA methyltransferases (DNMT3A and DNMT3B) and restore patterns of DNA methylation and expression of silenced tumor-suppressor genes in lung cancer, thus inhibiting tumorigenicity both *in vitro* and *in vivo* [27].

On the other side, many miRNAs are described to have an oncogenic function in NSCLC by targeting tumor-suppressor genes. For example, the miR-17-92 cluster targets PTEN which participates in the cell-survival signaling pathway [27]. It also targets the hypoxia-inducible factor 1

α (HIF-1α) which transactivates the genes involved in multiple biological processes such as angiogenesis, apoptosis, extracellular metabolism, cell proliferation, invasion and metastasis [28]. MiR-221 and miR-222 target PTEN and tissue inhibitor of metalloprotease-3 (TIMP-3), thereby enhancing survival and migration of NSCLC cells [29].

Overall, miRNAs have been suggested as key regulators of biological processes involved in lung cancer and could thus serve as potential therapeutic targets in this type of cancer [22].

CURCUMIN AND LUNG CANCER

Conventional therapies for lung cancer are poorly effective and burdened with serious adverse effects [30]. The anti-cancer properties of curcumin are mediated by modulation of several molecular targets that regulate the expression of transcription factors, inflammatory cytokines, enzymes, growth factors, receptors, adhesion molecules, antiapoptotic proteins, and cell cycle proteins, leading to cell apoptosis, inhibition of cell proliferation and migration, and also chemo- and radio-sensitization of lung cancer cells [31,32]. For example, this compound has been shown to increases ROS levels [33,34] and, in a dose-dependent manner, reduce the expression of DNA repair proteins and enhance p53 levels [35], which jointly result in the induction of apoptosis.

The most important pathway by which curcumin inhibits lung cancer cell proliferation is the JAK2/STAT3 pathway. This pathway also suppresses migration, invasion and angiogenesis [36,37]. JAK2/STAT3 is inhibited by curcumin also in cancer stem cells [38], which are implicated in tumor recurrence and in drug resistance; this activity leads to the inhibition of tumor growth *in vivo* [39].

Another mechanism by which curcumin inhibits the proliferation of lung cancer cells is induction of forkhead box protein O1 (FOXO1), a transcription factor that regulates cell proliferation, differentiation, and DNA damage repair [40,41]. Inhibition of cell proliferation by curcumin also results from epigenetic effects such as reactivation of silenced tumor-suppressor genes like RARβ that is induced by curcumin, leading to the inhibition of tumor growth [42].

The antineoplastic action of curcumin is also mediated by the inhibition of cancer cell migration. Curcumin downregulates early growth response protein 1 (EGR-1) via enhancement of cell-cell adhesion [43]. Moreover, this compound inhibits the production and activity of matrix metalloproteinases (MMPs) through several mechanisms such as downregulation of phosphokinase A, with consequent inhibition of MMP-9 production through NADPH oxidase-2 pathway [44], or the inhibition of the Rac1/PAK1 pathway, leading to the downregulation of MMP-2 and MMP-9 [45].

Finally, curcumin has chemosensitizing properties documented for many chemotherapeutic agents. Cisplatin, the most common chemotherapeutic agent used in NSCLC, has a biological cellular resistance that is generally associated with cellular DNA repair mechanisms [46], and this resistance has been shown to be reversed by curcumin. Furthermore, curcumin enhances the cisplatin-mediated inhibition of proliferation and induction of apoptosis by inhibition of the Fanconi anemia/BRCA pathway [47] and HIF-1α [48].

CURCUMIN AND MICRORNAS IN LUNG CANCER

Several lines of recent evidence have shown that the pharmacological effects of curcumin in lung cancer are also mediated by modulation of several miRNAs. Zhang et al., through qRT-PCR analysis, documented that curcumin down-regulates the expression of miR-21 in a dose-dependent manner in lung cell line A549, with a reduction in miR-21 expression of about 60% in cells exposed to 40 μM of curcumin. In these experimental model, flow cytometric analysis showed that curcumin at 20-40 μM increased the portion of apoptotic annexin V-positive cells by approximatively 2-5-fold, and inhibited cell proliferation and induced apoptosis. PTEN, the putative target of miR-21, was significantly elevated in curcumin-treated A549 cells, as determined by Western blot analysis [49].

Ye et al. documented through miR microarray that 15 μM of curcumin upregulate the tumor suppressive miR-192-5p and miR-215 in A549 cells (p53 wild type), but not in H1299 cells (p53

null). Curcumin upregulates and activates p53 in the p53 wild type H460, A427, and A549 NSCLC cells; the lack of p53 in H1299 cells impaired curcumin-mediated upregulation of miR-192-5p/215. As documented by a dual luciferase activity assay, X-linked inhibitor of apoptosis (XIAP) is a target of miR-192-5p/215. Therefore, curcumin induced apoptosis through activation of the p53-miR-192-5p/215-XIAP pathway in NSCLC cells [50]. Moreover, induction of miR-192-5p by curcumin inhibits cell proliferation and induces apoptosis through inhibition of the Pi3K/Akt pathway, a pathway implicated in growth factor-mediated cell survival [42].

Another miRNA that is modulated by curcumin is miR-186* which is an oncogenic molecule implicated in the downregulation of proapoptotic genes in lung cancer. Tang et al. [51] and Zhang et al. [52,53] studied the effect of curcumin on the expression of miR-186* in A549/DDP human lung cancer cells. Microarray analysis and qRT- PCR showed that curcumin may induce apoptosis by down-regulating miR-186* expression in these cells. The target of miR-186*, predicted using the Miranda database and confirmed by using dual luciferase reporter assays and Western blot analysis, was caspase-10, an initiator caspase in death receptor signaling, crucial for apoptotic signaling. This caspase was significantly increased in curcumin-treated lung cancer cells. Therefore, curcumin induces A549 cell apoptosis through the miR-186* pathway in a dose- and time-dependent manner, by increasing caspase-10 [51–53].

Another miRNA modulated by curcumin is miR-874, a tumor suppressive miRNA, which has been shown to target matrix metalloprotease-2 (MMP-2) in NSCLC cell lines. In fact, Ahmad et al documented that curcumin inhibits MMP-2 expression and activity through upregulation of miR-874 in A549 e H1299 cell lines [54].

Finally, Wu et al. documented with miRNA microarray analysis and qPCR that curcumin upregulates miRNA-let7c and miR-101 in A549 cells. Enhancer of zeste homolog 2 (EZH2), an oncogene that regulates cell cycle progression through activation of NOTCH signaling pathway was significantly downregulated in A549 cells with overexpression of miRNA-let7c and miR-101.

Therefore, the effect of curcumin in this miRNA may lead to inhibition of lung cancer cell growth [55].

Table 1. Modulation of miRNA expression by curcumin.

miRNA	Modulation	Cell line	References
miR-21	-	A549	Zhang *et al.* d
miR-192-5p	+	p53 wild-type H460, A427, A549	Ye *et al.*
miR-215	+	p53 wild-type H460, A427, A549	Ye *et al.*
miR-186*	-	A549	Zhang *et al.* and Tang *et al.* (Tang *et al.*, 2010a; Zhang *et al.*, 2010b; Zhang *et al.*, 2010d)
miR-874	+	A549, H1299	Ahmad *et al.*
let-7c	+	A549	Wu *et al.*
miR-101	+	A549	Wu *et al.*

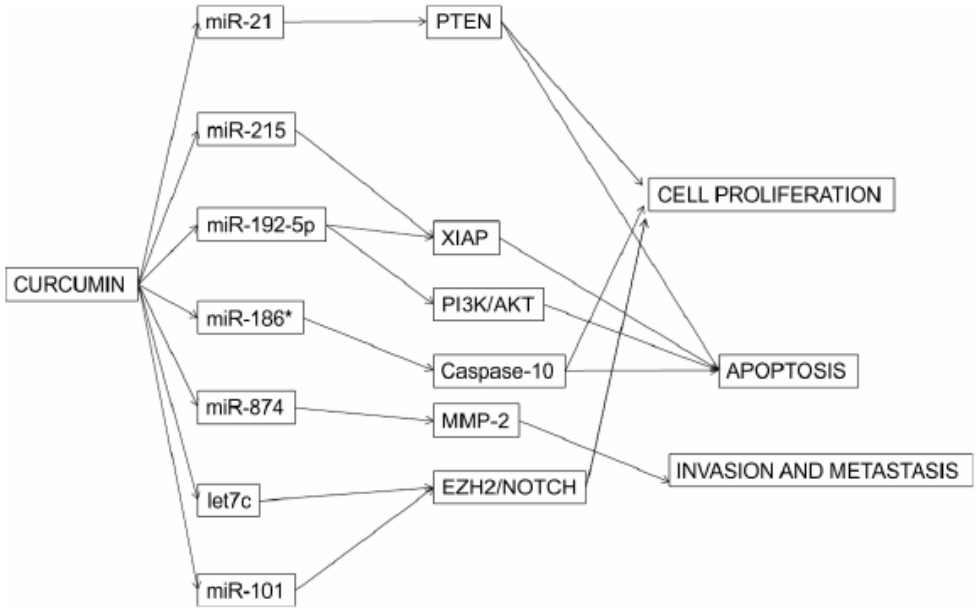

Fig. (1). MiRNA-mediated mechanisms of action of curcumin in lung cancer. PTEN: tumor-suppressor phosphatase and tensin; XIAP: X-linked inhibitor of apoptosis; PI3K/AKT: Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT; EZH2/NOTCH: enhancer of zeste homolog 2.

CONCLUSIONS

In the last years miRNAs have been demonstrated to play a primary role in the pathogenesis of lung cancer and emerged as candidate therapeutic targets. Recent studies have documented that curcumin's anti-cancer activity is also mediated by modulation of miRNAs. Further studies are necessary to explore this very promising field and the potential of curcumin to regulate oncogenic and tumor-suppressive miRNAs in the clinical setting as well in other pathological states due to imbalance in miRNA homeostasis.

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c) CURCUMIN AND TREATMENT OF MELANOMA: THE POTENTIAL ROLE OF MICRO-RNAS

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Review

Curcumin and treatment of melanoma: The potential role of microRNAs

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ABSTRACT

Melanoma is the most aggressive type of skin cancer and is characterized by poor prognosis in its advanced stages because treatments are poorly effective and burdened with severe adverse effects. MicroRNAs (miRNAs) are small non-coding RNAs that are implicated in several cellular processes; they are categorized as oncogenic and tumor suppressor miRNAs. Several miRNAs are implicated in the pathogenesis and progression of melanoma, such as the tumor suppressor miR-let7b that targets cyclin D and regulates cell cycle. Curcumin is a natural compound derived from *Curcuma longa* L. (turmeric) with anti-cancer properties, documented also in melanoma, and is well tolerated in humans. Pharmacological activity of curcumin is mediated by modulation of several pathways, such as JAK-2/STAT3, thus inhibiting melanoma cell migration and invasion and enhancing apoptosis of these cells. The low oral bioavailability of curcumin has led to the development of curcumin analogues, such as EF24, with greater anti-tumor efficacy and metabolic stability. Potential anti-cancer activity of curcumin and its analogues is also mediated by modulation of miRNAs such as miR21, that is implicated in cell cycle regulation and apoptosis through down-regulation of PTEN and PDCD4 proteins. Curcumin has a potential role in the treatment of melanoma, though further studies are necessary to explore its clinical efficacy.

MELANOMA AND MICRO-RNAs

Melanoma is one of the most frequent malignancy in United States and it represents the deadliest form of skin malignancy; furthermore, its incidence is rising. Advanced stages of melanoma are characterized by poor prognosis, mainly due to lack of effective treatments and to development of chemotherapy-resistance.[1]

MiRNAs are small, noncoding RNAs that are involved in several cellular processes, such as differentiation, proliferation, and apoptosis. These RNAs can be categorized into oncogenes and tumor-suppressor genes. Their action is mediated by a post-transcriptional silencing of target genes, through an imperfect binding of target mRNAs, that allows miRNAs to silence multiple genes.[2]

In the last years several miRNAs have been identified to be implicated in the pathogenesis and progression of melanoma, [3] such as miR-let7b, a tumor suppressor miRNA that is commonly downregulated in this cancer. MiR-let7b targets cyclin D, a key player in the control of cell cycle progression; and downregulation of this RNA leads to cell cycle acceleration and thus promotion of cell proliferation.[4] MiR-205 is another oncosuppressor miRNA downregulated in melanoma, and it is implicated in cell survival and invasion. MiR-205 expression correlates with zing-finger E-box binding homeobox 2 (ZEB2) downregulation and with E-cadherin upregulation, which regulate the epithelial-to-mesenchymal transition (EMT) and melanoma metastasis.[3] MiR-221/222 is an oncogenic miRNA cluster that is commonly overexpressed in melanoma. This cluster targets many genes, including p27, tissue inhibitor of metalloproteinase (TIMP) and phosphatase and tensin homolog (PTEN), thereby enhancing cell proliferation. Downregulation of miR-221/222 reduces cell growth and invasion.[2]

CURCUMIN AND MELANOMA

Melanoma therapy is characterized by poor efficacy, without evidence of significant improvement of prognosis; furthermore, it is burdened with several side effects that negatively affect patients' quality of life.

Curcumin is a yellow pigment derived from *Curcuma longa* that has anti-oxidant, anti-inflammatory, and anti-neoplastic activities and is well tolerated in humans. Anti-cancer activity of curcumin is mediated by modulation of several pathways, targeting multiple genes, transcription factors, inflammatory cytokines, enzymes, growth factors, receptors, adhesion molecules, anti-apoptotic proteins, and cell cycle proteins, leading to apoptosis and inhibition of cell proliferation and migration.[5] Potential anti-cancer activity of curcumin is also documented in melanoma.[6] One of the mechanisms through which curcumin can induce apoptosis in melanoma is activation of caspases 3 and 8.[6] Furthermore, curcumin has been demonstrated *in vitro* to inhibit melanoma cell migration, invasion and to enhance apoptosis through downregulation of JAK-2/STAT3 signaling

pathway.[7] Osteopontin (OPN) is a protein implicated in nuclear factor kappa B (NF-κB)-mediated induction of matrix metalloproteinase-2 (MMP-2) and tumor growth in melanoma. Curcumin *in vitro* downregulates OPN-induced pro-MMP-2 activation and inhibits OPN-induced cell proliferation and migration, extracellular matrix invasion, and induces apoptosis in these cells. *In vivo,* this compound suppresses pro-MMP-2 expression and the OPN-induced tumor growth, once injected in tumor site in mice.[6]

CURCUMIN AND MiRNAs IN MELANOMA

The biological effects of curcumin and its analogues in melanoma are also mediated by modulation of miRNAs. Dahmke et al. described that dietary intake of curcumin modulates the expression of 147 miRNAs in an engrafting mouse melanoma model. The most upregulated miRNA was mmu-miR-205-5p, whose expression was 135 times higher in melanoma cells of mices treated with curcumin compared with controls. This miRNA has been shown to reverse EMT in various tumor types. B-cell lymphoma 2 (Bcl-2) and proliferating cell nuclear antigen (PCNA) are predicted targets of mmu-miR-205-5p and are involved in cancer-related pathways, including apoptosis and proliferation. In the above-mentioned study, Bcl-2 and PCNA were significantly downregulated by curcumin, both *in vivo* and *in vitro*.[8] Diphenyldifluoroketone (EF24), a curcumin analog characterized by greater biological activity and bioavailability than curcumin, is documented to modulate miRNA expression in melanoma cells *in vitro* and *in vivo* (Figure). Zhang et al. showed that EF24 downregulates high mobility group AT-hook 2 (HMAG2), a key regulator of tumor-EMT, through upregulation of miR-33b, thus configuring EF24 as a potential anti-metastatic agent in melanoma therapy.[9] Another miRNA modulated by EF24 is miR-21, which is an oncogenic miRNA. EF24-mediated downregulation of miR-21 induces apoptosis of melanoma cells *in vitro,* inhibits lung metastasis and prolongs mice survival *in vivo* through induction of PTEN and programmed cell death 4 (PDCD4), which are implicated in cell cycle regulation and apoptotic pathways.[10]

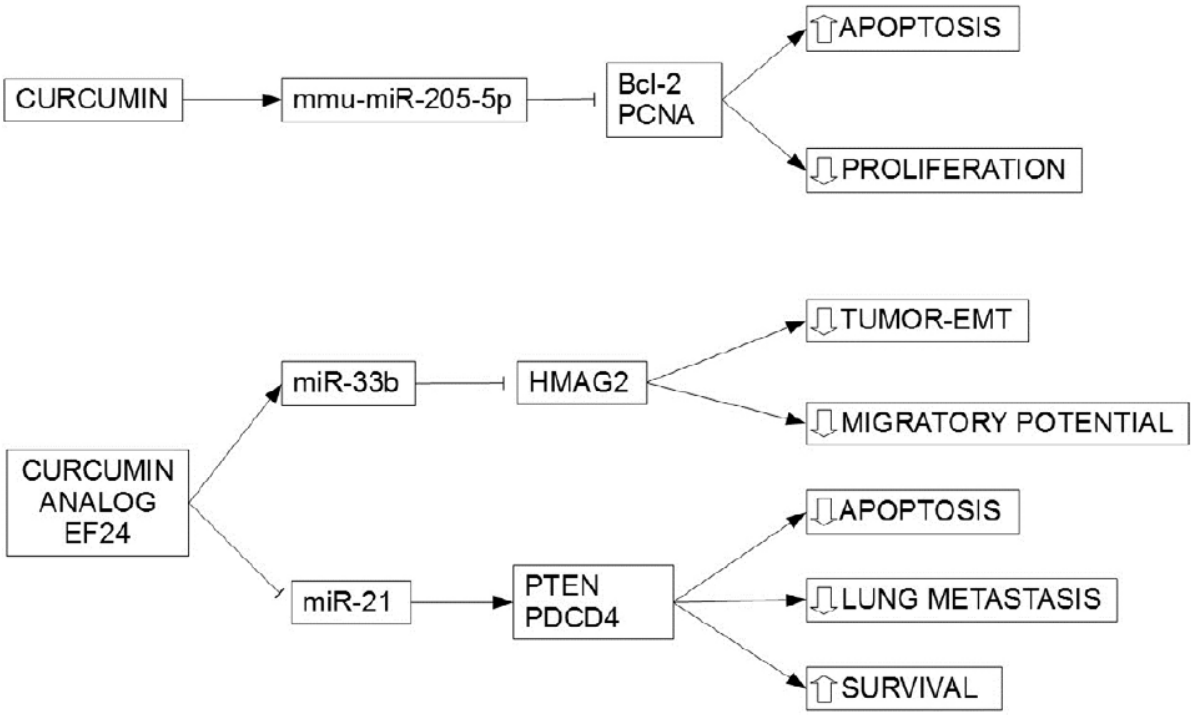

Fig. 1. Anti-tumor effects of curcumin and its analogue, EF-24, mediated by the alteration of microRNAs.

CONCLUSION

Melanoma is the most aggressive type of skin cancer and its poor prognosis is related to the limited efficacy of chemotherapy, that is also complicated by several adverse effects. Curcumin is a natural compound that is well tolerated in humans, with anti-cancer properties documented in melanoma. In addition to other pathways, curcumin acts through modulating miRNAs that are promising new targets for anti-cancer therapy. The most important limit for *in vivo* curcumin use is its low bioavailability when administrated orally. In this context curcumin analogues have been elaborated with greater anti-tumor activities, and fabricated delivery systems have been developed. Further studies are necessary to explore the efficacy of curcumin and its analogues in experimental models of melanoma, and the role of this phytochemical in regulating oncogenic and tumor-suppressive miRNAs.

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d. HEMOGLOBIN CONCENTRATION INFLUENCES N-TERMINAL PRO B-TYPE NATRIURETIC PEPTIDE LEVELS IN HOSPITALIZED OLDER ADULTS WITH AND WITHOUT HEART FAILURE

Hemoglobin Concentration Influences N-Terminal Pro B-Type Natriuretic Peptide Levels in Hospitalized Older Adults with and without Heart Failure

Diana Lelli, MD, Raffaele Antonelli Incalzi, MD, and Claudio Pedone, MD, PhD, MPH

OBJECTIVES: To investigate the relationship between hemoglobin and N-terminal pro B-type natriuretic peptide (NT-proBNP) concentration in hospitalized older adults with or without a diagnosis of heart failure (HF).

DESIGN: Cross-sectional study based on retrospective hospital records review.

SETTING: Geriatric acute care ward.

PARTICIPANTS: Individuals aged 65 and older (N = 226; mean age 81.1), with (n = 104) and without (n = 122) a diagnosis of HF.

MEASUREMENTS: Information was collected on demographic characteristics, comorbidities, and laboratory and echocardiographic data. The relationship between hemoglobin and NT-proBNP was evaluated using linear regression models adjusted for potential confounders.

RESULTS: A negative association was found between NT-proBNP and hemoglobin ($\beta = -0.226$, $P < .001$). The regression coefficient was −0.114 ($P = .04$) in the subsample with HF and −0.191 ($P < .001$) in the subsample without HF. After adjustment for potential confounders, the inverse association between hemoglobin and NT-proBNP was confirmed in the whole sample ($\beta = -0.182$, $P < .001$), in those with HF ($\beta = -0.136$, $P = .007$), and in those without HF ($\beta = -0.165$, $P = .003$).

CONCLUSION: Hemoglobin concentration should be taken into account in the interpretation of NT-proBNP in hospitalized older adults, especially those without HF. **J Am Geriatr Soc 65:2369–2373, 2017.**

Key words: heart failure; natriuretic peptides; elderly; anemia

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Natriuretic peptides are hormones that are secreted from the ventricular myocardium in response to wall stretching and have an important role in maintaining the homeostasis of individuals with heart failure (HF).[1]

These peptides have recently been recognized as cardiac biomarkers, and they are commonly used for diagnosis and prognosis stratification of HF in general [2–6] and in older adults in particular.[7] Several factors influence the concentration of natriuretic peptides; for example, it is lower in in obese individuals [8] and higher in those with renal insufficiency.[9] Some studies have found a negative correlation between hemoglobin and natriuretic peptide concentrations,[10,11] but very few included elderly adults, and none reported separate data from this population in which HF is most common.[12] Anemia has a prevalence of 20–30% in individuals with HF [13] and may influence natriuretic peptide concentration by activating the sympathetic nervous system, with a consequent increase of cardiac output and ventricular wall stress.[14] Aging is characterized by chronically high norepinephrine levels due to reduced uptake of this neurotransmitter [15] and chronic inflammation,[16] which stimulates the sympathetic nervous system.[17] This may cause a different correlation between anemia and natriuretic peptides in elderly than in young adults.

Only one study has compared the effects of anemia on natriuretic peptides in individuals with HF with the effects on those without a diagnosis of HF.[18] Like elderly adults, individuals with HF have chronic activation of the sympathetic nervous system,[19,20] and the anemia-mediated activation of this system with consequent increase of natriuretic peptide levels may be different than in individuals without HF.

The aim of this study was to investigate the relationship between hemoglobin and N-terminal pro B-type natriuretic peptide (NT-proBNP) concentration in hospitalized older adults with and without a diagnosis of HF.

METHODS

Medical records were reviewed of 226 individuals admitted to a geriatric acute care ward aged 65 and older with

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INTRODUCTION

Natriuretic peptides are hormones that are secreted from ventricular myocardium in response to increased wall stretching and have an important role in maintaining the homeostasis of heart failure (HF) patients (1).

In the latest years, these peptides have been recognized as cardiac biomarkers and they are commonly used for diagnosis and prognosis stratification of HF (2–6), also in older people (7). However, concentration of natriuretic peptides is influenced by several factors; for example, it decreases in obesity (8), and increases in renal insufficiency (9). Some evidences show a negative correlation between hemoglobin and natriuretic peptides concentration (10,11), but very few studies included elderly patients, and none reported separate data on this population, in which HF is most common (12). Anemia has a prevalence of 20-30% in HF patients (13), and may influence natriuretic peptides concentration by activating the sympathetic nervous system, with consequent increase of cardiac output and ventricular wall stress (14). Aging is characterized by chronic elevation of norepinephrine levels, due to reduced uptake of this neurotransmitter (15), but also to chronic inflammation (16) that stimulates the sympathetic nervous system (SNS) (17). This may cause a different correlation between anemia and natriuretic peptides in the elderly respect to young adults.

Furthermore, only one study investigated the effects of anemia on natriuretic peptides in HF patients compared to patients without diagnosis of HF (18). Like elderly patient, HF patients are characterized by chronic activation of the SNS (19,20), and the anemia-mediated activation of this system with consequent increase of natriuretic peptide levels may be different respect to patients without HF.

The aim of this study is to investigate the relationship between hemoglobin and NT-proBNP concentration in hospitalized older patients with or without diagnosis of HF.

METHODS

We reviewed 226 medical records of patients admitted to a geriatric acute care ward, aged 65 years or over with no exclusion criteria based on principal diagnosis, comorbidities, or pharmacological therapy. NT-proBNP and hemoglobin were measured on admission, independently of clinical characteristics. Echocardiography was available for all the patients included in the study. We classified participants by presence (n=104) or absence (n=122) of heart failure, defined according to Framingham criteria, or to ejection fraction ≤40%.

Acute HF exacerbation were identified on the basis of evidence from the clinical chart of acute worsening of symptoms and specific therapy (e.g., intravenous diuretics). We recorded demographic characteristics, discharge diagnosis, and selected comorbidities that may influence NT-proBNP values: diabetes, rheumatoid arthritis, hepatic cirrhosis, acute coronary syndrome, sepsis, hyperthyroidism, chronic obstructive pulmonary disease (COPD), atrial fibrillation, pulmonary hypertension, ischemic heart disease. We also recorded renal function estimated using the CKD-EPI formula, erythrocyte sedimentation rate **(**ERS), C reactive protein (CRP), body mass index (BMI), and arterial blood gas analysis. We collected the following echocardiographic parameters: ejection fraction (EF), aortic or mitral regurgitation, aortic stenosis, atrial enlargement, diastolic dysfunction, wall motion score index (WMSI), percent of akinesia extension. Systolic pulmonary artery pressure (PAPS) was recorded only in patients with tricuspid insufficiency and categorized into three groups: <35, 35-45, >45 mmHg.

Analytic approach: Descriptive statistics were used to compare participants with and without HF with respect to clinical and echocardiographic data, using mean and standard deviation for

continuous variables (with the exception of NT-proBNP, for which median and interquartile range were reported due to its skewed distribution), and proportions for categorical variables. The relationship between hemoglobin and NT-proBNP was evaluated calculating correlation coefficients; in this analysis NT-proBNP was log-transformed to obtain a normal distribution. The association between hemoglobin and log-transformed NT-proBNP concentrations was subsequently verified using a linear regression model adjusted for age, sex, estimated glomerular filtration rate (eGFR), oxygen saturation, COPD, ischemic heart disease, atrial fibrillation, diabetes, BMI, and ejection fraction. Iron deficiency was another potential confounder of the relationship under study; ferritin and serum iron concentration were not available for most patients, therefore we included mean corpuscular volume (MCV) as a proxy for this variable. In models with log-transformed dependent variables, the regression coefficients can be transformed into mean percent difference using the formula: ($e^{\square}$ * 100)-100. Missing values of BMI, related to high prevalence of patients bed constrained in our sample, were imputed using additive regression models. Since we hypothesized that haemoglobin concentration may differently affect NT-proBNP concentration in people with and without HF, all the analyses were stratified by this variable.

This study was notified to the Ethical Committee of the Campus Bio-Medico University.

RESULTS

The mean age was 81.1 years (SD 6.8), 39% of the participants were male. One hundred and four participants were classified in the HF group; they had a mean age of 82.8 years (SD 6.9) and 76% had an acute HF exacerbation. The mean age in the group without HF was 79.7 years (SD 6.4).

eGFR was lower in the HF group, with a mean value of 52.1 mL/min/1.73m^2 (SD 22.6), compared to 71 mL/min/1.73 m^2 (SD 20.2) in the subsample without HF. Compared to participants without

HF, those with HF had higher median NT-proBNP concentration (4207.5 ng/mL, IQR 6814 vs 460 ng/mL, IQR 1007.3) and lower mean peripheral oxygen saturation (93.7%, SD 4 vs 94.6%, SD 3.3).

In the HF subsample, 53% participants had HF as main discharge diagnosis; the second most frequent main discharge diagnosis was lung diseases (27%). In the subsample without HF, the main discharge diagnoses were lung diseases (18%), cancer (13%), cerebrovascular (11%), and gastrointestinal (10%) diseases. Atrial fibrillation was more prevalent in the HF subsample (53% versus 14%), as well as chronic ischemic heart disease (38% and 19% in the HF and non-HF subsample, respectively). Diabetes and COPD also were more prevalent in the subsample with diagnosis of HF. Mean hemoglobin concentration was higher in the non-HF subsample (11.9 g/dl, SD 2.2 vs. 11.3 g/dl, SD 2 in the HF subsample) (Table 1).

Table 1. General Characteristics of the Population

Characteristics	No Heart Failure, n = 122	Heart Failure, n = 104	Entire Sample, N = 226
Age, mean ± SD	79.7 ± 6.4	82.8 ± 6.9	81.1 ± 6.8
Male, %	38	40	39
Estimated glomerular filtration rate, mL/min per 1.73 m^2, mean ± SD	71.0 ± 20.2	52.1 ± 22.6	62.3 ± 23.2
Hemoglobin (g/dL, mean ± SD	11.9 ± 2.2	11.3 ± 2	11.6 ± 2.1
Mean corpuscular volume, fL, mean ± SD	90.0 ± 8.0	90.4 ± 9.0	90.1 ± 8.4
N-terminal pro B-type natriuretic peptide, pg/mL, median (interquartile range)	460.0 (1,007.3)	4,207.5 (6,814.0)	1,425.0 (3,804.5)
Peripheral oxygen saturation, %, mean ± SD	94.6 ± 3.3	93.7 ± 4	94.2 ± 3.7
Body mass index, kg/m^2, mean ± SD	27.2 ± 6.3	26.0 ± 5.3	26.6 ± 5.8
Discharge diagnosis, %			
Heart failure	0	53	24
Cardiovascular disease	7	6	7
Cerebrovascular disease	11	3	7
Lung disease	18	27	22
Cancer	13	3	8
Genitourinary disease	4	3	4
Gastrointestinal disease	10	5	8
Other	37	12	25
Comorbidities, %			
Atrial fibrillation	14	53	32
Diabetes mellitus	22	27	24
Chronic obstructive pulmonary disease	25	31	27
Ischemic heart disease	19	38	27

Echocardiography documented a mean ejection fraction of 46.8% (SD 12.3) in the HF subsample and of 57.5% (SD 3.9) in the non-HF subsample. In the HF subsample 60.6% participants had a preserved ejection fraction. This subsample also showed a higher prevalence of moderate or severe mitral or aortic insufficiency (42.3% vs 15.6% and 15.4% vs 7.4%, respectively), atrial enlargement (95.2% vs 63.1%), and pulmonary hypertension (76.6% vs 30.8%) (Table 2).

Table 2. Echocardiographic Characteristics of the Population

Characteristics	No Heart Failure, n = 122	Heart Failure, n = 104	Entire Sample, N = 226
Preserved ejection fraction (≥50%), %	97.5	60.6	80.5
Ejection fraction, %, mean ± SD	57.5 ± 3.9	46.8 ± 12.3	52.5 ± 10.3
Wall Motion Score Index, mean ± SD	1.1 ± 0.2	1.5 ± 0.5	1.3 ± 0.5
Acinesy extension, %, mean ± SD	2.7 ± 7.5	8.1 ± 14	5.1 ± 11.2
Left ventricular diastolic function, %			
Monophasic pattern	10.3	54.0	29.1
Grade I	87.1	35.6	65.0
Grade II	1.7	5.7	3.4
Grade III	0.9	4.6	2.5
Mitralic or aortic regurgitation, %			
Absent	1.6	0.0	0.9
Mild	75.4	42.3	60.2
Moderate	15.6	42.3	27.9
Severe	7.4	15.4	11.1
Aortic stenosis, %			
Absent	88.8	81.2	85.3
Mild	6.0	10.9	8.3
Moderate	5.2	4.0	4.6
Severe	0.0	4.0	1.8
Pulmonary hypertension, %			
Absent	69.2	23.4	47.5
Mild	22.1	35.1	28.3
Moderate	7.7	35.1	20.7
Severe	1.0	6.4	3.5
Atrial enlargement, %	63.1	95.2	77.9

The correlation coefficient between NT-proBNP and hemoglobin was -0.284 ($P<0.001$) in the whole sample, -0.202 ($P=0.039$) in the HF subsample and -0.309 ($P<0.001$) in the non-HF subsample (Figures 1 and 2).

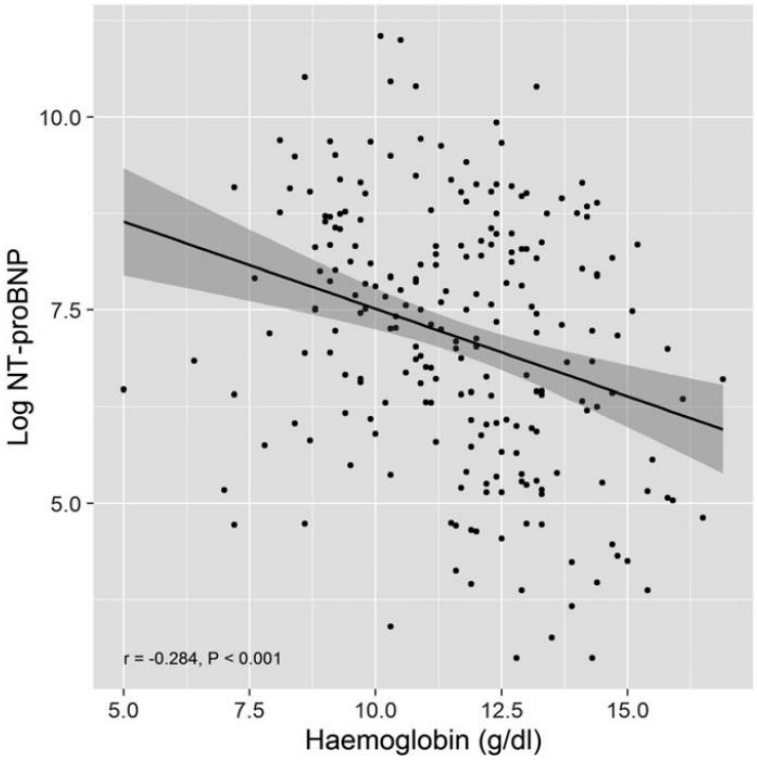

Figure 1. Correlation between hemoglobin and N-terminal pro b-type natriuretic peptide.

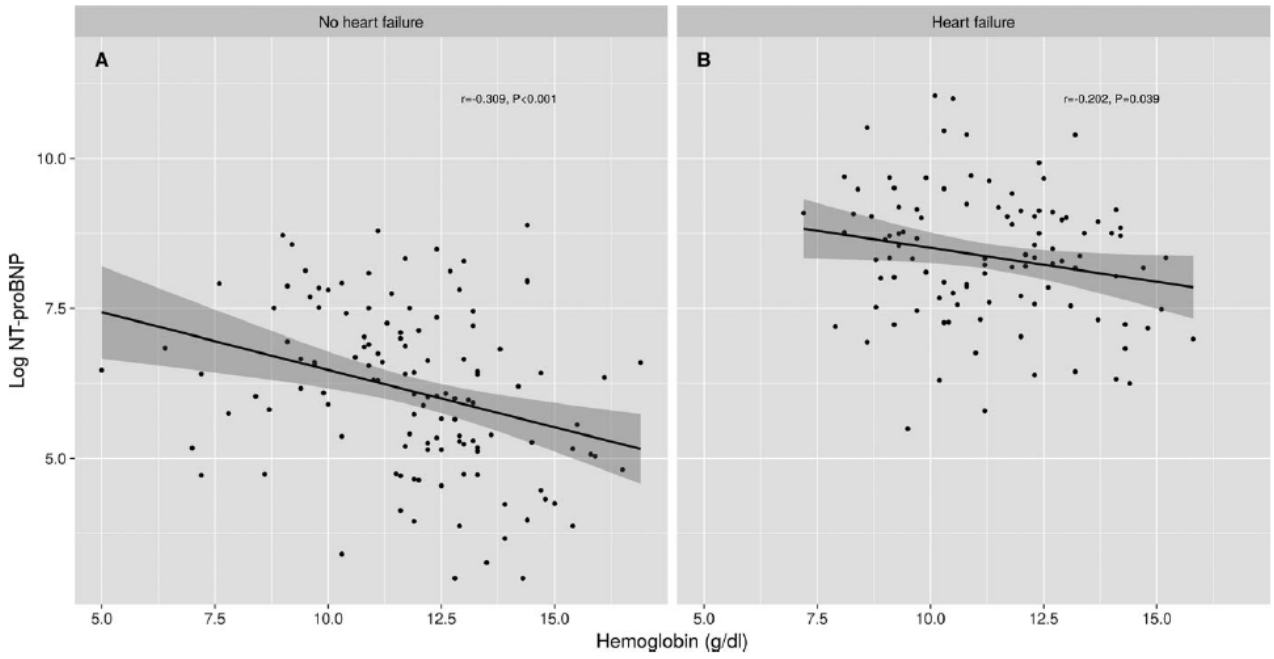

Figure 2. Correlation between hemoglobin and N-terminal pro b-type natriuretic peptide stratified for heart failure.

The unadjusted linear regression coefficient was -0.226 (P<0.001) in the total sample, -0.114 (P=0.039) in the HF subsample, and -0.191 (P<0.001) in the non-HF subsample. After adjustment for potential confounders (age, sex, BMI, eGFR, peripheral oxygen saturation, COPD, ischemic heart disease, atrial fibrillation, diabetes, ejection fraction, MCV), the inverse association between hemoglobin and NT-proBNP was confirmed, with a regression coefficient of -0.182 (P<0.001). After stratification for diagnosis of HF, the relationship was evident in both subsamples, with a regression coefficient of -0.136 (P=0.007) in the HF subsample and -0.165 (P=0.003) in the subsample without diagnosis of HF (Table 3).

Table 3. Adjusted Regression Linear Models

	No Heart Failure	Heart Failure	All Participants
	β (*P*-Value)		
Hemoglobin, g/dL	−0.165 (.003)	−0.136 (.007)	−0.182 (<.001)
Age	0.039 (.06)	0.034 (.02)	0.041 (.002)
Sex	−0.188 (.44)	0.028 (.90)	−0.159 (.36)
BMI, kg/m^2	−0.034 (.15)	−0.029 (.15)	−0.042 (.002)
Estimated glomerular filtration rate, mL/min per 1.73 m^2	−0.01 (.14)	−0.009 (.04)	−0.014 (.001)
Peripheral oxygen saturation	−0.012 (.78)	−0.013 (.62)	−0.033 (.09)
COPD	−0.014 (.96)	−0.315 (.13)	0.027 (.88)
IHD	0.013 (.96)	0.091 (.67)	0.161 (.39)
Atrial fibrillation	0.426 (.20)	0.426 (.03)	0.811 (<.001)
Diabetes mellitus	0.27 (.34)	−0.167 (.45)	0.148 (.42)
Ejection fraction	−0.066 (.03)	−0.029 (.001)	−0.057 (<.001)
MCV	0.007 (.64)	0.001 (.93)	0.007 (.45)

Models adjusted for age, sex, body mass index (BMI), creatinine clearance, peripheral oxygen saturation, chronic obstructive pulmonary disease (COPD), ischemic heart disease (IHD), atrial fibrillation, diabetes mellitus, ejection fraction, mean corpuscular volume (MCV).

DISCUSSION

Our study showed an inverse relationship between hemoglobin and NT-proBNP concentrations in a sample of hospitalized older people, both in subjects with and without diagnosis of HF. The importance of this result lies in the fact that this is the first study focused on patients with age ≥65 years, which is the age group in which HF is most prevalent and in which the diagnosis of HF may be more dependent on natriuretic peptides concentration due to the presence of other diseases, such as COPD, that could mimic HF (21). To our knowledge, only two studies had a sample with a mean age of about 70 years (10,22), and none over 80 years old. These studies, however, did not separately report data on older participants and were focused only on patients affected by HF.

Our results are in line with those obtained in younger samples. For example, Willis et al., in a sample with a mean age 54 years, found an independent inverse correlation between hemoglobin and NT-proBNP; however, echocardiography was available only for a small part of the sample, and patients with kidney failure were excluded from the study (23). The inverse correlation between hemoglobin and natriuretic peptides was also evidenced in a chronic kidney failure population without diagnosis of HF, but it was not adjusted for potential confounders (24).

The correlation between hemoglobin and natriuretic peptides has been also documented in populations with HF (10), both systolic (22), and diastolic (25), although in the latter study the analysis was not adjusted for potential confounders. Only one study in the literature evaluated the correlation between hemoglobin and natriuretic peptides in a population presenting in emergency department for acute dyspnoea, stratifying by HF. This study showed an inverse correlation only in patients with diastolic HF and in male patients without diagnosis of HF (18). However, the population characteristics of this study, including age, were not available, and echocardiography was performed only for a few patients.

The correlation between hemoglobin and natriuretic peptides may be explained by increased cardiac output and wall stress, consequent to decreased vascular peripheral resistances and increased heart

rate and left ventricular contractility. These effects are related to adrenergic response to low hemoglobin levels and anemia-related peripheral hypoxia. Furthermore, anemia and consequent reduction of plasma volume determinate activation of renin-angiotensin-aldosterone system, which causes retention of salt and water, contributing to the increase of pre-load and wall stress (14). An alternative explanation of the association between NT-proBNP and hemoglobin concentration is that iron deficiency, which is relatively common in patients with HF, is related to the severity of HF because it can affect myoglobin function, even in patients without anemia (26,27). In our sample, however, MCV did not affect the association between haemoglobin and NT-proBNP.

Interestingly, we found a slightly stronger association between hemoglobin and NT-proBNP concentrations in the subsample without HF. This may be explained by an increased activation of the sympathetic nervous system in response to low hemoglobin concentration in this subsample compared to patients with HF. In patients with HF, the complex pathophysiological structural and functional modifications related to chronic neuro-hormonal and adrenergic activation may reduce the impact of reduced hemoglobin concentration on increased wall stress and cardiac output, and consequently reduce the correlation between hemoglobin and natriuretic peptides.

The strength of this study is that it is the first study focused on older people; furthermore, we did not used exclusion criteria, making our sample representative of "real life" older patients. Finally, this is the first study on older subjects that analysed the independent correlation between hemoglobin and NT-proBNP making a comparison between subjects with and without HF, and that has echocardiography data for all patients.

Our study also has some limitations: it is based on chart review, and has a relative small size. Furthermore, we used NT-proBNP and not BNP, which is most commonly used in the clinical practice.

In conclusion, in a real-life sample of hospitalized older patients with or without diagnosis of HF, hemoglobin is independently and inversely associated with NT-proBNP levels, especially in

participants without diagnosis of HF. Our results show that on average a decrease of 1 g of hemoglobin may translate in a 17% increase in NT-proBNP values; therefore hemoglobin values should be taken into account in the interpretation of NT-proBNP levels to reduce the risk of misclassification of elderly patients without HF.

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5. DISCUSSION

Older adults are a population that is generally less studied compared to younger people, because of their complexity, that may influence the studied associations: they are often characterized by multiple comorbidities, frailty, disability, and poly-therapy. Thus, most of the clinical trials exclude these patients or select only those without much comorbidities or not disable, that are not representative of a "real-life" elderly population. In this context, the role of nutrition in older adults still has many open questions: only few observational studies, usually including patients without strict exclusion criteria are available on this topic. All the studies reported in this thesis had not strict exclusion criteria, thus reporting results generalizable to the older adults in the same setting.

Beside confirming that nutrition has a primary role in the outcomes of older adults, my results also indicate that the role of nutrition and micronutrients may have a different significance in this population with respect to younger people. In fact, I documented that, in a sample of community dwelling older adults followed-up 9 years, high sodium intake, positively associated with negative outcomes in younger people, has not impact on cardiovascular events and mortality, while a reduced intake is associated with an increased mortality. This association was more evident in frail people. Thus, this study highlighted for the first time that in this population is very important to ensure an adequate sodium intake and to avoid a sodium restriction, especially in frail older adults. Similarly, PUFA intake is associated with reduced mortality in adults, while the results of the above reported study documented that in community dwelling older adults PUFA intake is not associated with mortality. This study was the first documenting that in older adults there is not association between PUFA intake and serum concentration, thus explaining the negative results of the only one previous study on this topic and opening new challenges on the biological mechanisms at the basis of the association between intake and serum concentration in this population, and on the appropriateness of PUFA supplementation in this population.

Furthermore, the above reported studies documented that nutritional status and micronutrients significantly influence outcomes in older adults: in a sample of elderly patients admitted to rehabilitation units, 25(OH)vitamin D concentration was non-linearly and positively associated with functional outcomes, with an association more evident for 25(OH)D concentration up to 16 ng/ml. The results of this study suggest that the established cut-off point for deficiency, derived from the concentration at which vitamin D influence bone metabolism, is probably not representative of the effects of vitamin D concentration on muscular function. Furthermore, this study highlighted that 25(OH)vitamin D serum concentration estimation might contribute evaluating the chance of functional recovery of older adults after an acute event or after an elective orthopedic surgery. Further researches are needed to confirm these results, and intervention studies should assess the impact of interventions directed to increasing 25(OH)D levels in case of insufficient values in this population.

Functional gain in rehabilitation setting is influenced not only by 25(OH)vitamin D concentration but also by nutritional status: analyzing data from the same cohort, nutritional status was positively associated with physical function, and, considering only patients admitted after an elective orthopedic hip or knee replacement surgery for arthritis, malnutrition and risk of malnutrition were associated with a worse functional gain with respect to well-nourished. This was the first study that analyzed the role of nutritional status in influencing functional outcomes in older adults after elective orthopedic surgery, showing the potential impact of a nutritional screening before elective surgery. A protocol for an observational study at Campus Bio-Medico Teaching Hospital has been submitted to the Ethical Committee of this Institution, to confirm and expand these results. If they will be confirmed, a nutritional screening before surgery will be indicated, and malnutrition should be treated before the surgery in order to prevent disability after an elective hip or knee replacement surgery.

Nutritional status has an important role also in older adults affected by chronic heart failure: my study on this topic documented that in these patients it is negatively associated with physical function, independently from heart failure severity, appendicular skeletal muscle mass and caloric intake. These results are of interest because Mini Nutritional Assessment, one of the most common tools for malnutrition screening in older adults, that was used for the assessment of nutritional status in this study, catches not only the effects of a reduced caloric intake but provides a multidimensional evaluation, that in older adults is more effective in predicting outcomes than caloric intake alone. In this study, risk of malnutrition was associated with a 1-year 303% increased risk of heart failure exacerbation not requiring hospitalization compared to well-nourished, independently from ejection fraction; it was an outcome never studied before. Our results suggest that an early assessment of nutritional status using the Mini Nutritional Assessment should be performed in older adults affected by heart failure to identify patients at higher risk of negative outcomes. Further studies are needed to confirm these results and intervention trials including not only nutritional supplementation but also a multidimensional approach for the treatment of malnutrition are desirable.

Nutrition is important not only for primary prevention and improving outcomes: some micronutrients have a role in the treatment of several diseases; for example, recent studies documented that curcumin, a component of the spice Turmeric, commonly used in Ayurveda, thanks to its anti-inflammatory, anti-oxidant, anti-fibrotic and anti-neoplastic properties, may have a role in treating several diseases, including pulmonary diseases and lung cancer. Furthermore, it modulates microRNAs, single-strand non-coding RNAs implicated in post-transcriptional regulation of gene expression, thus having an important role in cell differentiation, proliferation, growth, mobility, and apoptosis. Despite the growing evidence of the effectiveness of curcumin, no extensive reviews were available on this topic. Thus, I carried out a narrative review reporting the effects of curcumin on the most important lung diseases, analyzing the pathogenesis of such

diseases and, on this basis, describing the pathogenetic pathways modulated by curcumin. Furthermore, I summarized the available studies on the role of curcumin in treating lung cancer and melanoma by modulating microRNAs. Despite the large number of *in vitro* and animal *in vivo* studies, very few clinical trials, and with contrasting results, were performed in humans. The main drawback of curcumin as a pharmacological agent is its poor bioavailability after oral administration, due to low intestinal absorption, rapid hepatic and intestinal metabolism, and rapid elimination via the bile and excretion into the feces. Thus, curcumin adjuvants (that reduce its metabolism) or derivates (nanoparticles, liposomes, micelles, structural analogues, etc.), have been evaluated to enhance curcumin's efficacy in vivo. However, whether these approaches to optimize the pharmacological action of curcumin are effective in humans remains to be explored and additional studies are needed to assess the clinical effectiveness of both curcumin and its analogs in human lung diseases.

Finally, diagnosis of heart failure, a chronic disease with poor prognosis and a prevalence that increases with age, could be challenging in older adults, in which it is common the coexistence of other diseases, such as COPD or chronic kidney failure, that could mimic heart failure signs and symptoms. Thus, when echocardiography is not available, natriuretic peptides are the most helpful diagnostic exam for a correct diagnosis. In this context, nutrition may have a role in influencing the correct diagnosis of heart failure: heart failure is characterized by micronutrient deficiencies, that may cause anemia, condition highly prevalent in these patients. In my study I documented that hemoglobin was inversely associated with NT-proBNP concentration, especially in patients without diagnosis of HF. It was the first study analysing Therefore, we can conclude that hemoglobin values should be taken into account in the interpretation of NT-proBNP levels to reduce the risk of misclassification of elderly patients without HF.