UNIVERSITY OF PIEMONTE ORIENTALE

DEPARTMENT OF HEALTH SCIENCES

Ph.D. PROGRAM IN FOOD HEALTH AND LONGEVITY

- XXXV CYCLE –

Ph.D. THESIS:

Healthy lifestyle and MEDITERRANEAN-DIET: fighting against OBESITY for healthy

ageing and cancer prevention.

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SUMMARY

Obesity has nearly tripled since 1975 worldwide, with 39% of adults being overweight in 2016, while 13% were obese. Non-communicable diseases (NCDs)are responsible for 74% of all deaths globally, with cardiovascular diseases accounting for most NCD deaths, followed by cancers, chronic respiratory diseases and diabetes. All the NCDs are related to obesity and most of the risk factors recognized that can be considered preventable: tobacco use, physical inactivity, alcohol abuse and unhealthy diets.

The aim of this PhD project is to uncover some links between obesity or overweight and cancer, one of the leading causes for mortality among the NCDs, specifically focusing on breast cancer for the exploratory project of this thesis.

Healthy lifestyle, including both healthy diet and physical activity engagement, improve body composition and nutritional status at different levels and can be considered pivotal for cancer and metabolic disease prevention and treatment.

In 2010, the Mediterranean diet (MD) was recognized as an Intangible Cultural Heritage of Humanity by the United Nations Educational, Scientific and Cultural Organization (UNESCO). Before and since then, increasing evidence supported the MD as one of the most effective healthy pattern for the prevention of NCDs, raising the scientific attention to a cultural pattern synthetized graphically by an iconic Pyramid.

(**Figure from**: Serra-Majem L, Tomaino L, Dernini S, Berry EM, Lairon D, Ngo de la Cruz J, Bach-Faig A, Donini LM, Medina FX, Belahsen R, Piscopo S, Capone R, Aranceta-Bartrina J, La Vecchia C, Trichopoulou A. Updating the Mediterranean Diet Pyramid towards Sustainability: Focus on Environmental Concerns. Int J Environ Res Public Health. 2020 Nov 25;17(23):8758. doi: 10.3390/ijerph17238758. PMID: 33255721; PMCID: PMC7728084)

Additionally, Mediterranean lifestyle emphasises together with a healthy dietary pattern, regular physical exercise, which constitutes another key pillar for health promotion.Health-promoting effects of exercise remain poorly understood together with the mechanisms regulating cellular senescence, a state of irreversible growth arrest.

Long-term moderate calorie restriction and endurance exercise training can decelerate biological ageing, promoting visceral fat loss, reducing inflammation and oxidative stress, helping with prevention of cancer, particularly breast cancer.

Moreover, during the years of this project the pandemic outbreak of Sars-Cov2 gave the opportunity to show how obesity, adipose tissue excess and also muscle mass depletion can be important not only for NCDs prevention, but also in the fight against communicable disease.

In the end, aiming to fight obesity and cancer, we present the results on metabolic improvements after 2 years of weight management through the implementation of Mediterranean diet in breast cancer survivors, with an exploratory analysis of the dropouts to identify possible predictors that can interfere with a long-term compliance to weight loss protocols.

SOMMARIO

L'Obesità è quasi triplicata dal 1975 in tutto il mondo, infatti circa il 39% degli adulti erano sovrappeso nel 2016, mentre il 13% era obeso. Le malattie non trasmissibili (NCDs) sono responsabili del 74% delle morti globalmente, con le malattie cardiovascolari che causano la maggior parte dei decessi per NCDs, seguite dal cancro, dalle malattie respiratorie croniche e dal diabete. Tutte le NCDs sono correlate all'obesità e alla maggior parte dei fattori di rischio riconosciuti che possono essere considerati prevenibili: fumo, sedentarietà, abuso di alcool e diete poco sane.

Lo scopo del progetto di questo dottorato è di svelare alcune delle connessioni tra obesità o sovrappeso e cancro, una delle principali cause di mortalità tra le NCDs, con un focus specifico sul carcinoma mammario per il progetto sperimentale della tesi.

Uno stile di vita sano, che include sia una dieta sana ed equilibrata che una attività fisica costante, migliora la composizione corporea e lo stato nutrizionale a differenti livelli, ponendosi in un punto centrale per la prevenzione e il trattamento del cancro e delle malattie metaboliche.

Nel 2010, la dieta Mediterranea (MD) è stata riconosciuta come Patrimonio Culturale Intangibile dell'Umanità dall' UNESCO (United Nations Educational, Scientific and Cultural Organization). Sin da allora e prima ancora, crescenti evidenze hanno supportato la MD come uno dei pattern alimentari più efficaci nella prevenzione delle NCDs,aumentando l'attenzione scientifica verso un pattern culturale che è stato sintetizzato graficamente con una iconica Piramide.

(**Figura da**: Serra-Majem L, Tomaino L, Dernini S, Berry EM, Lairon D, Ngo de la Cruz J, Bach-Faig A, Donini LM, Medina FX, Belahsen R, Piscopo S, Capone R, Aranceta-Bartrina J, La Vecchia C, Trichopoulou A. Updating the Mediterranean Diet Pyramid towards Sustainability: Focus on Environmental Concerns. Int J Environ Res Public Health. 2020 Nov 25;17(23):8758. doi: 10.3390/ijerph17238758. PMID: 33255721; PMCID: PMC7728084)

Inoltre, lo stile di vita Mediterraneoenfatizza, oltre ad un pattern alimentare salutare, un regolare esercizio fisico, che costituisce un altro pilastro per la promozione dello stato di salute. Gli effetti salutari dell'esercizio fisico rimangono ancora non completamente chiariti, come pure il meccanismo che regola la senescenza cellulare, uno stato di crescita irreversibile.

Una moderata restrizione calorica con esercizio fisico aerobico possono rallentare l'invecchiamento biologico, promuovendo la riduzione del grasso viscerale, riducendo l'infiammazione e lo stress ossidativo, supportando la prevenzione del cancro ed in particolare del carcinoma mammario.

In aggiunta, durante gli anni di svolgimento di questo Progetto, la pandemia da Sars-Cov2 ha dato l'opportunità di mostrare come ridurre l'obesità, l'eccesso di tessuto adiposo e la deplezione della

massa muscolare, possono essere strategie importanti non solo per la prevenzione delle NCDs, ma anche nella lotta alle malattie infettive.

In conclusione, con lo scopo di combattere obesità e cancro, vengono presentati I risultati del Progetto sullo studio dei miglioramenti metabolici in seguito a 2 anni di trattamento dietoterapico con Dieta Mediterranea nelle pazienti diagnosticate con carcinoma mammario al termine della terapia, con un'analisi esploratoria dei "dropout", al fine di identificare possibili predittori che possano interferire con una compliance a lungo termine a protocolli per il calo ponderale.

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GENERAL INTRODUCTION AND RATIONALE

1.1 THE IMPACT OF OBESITY ON HEALTH

Obesity has reached pandemic proportion both in Europe, with 39% of adults being overweight in 13% obese in 2016 (1), while in USA 42.4% of adults were obese and 9.2% were severely obese in 2018 (2). This significantly increasing trend of obesity, which has nearly tripled since 1975 worldwide, is alarming for the high risk to develop several chronic non-communicable diseases (NCDs), ultimately contributing to reduced survival.

Recently, obesity has been recognized as major risk factor for cancer, which is the second leading cause of death for NCDs, accounting for 9 million of people annually, and constituting over 80% of premature deaths for NCDs together with cardiovascular diseases (17.9 million), respiratory diseases (3.9million), and diabetes (1.6 million), globally. Obesity is a comorbidity related to all of these 4 groups of diseases and is recognized as one important modifiable risk factor for NCDs, together with tobacco use, physical inactivity, alcohol abuse (1).

Obesity and overweight are characterised by excessive body fat deposition, which promotes a pro-oxidative microenvironment with chronic low-grade systemic inflammation, increasing the risk of metabolic and cardiovascular diseases, and ultimately all-cause mortality. Healthy dietary patterns, weight loss intervention, and reduction of abdominal adiposity, instead, are related to a lower risk of cancer recurrence and all-cause mortality [3].The dysfunction of visceral fat may be reversed by weight loss, improving those factors contributing to the development of cancer such as insulin resistance, pro-inflammatory molecules and metabolites, circulating growth factors hormones and adipokines (4-6).

The third expert report on "Diet, nutrition, physical activity and breast cancer" of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) [dietandcancerreport.org] for preventing cancer generally recommends maintaining a healthy lifestyle by being physically active and eating a healthy diet for proper weight management [7].

1.2. AIMS OF THE Ph.D. PROJECT

The relation between overweight/obesity and cancer is still not fully clarified. Due to the high prevalence of obesity and the increasing prevalence of cancer, the aim of the project was to unravel possible interconnections between these two highly mortal diseases. Modifying behavioural factors to prevent obesity, can also have beneficial effects on obesity-related comorbidities, and as a consequence can slow cellular senescence with the ultimate aim of cancer prevention and healthy ageing.

During the years of this project, in 2020, Sars-Cov2 pandemic outbreak gave the opportunity to show how obesity, adipose tissue excess and also muscle mass depletion have a strong impact not only on NCDs prevention, but also in the field of communicable diseases. Evidence accumulated during the COVID-19 pandemic on obesity and the importance of a healthy body composition are reported in _Chapter 2._

The metabolic impairments and chronic low-grade systemic inflammation in the obese state sustains a pro-inflammatory microenvironment, increasing cytokine production and risk of cytokine storm during Sars-Cov2 sepsis or other secondary infections. Moreover, an impaired immune system and altered response to viral infection in obesity lead to a greater susceptibility to infections, longer viral shedding and greater duration of illness and severity of disease. High mortality and worse clinical outcomes were shown in patients with sarcopenic obesity, a state of coexistence of excessive fat mass and low mass and function of muscle mass (8).

Given the increase in prevalence of obesity worldwide, it is now important to recognize it as a preventable risk factor for cancer, estimated to be responsible for ~14% and ~20% of all cancer-related deaths in men and women, respectively (9). Excessive expansion of visceral adipose tissue contributes to a tumour microenvironment exerting both local and systemic _stimuli_ with insulin resistance and altered hormonal pathways promoting tumour growth, progression and infiltration(10).

Weight management and physical activity have significantly been proven to be protective factors for most types of cancer(11). Positive effects of exercise on weight loss and hypothesis on its effects on slowing cellular senescence are reported in _Chapter 3._

Our results are preliminary and limited, colon mucosa cells were selected as model of senescence phenotypes, being highly proliferating cancer prone tissues easy to collect. Our results suggest that

endurance exercise training is more powerful than caloric restriction to counteract the age-dependent deterioration of cellular epigenetic homeostasis and genomic stability.

Eventually, evidence is available on the impact of obesity in breast cancer (10-12) and on the preventative effects of the Mediterranean diet (MD) on NCDs (13-14). In *Chapter 4* we aim to explore how long-term regulation of various metabolic, inflammatory and ageing pathways could ultimately improve prognosis among breast cancer survivors (BCS).

Improvements in body composition by weight loss may reverse visceral fat dysfunction, protecting from development and recurrence of cancer. After reviewing current literature on healthy eating patterns, we identified MD as one of the most effective dietary patterns, exerting beneficial effects on metabolic parameters for cancer prevention and other obesity-related NCDs (13-14). At the same time, we noticed how there is a lack of evidence on the secondary prevention for all types of cancer. Therefore, being breast cancer (BC) the most common cancer in women, and being BC survivors a cohort of women showing a 30% excess risk for second malignancies (12), we focused our attention on the BC field. Indeed, overweight or obesity are the second preventable cause of cancer, related to 33% of postmenopausal breast cancers that could be prevented by lifestyle modification.

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2. **COVID-19 and the importance of a healthy body composition**

Obesity increases the risk to develop several NCDs, ultimately contributing to reduced survival. Recently, obesity has been recognized as a major risk factor for coronavirus disease-19 (COVID-19), contributing to worse outcomes in those with established COVID-19 (1-2).

Metabolic syndrome and excessive fat accumulation might impair the immune system efficiency, causing a chronic low-grade systemic hyperinflammatory state (3-4). Moreover, an impaired immune system with altered response to viral infection in obesity, for both innate and adaptive immune response, lead to an increased susceptibility to infections, longer viral shedding and greater duration of illness and severity of disease.

Obesity state shows a cluster of features that could explain the severity of disease presentation and worse outcomes: metabolic imbalance such as hyperinsulinemia and hyperleptinemia; immune system dysfunction, with impaired responses of B and T cell; a pro-inflammatory microenvironment characterised by altered production of adipokines (adipose-tissue derived cytokines), and inflammatory cytokines with high level of interleukin (IL)-6 and tumour necrosis factor-α (TNF-α). These features might also explain how COVID-19 cytokine storm causes septic shock during severe sepsis, and eventually death in the setting of obesity [5].

High mortality and worse clinical outcomes were shown in patients with sarcopenic obesity, a state of coexistence of excessive fat mass and low mass and function of muscle mass (6). Impaired body composition (fat accumulation and lean mass loss) plays a pivotal role in the response to the infection both on the short term, for immunological and inflammatory reaction to the virus, metabolic response and respiratory distress, and on the long-term outcomes affecting time required for a complete recovery, long-term disabilities, and eventually risk of death.

In adults with obesity, excessive fat expansion at visceral, epicardial and liver regions, constitutes increased risk of severe COVID-19 even in subjects younger than 40 years old (7-8).

In the end, acute state of inflammation and bed rest contribute to further loss of lean body mass, for increased energy and protein requirements (9). Remarkably, inactivity negatively impacts on muscular, cardiovascular, metabolic, endocrine and nervous systems, especially in subjects with sarcopenic obesity, by overlapping of chronic low-grade with acute-state inflammation, and aggravating lean mass catabolism (10).

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2.1 Obesity pandemic during COVID-19 outbreak: Narrative review and future considerations

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ABSTRACT

The high prevalence of obesity and obesity-related comorbidities has reached pandemic proportions, particularly in Western countries. Obesity increases the risk to develop several chronic noncommunicable disease, ultimately contributing to reduced survival. Recently, obesity has been recognized as a major risk factor for coronavirus disease-19 (COVID-19)-related prognosis, contributing to worse outcomes in those with established COVID-19. Particularly, obesity has been associated with higher hospitalisation rates in acute or intensive care and greater risk for invasive mechanical ventilation than lean people.

Obesity is characterised by metabolic impairments and chronic low-grade systemic inflammation that causes a pro-inflammatory microenvironment, further aggravating the cytokine production and risk of cytokine storm response during Sars-Cov2 sepsis or other secondary infections. Moreover, the metabolic dysregulations are closely related to an impaired immune system and altered response to viral infection that can ultimately lead to a greater susceptibility to infections, longer viral shedding and greater duration of illness and severity of the disease.

In individuals with obesity, maintaining a healthy diet, remaining physically active and reducing sedentary behaviours are particularly important during COVID-19-related quarantine to reduce metabolic and immune impairments. Moreover, such strategies are of utmost importance to reduce the risk for sarcopenia and sarcopenic obesity, and to prevent a reduction and potentially even increase cardiorespiratory fitness, a well-known independent risk factor for cardiovascular and metabolic diseases and recently found to be a risk factor also for hospitalizations secondary to COVID-19. Such lifestyle strategies may ultimately reduce morbility and mortality in patients with infectious disease, especially in those with concomitant obesity.

The aim of this review is to discuss how obesity might increase the risk of COVID-19 and potentially affect its prognosis once COVID-19 is diagnosed. We therefore advocate for implementation of strategies aimed at preventing obesity in the first place, but also to minimise

the metabolic anomalies that may lead to a compromised immune response and chronic low-grade systemic inflammation, especially in patients with COVID-19.

Graphical Abstract : Factors associated with increased severity and risk of death for COVID-19 in obesity.

INTRODUCTION

In 2020 two pandemics collided: obesity, a chronic noncommunicable disease, and the corona virus disease (COVID-19), a pandemic infection caused by the virus Sars-CoV-2. Obesity and COVID-19 often share similar comorbidities, namely metabolic, cardiovascular or pulmonary. Indeed, hypertension, diabetes mellitus (DM) and chronic obstructive pulmonary disease (COPD) are highly prevalent in patients hospitalized for COVID-19. Importantly, patients affected by one of more of the above listed conditions are more likely to require invasive mechanical ventilation (IMV) in intensive care unit (ICU). In addition, clinical management of individuals with obesity during hospitalization, intubation, mechanical ventilation, imaging, positioning and nursing in general can be difficult, and those difficulties are further augmented during COVID-19, with overloaded acute and intensive care units.

Before Sars-CoV-2, obesity was constantly increasing, reaching a worldwide prevalence of 11% for men and 15% for women. In Europe and USA, which have been strongly hit by COVID-19, however, obesity reaches 25.3% (WHO data) and 42.4%, respectively [1]. Age was initially considered the major risk factor for reduced survival in patients with severe COVID-19 due to their higher likelihood of having aging-associated comorbidities, such as DM, hypertension, obstructive sleep apnea syndrome (OSAS) or respiratory conditions, without including obesity to this list [2]. However, obesity has been now recognized as a major risk factor for worse prognosis, independent of age, proposing it as therapeutic target in patients with COVID-19.

METHODS

We have searched for "BMI" OR "Body Mass Index" OR "Obesity", AND "COVID-19" OR "SARS-Cov-2" OR "coronavirus disease" on database as PubMed, Google Scholar, MEDLINE, EMBASE, Scopus. With the same keywords, we have also used a dedicated tool in Pubmed: "LitCovid " (https://www.ncbi.nlm.nih.gov/research/coronavirus/). We have excluded preprint articles published on Medrixv and BioRixv, as the scope of our review is narrative and not a

systematic revision of the published literature that, at this time, would be incomplete while the global pandemic is still affecting many countries and data are rapidly changing.

We included publications reporting data on obesity (e.g., prevalence) or association between BMI and clinically relevant data such as mortality, severity and outcomes of disease among laboratory confirmed COVID-19 subjects.

Obesity and COVID-19

Obesity and severe obesity are associated with a greater severity of the disease and related risk of hospitalization, worse clinical surrogate outcomes, such as lower SaO2 and PaO2 requiring IMV, longer length of hospital stay and longer time to achieve oxygen weaning [[3], [4], [5], [6], [7], [8], [9], [10], [11]].

Early data from China [12,13] showed that frequent comorbidities in patients diagnosed with COVID-19 were hypertension, DM, COPD, and coronary heart disease, without details on body mass index (BMI) [14]. Additional data including BMI became available and clearly showed that obesity was not only highly prevalent, but also associated with reduced survival. Data reported by COVID-19–Associated Hospitalization Surveillance Network (COVID-NET) show that almost 90% of patients with COVID-19 hospitalized for 1 month had at least one or more underlying conditions, being hypertension the most common (49.7%), closely followed by obesity (48.3%), chronic lung disease (34.6%), DM (28.3%), and cardiovascular disease (27.8%). In the age range 18–64 years old obesity was the most prevalent comorbidity, and within those aged 50–64 years obesity was even more prevalent than hypertension and DM [15].

In **Fig. 1** COVID-NET data are shown up to December 31, 2020 (Fig .1, a-b).
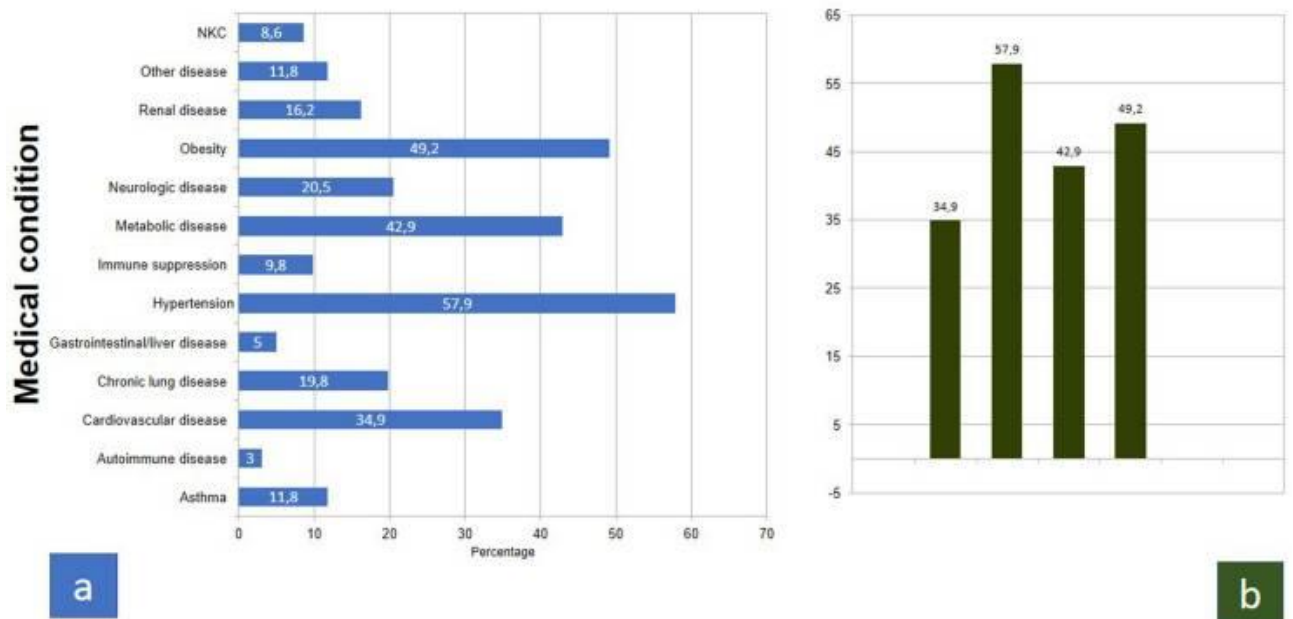
Weekly updated Data from COVID-NET [16] as of December 31, 2020, a) prevalence of medical conditions in hospitalized patients; b) focus on the most prevalent (>30%) conditions (same data showed in a).

Similar findings were confirmed in cluster of specific geographic area: Richardson reports the most common comorbidities being hypertension 56.6%, obesity 41.7%, and diabetes 33.8% in 5700 subjects hospitalized for COVID-19 in New York City (NYC) area within a month [17]. Consistently, data collected during the month of March in NYC by Petrilli's group in more than 5279 patients, confirm that age >44 years and morbid obesity (BMI ≥40 kg/m2) were strong predictors for hospitalization. Specifically, in those 75 years or older, odds ratio (OR) for hospitalization was

37.9 (95% CI, 26.1–56); age 65–74 years OR was 8.7 (95% CI, 8.7–11), and BMI ≥40 kg/m2 OR was 2.5 (95% CI, 1.8–3.4) [18].

Another report by Lighter et al. highlights the major impact of obesity in the age group younger than 60 years, in a cohort from NYC positive for COVID-19, that was fairly representative of USA population [19], with 21% of the total cohort having a BMI 30–34 kg/m2, and 16% BMI ≥35 kg/m2. Patients <60 years of age and with a BMI between 30 and 34 kg/m2 were two times more likely to be admitted to acute or critical care, while patients with a BMI ≥35 kg/m2 and aged <60 years were 2.2 times more likely to be admitted to acute care and 3 times to ICU, compared to individuals without obesity. Subjects <60 years of age in the general population without obesity were believed to be a lower risk group for COVID-19 with a more favorable prognosis. However, especially in Western countries characterized by the high prevalence of obesity, Sars-Cov-2 is spreading rapidly among the population. Obesity has been, in fact, confirmed as an independent risk factor even in younger individuals in other reports: one from an American cohort from California in which a higher level of care was required by patients with obesity (OR 2.0, P = 0.021) [4]; and one in which BMI >30 was significantly associated with higher risk for mortality, IMV and hospital admission (OR 95%CI of 6.29, 6.01, 2.61) [5].

Importantly, in a French retrospective analysis of patients admitted to ICU for SARS-CoV-2 infection, a greater BMI was positively associated with the severity of COVID-19, with nearly 90% of patients with class II obesity or greater (BMI ≥35 kg/m2) requiring IMV. The relation of obesity with SARS-CoV-2 was confirmed by using an historical ICU control group admitted for non-SARS-CoV-2 severe acute respiratory syndrome in the previous year [20]. Noteworthy, another French study reports a lower prevalence of obesity in ICU and less people requiring IMV, explaining that this difference can be due to a lower prevalence of obesity in that specific area, which was lower than a half compared to the population of the previously discussed study. Even if the overall prevalence of obesity was lower, however, in those with severe obesity the risk for IMV remained significantly greater than in the leaner counterparts (81.8% vs 41.9%, respectively) [21].

A recent analysis investigated the association between COVID-19 and metabolic-associated fatty liver disease (MAFLD) in a Chinese group of patients with obesity. Individuals with severe obesity and MALFD patients had more severe COVID-19 disease, however, the presence of obesity in those with MAFLD was associated with approximately a 6-fold increased risk of severe COVID-19. Remarkably, almost 90% of those with severe illness had obesity, compared with a lower prevalence of obesity (57%) in non-severe COVID-19. The association of obesity and COVID-19 severity remained significant, even after statistical adjustments for age, sex, smoking, DM, hypertension, and dyslipidemia [22].

In another Chinese report, Peng et al. described a statistically higher BMI in cardiovascular disease patients with a severe form of COVID-19 infection (27.0 ± 2.5 versus 22.0 ± 1.3, respectively). Furthermore, among the non-survivors, 88.2% of patients had a BMI >25 kg/m2, significantly higher than survivors (18.9%) [23].

Data available from Italy, to date, report a lower prevalence of obesity among deceased individuals, however, the overall age of Italian patients diagnosed with COVID-19 tended to be higher compared to other countries. Table 1 lists the pre-existing conditions (with a prevalence >10%) in the 27,955 deceased Italian people who tested positive for Sars-CoV-2, accessed at the institutional website on the 7th of May, 2020. Among this group, the mean number of comorbidities was 3.4 ± 1.9. Specifically, 3.9% had no comorbidity, 15% reported 1 comorbidity, 21.3% had 2 and 59.9% 3 or more comorbidities.

Table 1

Prevalence of comorbidities in the Italian 35,563 deceased people positive for Sars-CoV-2, accessed   at https://www.epicentro.iss.it/en/coronavirus/bollettino/Report-COVID-2019_1_march_2021.pdf [24] on the 1st of March, 2021. Modified selecting disease with a prevalence >10%.

	Women (43.9%)	Men (56.1%)	Total (n = 96141)
Mean Age	86 years	80 years	83 years
Hypertension	67.9%	64.1%	65.7%
Ischemic heart disease	23.3%	31.1%	27.9%
Atrial fibrillation	25.5%	23.4%	24.3%
Heart failure	17.6%	14.2%	15.9%
Stroke	12.6%	10.9%	11.6%
Type-2 Diabetes	27%	30.8%	29.3%
Dementia	32.4%	17.7%	23.6%
COPD	14.1%	19.4%	17.3%
Active cancer in last < 5 years	15.1%	17.7%	16.7%
Chronic kidney disease	19.8%	22.2%	21.2%
Obesity	10.9%	11.1%	11%

Legend: COPD chronic obstructive pulmonary disease.

Moreover, Table 2 summarizes the most prevalent pre-existing conditions across some countries.

Table 2

A comparison of preexisting medical conditions between countries with the highest prevalence COVID-19 (China, USA, Italy, and France).

Table 2
A comparison of preexisting medical conditions between countries with the highest prevalence COVID-19 (China, USA, Italy, and France).

	Median Age (years)	Obesity (%)	Diabetes (%)	Hypertension (%)	CVD (%)	Lung-disease (%)
China (Guan et al. 12)	47 (58.1%M)					
Tot			7.4	15	2.5	1.1
Non-severe			- 5.7	- 13.4	- 1.8	- 0.6
Severe			- 16.2	- 23.7	- 5.8	- 3.5
COVID-NET (16, up to August 29th)		47.9	41.5	56.7	32.5	18.7
COVID-NET [15]	Overall	48.3	28.3	49.7	27.8	34.6
	- 50-64	- 49	- 32.1	- 47.4	- 19.6	- 28.3
	- >65	- 41	- 31.3	- 72.6	- 50.8	- 38.7
NYC (Richardson et al., 17)	63 (60.3%M)	41.7	33.8	56.6	18	17.3
NYC (Petrilli et al., 18) - tot		35.3	22.6	42.7	52.1	14.9
Hospitalized	54 (49.5%M)	39.5	34.7	62	70	16.5
Non hospitalized	(61.2%M)	30.8	9.7	21.9	32.2	13.1
NYC (Cummings et al.,3)	62 (67% M)	46	36	63		
France (Simonnet et al., 20)	60 (73%M)	BMI 29.6 kg/m^2				
IMV		31.1 kg/m^2	- 27	- 56		
Non-IMV		27 kg/m^2	- 13	- 32		
China (Zheng et al., 22), tot	47		24.2	28.8		
		with obesity	- 31.1	- 35.6		
		without obesity	- 9.5	- 14.3		
Italy (ISS, 24)*	80 (57.6%M)	10.4	29.5	65.8	44	17.1

All data are in %, except age in years.
Legend: *Refers to data of deceased population positive for Sars-CoV-2; NYC New York City, CHD coronary heart disease, **IMV** invasive mechanical ventilation.

2.1.1. Obesity and susceptibility to viral infections: the basis for a more severe COVID-19 SARS-CoV-2 binds to the receptor angiotensin converting enzyme 2 (ACE-2) to enter the cells. The expression of this receptor in particular tissues, including adipose tissue and lungs, is increased in obesity, in relation to leptin resistance and upregulation of SOCS-3 (suppressor of cytokine signaling-3), a gene involved in regulation of inflammation and inhibitor of leptin signalling. At the same time, SARS-CoV-2 affects the expression of genes related to lipid metabolism in epithelial cells, having a possible role in white fat differentiation. These pathways suggest that individuals with obesity may have a higher susceptibility to Sars-CoV-2 infection, and it could potentially also explain, at least in part, the increased risk for severe complications once COVID-19 is diagnosed [25,26].

BMI positively correlates with infectious virus shedding in aerosol of cases affected by influenza virus, and vaccination coverage is less efficient in individuals with obesity [27]. Moreover,

patients with obesity show a more prolonged duration of illness as well as a greater risk for severe influenza-like illness and higher respiratory mortality during previous pandemic of viral infections, consistently with recent data on COVID-19-related mortality. This was confirmed by a large body of evidence, which has reported an impaired immune response to influenza or influenza-like viruses in obesity. Notably, subjects with obesity shed influenza A virus for a longer period of time than subjects without obesity, having a viral shedding 42% longer when symptomatic and up to 104% longer in asymptomatic or pauci-symptomatic subjects [28]. Therefore, obesity plays a crucial role in viral transmission, significantly increasing the chance to spread influenza and influenza-like diseases in countries where the prevalence of obesity is high.

Metabolic syndrome might impair the immune system efficiency, causing a chronic hyperinflammatory state in obesity that could explain these data [29,30].

Immune system dysfunction, caused by a chronic low-grade systemic inflammation in metabolic conditions such as hyperinsulinemia and hyperleptinemia, increases vulnerability to infections altering both the innate and the adaptive immune response [29,30]. Indeed, the impaired responses of B and T cell in obesity may cause an increased susceptibility and possibly a delayed resolution of viral infections [30]. Excess adipose tissue can promote this pro-inflammatory microenvironment characterized by the production of adipokines (adipose-tissue derived cytokines), with increased leptin and reduced adiponectin. This chronic imbalance between high leptin, with well-known pro-inflammatory features, and low adiponectin, an adipokine with anti-inflammatory properties, induces macrophage production of high level of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), possibly triggering and even worsening the COVID-19 inflammatory cytokine storm [30,31]. The adipokine imbalance and the lack of the adiponectin negative feedback, might also explain how the cytokine storm causes septic shock during severe sepsis, and eventually death in the setting of obesity [31].

In addition to immune and metabolic impairments, pulmonary ventilation and gas exchange in obesity may be compromised as a result of a reduced diaphragmatic excursion and relative

increase in anatomical death space. COPD and OSAS are common comorbidities in obesity, and hypoxia may aggravate the pro-inflammatory state described above. Accordingly, Petrilli et al. highlighted that the degree of oxygen impairment and markers of inflammation were the strongest predictors of poor outcomes during COVID-19 hospitalization [18]. An additional condition recognized in COVID-19 that is also characteristic of obesity, is the increased risk for thromboembolism, resulting in a further increased risk for mortality [32].

Physical activity, cardiorespiratory fitness and immune system

Physical activity (PA) plays a key role in our mental and physical health. According to the World Health Organization (WHO), globally, 1 adult in 4 is not active enough and more than 80% of the adolescent population is insufficiently physically active. Insufficient PA leads to high risk of sarcopenia and reduced cardiorespiratory fitness, can cause noncommunicable disease and depression [33], as well as increasing the risk for weight gain (https://www.who.int/news-room/fact-sheets/detail/physical-activity). Of note, these conditions have been shown to correlate with a higher incidence of complications [34] and death during COVID-19 infection (https://www.epicentro.iss.it/en/coronavirus/sars-cov-2-analysis-of-deaths). Even children are at risk of weight gain for social distancing and stay-at-home requirements, with reduced opportunities of PA and increased sedentary behaviors [35].

Regular PA is a simple and effective way to deal with stress and frustration, and bad quality of sleep, especially during the current COVID-19 lockdown [36,37]. PA exerts positive effects on insulin resistance and immune response by inhibiting inflammatory cytokines pathway and macrophage activation [38-40] and by modulating inflammation and improving vaccination outcomes in the elderly [41]. In addition, PA can enhance antioxidant defense and reduce oxidative stress [30], acting as a strong non-pharmacological immunomodulatory intervention and as modifier of the adipokines imbalance. Therefore, the immune system is affected by regular exercise with a clear inverse relationship between moderate exercise training and illness risk [42].

Although the lockdown is the principal strategy to contain the virus spread, it can promote sedentary behaviors, reducing regular PA and energy expenditure and increasing the risk of potential worsening of chronic health conditions and sarcopenia, potentially leading to worse outcomes in COVID-19 infections [13]. Therefore, it is strongly recommended to maintain appropriate level of PA, even at home during quarantine also to counteract the loss of muscle mass and muscle functionality and therefore the risk for sarcopenia, and to maintain adequate immune system functions during such a current difficult period [43,44]. It is possible to improve cardiorespiratory fitness at home by performing safe, simple, and easy exercises, as stretching and strengthening exercises, activities for balance and control, or a combination of them (e.g. walking in the house, alternating leg lunges, stair climbing, stand-to-sit and sit-to-stand using a chair and from the floor, chair squats, and sit-ups and pushups). The use of eHealth and exercise videos to encourage and deliver PA through the Internet, mobile technologies, and television are other strategies [45,46]. In children, the schools should plan physical education classes delivered through video to promote PA and possibly improve cardiorespiratory fitness even while staying at home [35]. Finding a personal program to follow a proper PA would be particularly important during quarantine with stay-at-home requirement, when the psychological and physical burden can be heavy, especially in inactive or sedentary subjects.

At last, metabolic improvement, reduced systemic inflammation and improved immune system achieved through exercise training, in subjects with obesity even in absence of weight loss, may reduce morbidity and mortality during influenza and influenza-like disease [47]. To maintain appropriate level of cardiorespiratory fitness, mental health, muscle mass and thus energy expenditure and body composition, Barazzoni et al. recommend to engage in exercise every day >30 min, or every other day > 1 h [48,49].

Psychoneuroimmune implication of COVID-19

The COVID-19 pandemic affects also psychological well-being, triggering a wide range of psychological problems, such as panic disorder, anxiety and depression.

Researchers at Shanghai Mental Health Center developed a Covid-19 Peritraumatic Distress Index (CPDI), a national online survey to assess Covid-19 distress level. Starting from January 31st 2020, this first nationwide large-scale survey of psychological distress in the general population of China collected 52,730 valid questionnaires by 35.27% males and 64.73% females. Almost 35% of them experienced psychological distress (50,364). In Italy numerous studies are currently ongoing (https://ec.europa.eu/eusurvey/runner/COVIDSurvey2020).

Multiple studies on psychoneuroimmunology describe how emotional distress through the nervous system can impair immunity and influence recovery from diseases [50]. Quarantined positive COVID-19 subjects may be at greater risk for depression, fear, guilt and anger related to the disease itself; however, even individuals without COVID-19 can be psychologically affected by the pandemic. The dramatic increase of public fears and decrease in social and economic activities may, in fact, trigger psychosocial distress [51]. Subjects with obesity who are already stigmatized, during self-quarantine and social distancing are, in fact, experiencing higher rates of depression [52]. Moreover, the awareness of the high risk for severe complications of COVID-19 in obesity can increase the psychological burden. Psychosocial distress in obesity can be due to multiple reasons, such as quarantine, duration of social and family distancing, social media reports, fear of infection, provision of protective-supplies, financial crisis, and stigma [49].

To reduce the negative impact of these stressors on mental health, different strategies can be implemented, including optimization of remote clinical mental support using telemedicine, promoting virtual connections with family and friends to facilitate social relationships and emotional support, and promoting entertainment activities (e.g., books, games, indoor hobbies and physical activity, phones, internet access), but also reinforcing the concept that personal commitment in social distancing and quarantine are useful strategies to reduce the risks related to Sars-Cov2 infection [53]. Finally, psychological support should be routinely implemented to enhance psychological resilience and to eventually improve psychoneuroimmunity during COVID-19 [51].

Dietary quality and vitamin supplementation

Some vitamins and micronutrients are known to have a role in the immune system and subjects with obesity are often in deficiency or insufficiency states due to unhealthy diets. Subjects with obesity often show low blood levels of Vitamin D, that is recognized to exert positive functions on inflammation and to protect against respiratory tract infection [54].

Many other micronutrients are involved metabolic and immunological pathways, therefore an assessment of trace element and vitamins such as Iron, Zinc, Selenium, Vitamin A, E, B6 and B12 might be indicated to tailor medical nutrition therapy and the related need for dietary supplementations [48]. A special attention to protein and energy intake should aim to prevent or even treat sarcopenic obesity, especially in those subjects discharged after hospitalization or in any older obese patients. Essential aminoacid supplementation may support nutritional management, if required. Importantly, reducing the consumption of foods with pro-inflammatory properties, such as added sugars and saturated fats, and possibly increasing the consumption of foods rich in unsaturated fatty acids with well-known anti-inflammatory properties, might represent a useful strategy [54].

Study limitations

Our manuscript is not without limitations, particularly it analyzed reports with a wide variability of data, including national reports on deceased subjects, as in the case of Italy [15], but also observational studies from different countries including outcomes on both hospitalized and not-hospitalized patients. Moreover, severity of disease was differently classified, as per requirement of IMV. Notably, most publications on COVID-19 at the beginning of the pandemic did not mention obesity among comorbidities. It is not clear if this missing data was less relevant at the beginning of the outbreak, or obesity relevance was just reported later during COVID-19 pandemic.

Future analysis will be necessary to compare multiple data on COVID-19 outbreak from different countries and health systems in subjects with obesity and identify the most appropriate therapeutic strategy in this population.

CONCLUSION

Taken together, this review highlights the importance of prevention of obesity in the first place. Subjects with obesity may, in fact, need to extend the quarantine period and to take extra-precautions during viral pandemic such as COVID-19, adopting preventive measures such as social distancing, appropriate hygiene, and wearing face masks in public settings. More importantly, the best practice to prevent worse outcomes and to lower the mortality of acute and chronic diseases would be to lower the burden of obesity, not only during the state of emergency, but as prevention in possible future viral pandemics.

Finally, the nutritional management after hospitalization for COVID-19 patients, especially after ICU stay or intubation, represents a major opportunity to improve post-discharge quality of life, and this remains challenging in patients with obesity. Hospitalization-related or disease-related complications may have worse outcome in obesity after a long inpatient stay, such as dysphagia, loss of muscle mass and function (sarcopenia and sarcopenic obesity) [55] and impaired mobility, or secondary infections.

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2.2　　　　　　　　COVID19 and nutrition:
"COVID pandemic and short- and long- term alterations of body composition"

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ABSTRACT

COVID-19 can occur in a range of presentation from asymptomatic to severe forms. Among the major risk factors for worse severity, malnutrition for excess or undernutrition and body composition play a role in the response to the infection. Fat accumulation and lean mass loss can affect whole-body functioning: on the short term, susceptibility and immunological response to the virus, inflammatory reaction, metabolic and respiratory distress, and on the long term disease outcomes such as LOS, time required for a complete recovery, risk of ICUAW and long-term disabilities, or eventually risk of death.

This manuscript aims to review evidence collected during COVID-19 pandemic, and to provide information on the impact of body composition on severity and outcomes of the disease both on short and long term, analysing methods used for body composition assessment. Malnutrition screening tools will be discussed to screen, diagnose, and treat those group of patients at higher risk of COVID-19 severe presentation, worse outcomes, and long-term disabilities or even death.

Subjects with malnutrition, sarcopenia, obesity and/or sarcopenic obesity, and elderly with impaired body composition or risk of malnutrition require tailored MNT to improve outcomes of the critical illness and to prevent the risk of developing chronic impairments.

KEYWORDS

"Coronavirus disease 2019" (COVID-19) outbreak was declared pandemic on March 11th, for the global diffusion of the novel "severe acute respiratory syndrome coronavirus 2" (SARS-Cov-2) causing the disease. COVID-19 can occur in a wide range of presentation from asymptomatic forms to mild or severe forms. Severe illness presentation requires hospitalization in acute or intensive care units (ICU) with respiratory support and invasive mechanical ventilation (IMV). Major risk factors of a more severe presentation and risk of death are age, male sex, and the presence of one or more chronic comorbidities, such as cardiac, kidney, pulmonary diseases, hypertension, diabetes, and obesity [1-2].

The body composition of the organism exposed to the virus plays an important role in the response to the infection, both on the short term, considering susceptibility and immunological response to the virus, inflammatory reaction, body condition related to metabolic and respiratory distress, but also on the long term needed for a complete recovery after weeks of illness. Malnutrition for excess or undernutrition can cause fat accumulation and lean mass loss affecting the quality of body composition and whole-body functioning.

This manuscript aims to review evidence collected during COVID-19 pandemic emergency, which is still ongoing worldwide, and to provide information on the impact of body composition on severity and outcomes of the disease both on short and long term, analyzing methods used for its assessment.

Another important assessment for patients undergoing acute respiratory distress syndrome (ARDS) is to analyze the risk or presence of malnutrition at the time of hospitalization, home discharge, and during the recovery from an illness requiring weeks in bed or home confinement.

During pandemic emergency, assessing body composition is particularly difficult in such overwhelmed clinical scenario. Nevertheless, some studies managed to collect data through computer tomography (CT) scan performed for the clinical assessment of pneumonia at chest level, sometimes extending imaging to the abdominal region. While on a regular basis, bioimpedentiometry (BIA) is considered a feasible and repeatable method for assessment and monitoring of its changes.

Malnutrition prevalence and screening

Screening tools to assess the presence or risk of malnutrition are multiple and can be chosen depending on the ideal clinical or validated setting (TABLE 1, [3**-9]). The high prevalence of malnutrition in patients hospitalized for critical illness requires a prompt admission assessment to manage clinical outcomes and recovery, while some patients at risk can become malnourished along the hospitalization. Moreover, elderly subjects are a group at higher risk of malnutrition and at the same time worse COVID-19 outcomes compared to the rest of the population.

A Chinese cohort revealed presence of malnutrition, assessed by mini nutritional assessment (MNA), in elderly patients (age >65 years) hospitalized with COVID-19 higher than expected (52.7%) with 27.5% of them at risk of malnutrition, especially those having diabetes, low calf circumference, and low albumin [5]. This could be explained by the acute state of the disease, as it will be further discussed.

The relevance of malnutrition risk or presence as prognostic factor in COVID-19 patients is confirmed by higher mortality, especially for those admitted to ICU. Indeed, those patients classified malnourished at the mNUTRIC score had double the chance to die during a mean hospitalization of 28 days for higher incidences of ARDS, acute myocardial injury, secondary infection, shock and higher chance to need vasopressors [6*].

Assessing by particular questionnaire or screening tools the risk or presence of malnutrition, can only in part predict impairment in the body composition state in subjects during the acute phase of the disease, but it also has a prognostic value for the recovery phase. Therefore, tools to assess the nutritional state in subjects even not requiring hospitalization, have been proposed in primary care to be performed also remotely [7]. Higher risk of malnutrition is related to significantly longer length of stay (LOS), higher hospital expenses, worse disease severity [8].

Methods to assess body composition

Although highly necessary for a clinical and prognostic assessment of subjects, especially if affected by acute illness, screening tools do not provide information on body composition. Malnutrition can, indeed, overlap with altered body composition in addition to sarcopenia and/or cachexia [9], but so far no validated screening tool is able to properly assess and distinguish

between these different conditions. Body composition changes with aging affects health state to the extent that alterations, such as sarcopenia, show high mortality in geriatric patients with acute illness [10].

Multiple methods can be uses to analyse the body different compartments and each method has its sensibility, but this review will not analysethis specific details, as the method of choice reported in each study has been chosen mainly by clinical convenience and experience at each different centre. The studies reported, therefore, are mainly retrospective or cross-sectional with data collected during the emergency of the pandemic.

BMI

The simplest methods to provide information on the body would be body mass index (BMI) calculation, although sometimes performing collection of height and weight in bedridden patients results difficult. A positive relation between BMI and worse severity and outcomes in COVID-19 will be further discussed (see later "Obesity and Sarcopenic Obesity"). However, BMI do not provide a detailed characterization of fat and lean body mass compartments or any information on their distribution.

BIA

Bioelectrical impedance-analysis (BIA) represents a practical tool to provide information on skeletal muscle mass (SMM), easily performed in subjects hospitalized or outpatients at relatively low-cost. BIA measures the opposition to an alternating current at different frequency, usually between 50 kHz and 250 kHz, passing through body compartments (resistance) and the delay in conduction by membranes (reactance). Different prediction equations have been developed to quantify body mass compartments, especially water and cellular mass. These equations, including variables like gender, age, weight, and height, require validation to be used in specific populations [11]. The phase angle, showing the relation between reactance and resistance, is considered an important marker of cellular integrity, also described as an independent predictor of long-term mortality in ICU admitted patients [12].

The importance of this measurements is off-balanced by the large variation in over or dehydration, as frequently happens in critically ill subjects. Nevertheless, BIA measurements were found acceptable for critically ill geriatric patients [13], but more reliable and reproducible in euvolemic subjects.

Performing BIA measurements, a group in Netherlands found no independent association of body composition values (fat mass, visceral fat area, and fat-free mass) with disease severity comparing ICU against ward admitted COVID-19 subjects. However, a composite score composed of mortality, morbidity, and ICU admission, revealed that a low phase angle was associated with COVID-19 severity [14*]. Fluid overload, defined as extracellular-water-ratio (ECW/TBW), was also associated with higher risk of mortality, particularly in ICU patients.

ULTRASOUND

Performing ultrasonography (US) is another costless and repeatable method to assess adipose subcutaneous tissue and muscle quality and quantity of SMM, but it is highly operator dependent, therefore easily biased. Muscle mass modifications, assessed mostly on lower limb and sometimes upper limb, have been described in patients after long-stay ICU, by US imaging showing non-homogenous features of muscle tissue with reduced mass [15]. However, pandemic did not allow such measurement and no US study in COVID-19 has been reported.

DEXA and MRI

Dual-energy X-ray absorptiometry (DEXA) and Magnetic resonance imaging (MRI) are two techniques with low-dose or no radiation exposure, easy to perform, able to accurately measure fat and fat-free mass, but expensive for clinical or routine study. Although these methods are considered the most accurate and precise, together with computed tomography scan, to evaluate body composition and skeletal muscle quantity and quality, no study was able to collect DEXA and MRI imaging in COVID-19 subjects during the early phase of the pandemic.

CT

Conversely, some COVID-19 studies report data analysis of computed tomography (CT) scans at the chest level, which represents a diagnostic tool for pneumonia, weather positive or not to SARS-CoV-2. Different measures to estimate body composition by CT have been used, mainly to quantify subcutaneous fat mass and visceral adiposity. For instance, the ratio of waist circumference per paravertebral muscle circumference (FMR) as potential surrogate of body composition and obesity, provides prognostic value for the patient outcome and the need of an ICU treatment in a 22-day follow-up [16*]. Accordingly, total abdominal and visceral adipose tissue (VAT) is associated with worse COVID-19 severity as assessed by a lung severity score (LSS) and need for hospitalization, ICU or IMV [17**-18*]. VAT can be reliably estimated by chest CT, at the level of the first lumbar vertebra (Th12-L1; L1-L2), instead of lower levels (L4-L5), to provide quantification of visceral fat area (VFA) and upper abdominal circumference. Moreover, CT single axial slice at L3 vertebral body assessing VAT, subcutaneous adipose tissue (SAT), total adipose tissue (TAT), and VAT/TAT, along with age, gender, sex and BMI can be used in a clinical model to predict the need for hospitalization, better than using BMI alone [18*]. Increment by ten square centimeters of VAT increases 1.37-fold and a 1.32-fold the likelihood of ICU treatment and mechanical ventilation, while each additional centimeter of circumference increases these chances 1.13-fold and 1.25-fold [19]. Another CT study reveals that, independently of BMI, age and sex adjusted, every mm of VAT increases 1.16 times (95% CI 1.07–1.26; P ,0.0001), the risk of ICU admission for COVID-19, that was associated with a 30% higher VAT and a 30% lower SAT, independent of age and sex [20**].

CT-scan is paramount in assessing fat distribution by visceral adiposity as described through VAT/SAT ratio, and also intramuscular fat (IMF), both described as independent risk factors of critical illness (odds ratio: 2.47; 95% CI: 1.05-5.98, P=0.040 and 11.90 95% CI: 4.50-36.14; P < 0.001) with increased risk of mechanical ventilation and death [21**]. Furthermore, when analysing the younger cohort of subjects (< 60 years), high visceral adiposity and high IMF deposition show even higher risk for critical COVID-19 illness (OR: 7.58, 95% CI: 1.7-42.21, P = 0.011), (OR: 18.67, 95% CI: 3.64-147.42, P = 0.001) [21**].

Obesity and Sarcopenic Obesity

In adults with obesity, increased risk of severe COVID-19 is reported not only in subjects younger than 60 years old [21**], but also younger than 40 years old [22*] with CT findings of excessive fat expansion at visceral and epicardial regions. In addition, increased liver fat [22*-23] with metabolic associated fatty liver disease (MAFLD) increases more than six times the chance of disease severity.

Ectopic fat deposition can drive organ malfunction, in pro- inflammatory environment with increased adipokines and inflammatory cytokines like TNF-alpha, leptin, and IL-6, leading to impaired immune response to SARS-CoV-2 infection and severe complications.

Excessive fat mass often hides a loss of lean body mass in subject with obesity, that after a long ICU hospitalization are at higher risk to develop sarcopenia. A report of body composition imaging after 20 days of bed-rest in ICU reveals a loss of both fat (9%) and lean mass, but increased liver fat attenuation, due to prolonged increased energy expenditure, insulin resistance, and chronic inflammatory state [23]. Sarcopenic obesity, a state of coexistence of excessive fat mass and low mass and function of muscle mass, is associated with worse clinical outcome [24**].

Undoubtedly, worse illness severity and clinical outcomes, defined as need for hospitalization (OR 1.76), ICU admission (OR 1.67), need for IMV (OR 2.19), longer LOS, or death (OR 1.37), are reported for subjects with obesity up to 39% higher risk compared to non-obese population [25-26*]. However, fat distribution and muscle mass quality are better predictor of disease than BMI and total fat.

Sarcopenia, weight loss and ICUAW

Sarcopenia, a deterioration of muscle mass and function occurring in subjects with obesity or older adults, contributes to the compromised respiratory function in acutely ill COVID-19 patients. Impaired muscular quality affecting unfavourable outcomes can be present in subjects already malnourished at the time of the admission or can also develop during hospital stay [27**-28]. Notably, muscle wasting occurs rapidly, characterized by fibre denervation at the neuromuscular junction and upregulation of protein breakdown, not counteracted by muscle protein synthesis,

already suppressed within two-three days of inactivity [29**]. Indeed, after 10 days the loss of muscle mass is about 6% and after 30 days about 10%.

Therefore, prompt evaluation of nutritional status and body composition is a crucial step in patients with COVID-19 both in clinical and community setting. Indeed, weight loss higher than 5% and risk of malnutrition can occur even after discharge, during recovery phase, or even in patient managed at home [27**], often in relation to disease duration.

Acute state of inflammation and bed rest contribute to the loss of lean body mass, for increased energy and protein requirements, with subjects in ARDS losing up to 18% of the body weight [27**]. Whether inactivity is simultaneous to positive energy balance, fat mass deposition can occur, together with impaired glucose homeostasis, especially for reduced muscle insulin-sensitivity, consequently promoting the pro-inflammatory state of the disease, and increasing the risk of cytokine storm [29**]. Remarkably, inactivity negatively impacts on muscular, cardiovascular, metabolic, endocrine and nervous systems, both at short and long term, by overlapping of chronic low-grade with acute-state inflammation, and aggravating lean mass catabolism.

During or after prolonged stay in ICU, a muscular weakness due to neuropathy, myopathy and muscle atrophy caused by critical illness and pharmacological intervention, has been described as intensive care unit-acquired weakness (ICUAW) [15]. The loss of muscle mass reaches up to 80% of elderly ICU patients, and in the long term up to 30% report transient disabilities, for instance dysphagia, sometimes permanent disabilities. ICUAW constitutes an important predictor of long-term mortality and morbidity [15, 30**], affecting nutritional status and body composition on both short and long term.

Conclusion and Medical Nutrition Therapy (MNT)

In conclusion, MNT should be tailored and promptly applied to prevent or treat malnutrition in adults with suspected or confirmed COVID-19 infection in both clinical and community setting. Regular assessments for body composition and malnutrition screening tools should be performed to evaluate prognostic features especially in high risk groups, as described for older adults, subjects with obesity and/or sarcopenia, malnourished or with chronic comorbidities. Frail subjects

require bed rest along the hospitalization or even after discharge and home confinement often forces to inactivity, sedentarism, social distancing, with negative impact on psychological and nutritional state.

In post-COVID-19 patients, a specific nutritional and motor rehabilitation should be provided to prevent and/or treat malnutrition, improving body composition to help a quick recovery, reduce complications or mortality, and improve both short- and long-term prognosis [30**]

TABLE 1: Malnutrition screening tools: description of item and scores to assess presence or risk of malnutrition used in different hospital and community settings.

Screening Test	Approach	Setting	Description of Items	Score
MUST [1,8]	3 questions	Hospital, home care, community	0-1-2 points for each question: - BMI - Unintentional weight loss - Reduced food intake or acute illness	Risk of malnutrition ($\geq 2/6$): 0 low 1 medium 2 high
MNA [1-5,8]	18 domains	Hospital, home care, community	Same as MNA-sf plus: lives independently, medication, pressure ulcers, number of meals, intake protein, fruit, vegetable, fluids intake, mode of feeding, self-view of nutritional status, self-assessment of health status, mid-arm circumference, calf circumference in cm.	24 no risk 17–23.5 risk < 17 malnutrition
MNA-sf [4,8]	6 domains	Hospitalized/ institutionalized elderly	- BMI - Unintentional weight loss - Reduced food intake - Mobility - Physical stress/acute illness - Cognitive status	12–14 normal 8–11 risk 0–7 malnutrition
NRS-2002 [4,8]	2 levels (same items): - I risk screening - II grading	Hospital	-BMI -Unintentional weight loss -Reduced food intake -Disease severity - Age $\geq$ 70 years	Risk $\geq$ 3 (level 1) Grading severity (level 2): 1 mild 2 moderate 3 severe
(m)NUTRIC [1,6]	Health status	ICU	- Age - Illness - Inflammation	≥ 5 high risk <5 low risk
NRI [4,8]	= (1.519 × serum albumin (g/L) + 41.7 × (present weight/usual weight)	Hospital/home care	- Albumin - Weight	Risk of malnutrition < 83.5 high risk 83.5–97.5 moderate 97.5–100 mild risk > 100 no risk
GNRI [4]	= (14.89 × albumin (g/dL)) + (41.7 × (body weight/ideal body weight))	Hospitalized elderly	- Albumin - Weight	Risk of malnutrition < 82 high 82–91 moderate 92–98 low > 98 no risk
R-MAPP [7]		Primary care (remote)	Composed of 2 validated test: MUST + SARC-F [8]	Treat if MUST $\geq$ 2 and SARC-F $\geq$ 4
GLIM [3]	2-step: - I risk screening - II assessment severity of malnutrition	Clinical settings	3 phenotypic criteria (unintentional weight loss, low BMI, reduced muscle mass) + 2 etiologic criteria (reduced food intake or assimilation, and inflammation or disease burden)	Malnutrition diagnosis at least 1 phenotypic + 1 etiologic criterion AND Malnutrition Grading: moderate-severe

Abbreviations: MUST Malnutrition Universal Screening Tool; MNA Mini Nutritional Assessment; MNA-sf Mini Nutritional Assessment-short form; NRS-2002 Nutritional Risk Screening tool 2002; (m)NUTRIC score (modified) Nutrition Risk in the Critically ill score; NRI Nutritional Risk Index; GNRI Geriatric Nutritional Risk Index; R-MAPP Remote – Malnutrition APP (also named Remote Malnutrition in Primary Practice), SARC-F (5-item questionnaire: Strength, Assistance with walking,

Rise from a chair, Climb stairs and Falls); GLIM Global Leadership Initiative on Malnutrition, ICU intensive care unit, BMI body mass index

KEY POINTS

•   Malnutrition and body composition assessment are pivotal during a critical illness as prognostic factor for worse disease outcomes and high risk of long-term chronic disabilities.

•   During COVID-19 outbreak, severe form of disease presentation and higher risk of mortality were found in specific population groups such as elderly, sarcopenic, or subjects with obesity and malnutrition.

•   Methods to analyse body composition could be performed even during the COVID-19 emergency to assess fat accumulation (in obesity, MAFLD,…) and lean mass loss (in sarcopenia, cachexia, elderly subjects,…), both affecting short and long-term outcomes.

•   Tailored MNT is required in each high-risk group to prevent or treat the specific impairment during critical illness (e.g. COVID-19) to improve the short and long-term outcomes of the disease preventing chronic disabilities (e.g. ICUAW).

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3. Healthy lifestyle: the impact of exercise training on healthy ageing and cancer prevention

Within the burden of NCDs, 1.6 million deaths annually can be attributed to insufficient physical activity (WHO factsheets).

Although epidemiological evidence suggests an inverse association between physical activity and risk for cancer, in particular for colon and breast cancer(1-2), regulatory mechanisms remain poorly understood. Weight management and physical activity have significantly been proven to be protective factors for most type of cancer (3-4), showing positive effects of exercise on weight loss and improvements on insulin resistance and insulin signalling (5-6).

Several metabolic and inflammatory factors are involved in the health-promoting effects of exercise training. Regular exercise training promotes visceral fat loss, reduces inflammation and oxidative stress (5-6), moreover promotes DNA and cellular repair, proteostasis, replicative stress resistance, and apoptosis of permanently damaged cells (7).

Long-term regulation of various metabolic, inflammatory and ageing pathways could ultimately improve prognosis among breast cancer survivors (8).

Cellular senescence—a state of irreversible growth arrest—is considered a basic mechanism of ageing. Ageing is associated with progressive metabolic and hormonal alterations that translate into significant gene expression and epigenetic adaptations, including changes in DNA methylation (9-10).Recently, deviation of epigenetic from chronological age (i.e., epigenetic age acceleration) has been shown to strongly and robustly predict longevity and healthy ageing (11-12) and have been studied as potential biomarkers of epigenetic age, as a proxy of a person's biological age (13).

We investigated some biomarkers as evidence of decelerating biological ageing with beneficial effect of long-term moderate (non-extreme) caloric restriction (CR) and regular endurance exercise training in in humans, concluding that long-term moderate CR and endurance exercise training can decelerate biological ageing of colon mucosa via epigenetic mechanisms that affect both the inflammatory processes leading to mucosal injury and the regenerative capacity of the epithelium.

On the other hand, the epigenetic mutation load (EML), defined as the total number of whole-genome stochastic epigenetic mutations is considered a biomarker of risk for age-related diseases rather than a proxy for longevity. In fact, a higher EML has been associated with an

increased risk of several common cancers and neurodegenerative disorders(13-14).

Therefore, studying the effects of exercise training on DNA methylation and EML could provide information on the age-dependent deterioration of cellular epigenetic homeostasis and genomic stability, and on the risk of developing age-related diseases.

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3.1 Long-term intensive endurance exercise training is associated to reduced markers of cellular senescence in the colon mucosa of older adults

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ABSTRACT

Regular endurance exercise training is an effective intervention for the maintenance of metabolic health and the prevention of many age-associated chronic diseases. Several metabolic and inflammatory factors are involved in the health-promoting effects of exercise training, but regulatory mechanisms remain poorly understood. Cellular senescence—a state of irreversible growth arrest—is considered a basic mechanism of aging. Senescent cells accumulate over time and promote a variety of age-related pathologies from neurodegenerative disorders to cancer. Whether long-term intensive exercise training affects the accumulation of age-associated cellular senescence is still unclear. Here, we show that the classical senescence markers p16 and IL-6 were markedly higher in the colon mucosa of middle-aged and older overweight adults than in young sedentary individuals, but this upregulation was significantly blunted in age-matched endurance runners. Interestingly, we observe a linear correlation between the level of p16 and the triglycerides to HDL ratio, a marker of colon adenoma risk and cardiometabolic dysfunction. Our data suggest that chronic high-volume high-intensity endurance exercise can play a role in preventing the accumulation of senescent cells in cancer-prone tissues like colon mucosa with age. Future studies are warranted to elucidate if other tissues are also affected, and what are the molecular and cellular mechanisms that mediate the senopreventative effects of different forms of exercise training.

Regular physical exercise is one of the key pillars for health promotion since ancient times, although our ancestors did not know the biological processes responsible for its beneficial effects. Data from animal and human randomized trials indicate that aerobic exercise training improves glucose tolerance, insulin sensitivity and lipid metabolism through multiple mechanisms, including mitochondrial biogenesis, increased expression of the insulin responsive glucose transporter type 4 (GLUT4) and lipoprotein lipase in the skeletal muscle[1,2]. Regular exercise training also promotes visceral fat loss, reduces inflammation and oxidative stress, and improves left ventricular diastolic function in overweight men and women[1,3–6].

Accumulating data show that physical activity evokes profound metabolic and molecular responses not only in key metabolic organs (skeletal muscle, adipose tissues, and liver), but also in tissues at high risk of neoplastic transformation. Epidemiological studies suggest an inverse association between physical activity and risk for 13 different types of cancer, in particular for colon and breast cancer[7–9]. Regular exercise training can also improve prognosis among breast and colorectal cancer survivors[10,11] by long-term regulation of various metabolic, inflammatory and aging pathways that promote DNA and cellular repair, proteostasis, replicative stress resistance, and apoptosis of permanently damaged cells[12].

One of the fundamental cellular mechanisms regulating aging and tumor development is cellular senescence[13]. Cellular senescence is a state of irreversible proliferative arrest triggered by diverse DNA or mitochondrial damages to prevent propagation of damaged cells[14]. Senescent cells are characterized by the engagement of the Cyclin-dependent kinase inhibitors p16Ink4a (p16) and p21CIP1 (p21)[15], enhanced lysosomal activity, and a hypersecretory phenotype known as Senescence-Associated Secretory Phenotype (SASP). The SASP remain highly heterogeneous and dependent on various intrinsic and extrinsic factors[16,17]. However, persistent senescent cells can cause chronic low-level inflammation and aberrant tissue growth and remodeling via SASP factors[18–23].

Lifestyle factors can have consequences on induction and accumulation of cellular senescence. For example, caloric restriction (CR), a well-known and highly conserved anti-aging and anti-cancer intervention, is associated to reduced accumulation of senescent cells in both mice and humans[24,25]. Interestingly, we have also recently shown that, at least in mice, high-protein and high-fat diets lead to premature hepatic accumulation of hyper-inflammatory senescent cells[26]. Besides dietary approaches, it has been shown that a 12-week exercise program reduces circulating senescence biomarkers in older adults[27] and a recent human study suggests that the number of senescent cells of the adipose tissue is inversely correlated to physical function in older women[28]. However, whether regular vigorous aerobic exercise can prevent accumulation of age-associated senescence, especially in highly proliferating cancer prone tissues, remains controversial[29]. Here, we studied the effects of chronic intensive endurance exercise training on cardiometabolic health and candidate biomarkers of cell senescence in colon mucosa biopsies of master athletes who ran an average 48 miles/week (range 30 to 90 miles/week) for an average of 21 years (range 5–35 years).

Participants in this study were endurance runners (mean age 57 ± 10 years) consuming usual American diets (EX); age- and sex-matched sedentary (regular exercise < 1 h per week) controls eating Western diets (WD-o); and very young (mean age 24.3 ± 2 years) sedentary controls (WD-y) who should have negligible numbers of senescent cells. Average calorie intake in the EX group was 2806 ± 618 kcal/day, 13 and 7% higher than in the WD-o (2443 ± 407 kcal/day) and WD-y (2618 ± 712 kcal/day) groups, respectively ($p < 0.05$ for EX vs. WD-o). The percentages of total energy intake derived from protein, carbohydrate, and fat were similar among the groups: 15.7%, 51.8%, and 32.5%, respectively, in EX; 15.1%, 50.4%, and 32.8%, in WD-o; 17.2%, 48.2%, and 33.4% in WD-y.

In Table Table1,1, we reported the study sample's summary statistics, including the distribution of age, sex, body mass index, DXA body fat percentage and lean mass, and a range of fitness and cardiometabolic parameters. BMI, body fat, resting heart rate, LDLc, total cholesterol HDL ratio, triglycerides, triglycerides to HDL ratio, fasting glucose, fasting insulin, HOMA-IR, and total white blood cell count were significantly lower in the EX group than in the WD-o group ($p < 0.05$). As

expected, EX volunteers had significantly higher VO2max and HDLc than WD-o participants (p < 0.05).

Table 1

Characteristics of the study subjects.

	EX group (n = 44)	WD-o group (n = 44)	WD-y group (n = 6)	Among group P
Age (years)	57 ± 10	57 ± 9	24.3 ± 2[b,c]	<0.001
Sex (M:F)	37:7	37:7	4:2	–
Height (m)	1.75 ± 0.1	1.76 ± 0.1[a]	1.79 ± 0.1	NS
Weight (Kg)	70.0 ± 10	78.8 ± 14[a]	82.6 ± 13[b]	<0.001
BMI (Kg/m²)	22.7 ± 4	25.3 ± 2.7[a]	25.7 ± 1[b]	<0.001
Body fat (% body weight)	14.8 ± 6.5	25.2 ± 6.6[a]	17.9 ± 8.2[d]	<0.001
Lean mass (kg)	56.1 ± 8	54.7 ± 11	63.3 ± 14	NS
Resting heart rate (b/min)	52 ± 8	63 ± 10[a]	66 ± 12[b]	<0.001
VO2max (ml/Kg/min)	51 ± 10	33 ± 7[a]	–	<0.001
SBP (mm Hg)	126 ± 19	129 ± 14	129 ± 11	NS
DBP (mm Hg)	73 ± 10	79 ± 9[b]	78 ± 11	0.055
LDL-c (mg/dl)	92 ± 22	115 ± 28[a]	94 ± 24	0.004
HDL-c (mg/dl)	68 ± 17	55 ± 15[a]	64 ± 18	<0.001
Triglycerides (mg/dl)	64 ± 22	120 ± 71[a]	61 ± 33[d]	<0.001
TChol/HDL ratio	2.6 ± 0.5	3.8 ± 1.0[a]	2.9 ± 0.9[d]	<0.001
TG/HDL ratio	1 ± 0.4	2.5 ± 2.0[a]	0.9 ± 0.6[d]	<0.001
Fasting glucose (mg/dl)	90 ± 8	94 ± 9[b]	82 ± 5[c]	0.001
Fasting insulin (mg/dl)	3.0 ± 2.3	7.6 ± 5.4[a]	6.6 ± 3	<0.001
HOMA-IR	0.7 ± 0.6	1.8 ± 1.3[a]	1.3 ± 0.6	<0.001
WBC (K/cumm)	4.5 ± 1.2	5.8 ± 1.6[a]	4.9 ± 0.5	<0.001
hsCRP (mg/L)	0.7 ± 0.6	1.8 ± 1.3	0.8 ± 0.3	NS

All values are means ± SD.

Significantly different from EX group, [a]P ≤ 0.003, [b]P ≤ 0.05.

Significantly different from WD-o group, [c]P ≤ 0.003, [d]P ≤ 0.05.

Characteristics of the study subjects.

To investigate the effects of long-term EX on biomarkers of cell senescence, we collected colon mucosa biopsies in a subset of 11 middle-aged (58.6 ± 8.3 years), weight-stable and lean (BMI, 24.5 ± 2.8 kg/m2) master athletes, 10 age- and sex-matched nonobese (BMI, 27.1 ± 2.3 kg/m2) and

6 sedentary young (24.3 ± 2 years) and lean (BMI, 25.7 ± 1 kg/m2) control subjects (Supplementary table 1). Because p16 is still considered one of the most relevant cell senescence markers in human specimens, and because p16 measurements were included in most of the previous studies on the effect of physical exercise on senescence markers, we measured its mRNA abundance. As expected, p16 levels were markedly higher in older than younger sedentary individuals consuming Western diets (Fig. (Fig.1A).1A). Strikingly, this upregulation was significantly blunted in endurance runners (Fig. (Fig.1A).1A). p21 is another important regulator of cell cycle arrest often dysregulated during senescence. Similar to what observed for p16, p21 levels were upregulated in older sedentary individuals compared to young sedentary or endurance runners (Fig. (Fig.1B).1B). However, the difference in expression between groups did not reach statistical significance. The detrimental functions of cellular senescence are, at least partly, mediated by pro-inflammatory secreted factors. The composition of pro-inflammatory SASP is variable, but IL-6 remains one of the most consistent SASP factors13. In accordance, IL-6 mRNA levels of colon mucosa were significantly higher in old sedentary individuals consuming Western diet, whereas IL-6 levels in master athletes where low and similar to those of very young sedentary people consuming Western diets (Fig. (Fig.1C).1C). We then measured levels of two additional SASP factors, IL8 and MMP3. We observed a trend for the upregulation of MMP3 and IL8 in older sedentary individuals compared to young sedentary and endurance runners, but there was no statistical significance (Fig. 1D, E). p16 mRNA levels correlated linearly with p21 (r = 0.758; p < 0.001) and IL-6 (r = 0.798; p < 0.001) mRNA levels (not shown). To evaluate potential links between the senescence burden and metabolic alterations, we then studied the association of p16 levels with various metabolic parameters. Strikingly, p16 mRNA levels were linearly correlated with the triglycerides to HDL ratio, a well-accepted marker of metabolic syndrome, coronary heart disease and colon adenoma risk (Fig. (Fig.1F1F)30–32. Interestingly, in patients with early-stage colorectal cancer, the combination of obesity and low HDL-cholesterol and high triglycerides levels predicts worst cancer survival33.

Fig. 1
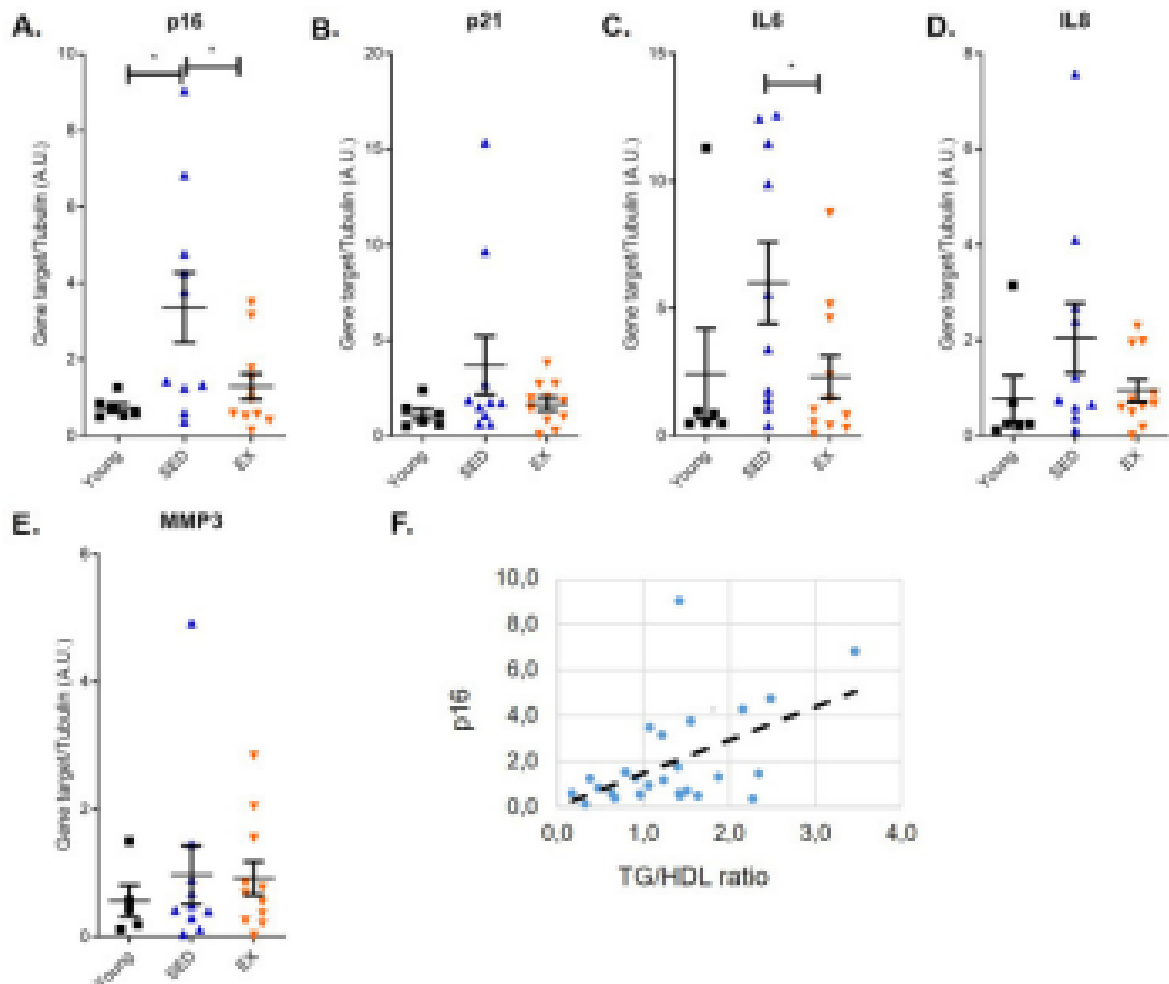
Expression of senescence-associated genes in the colon mucosa of master athletes and sedentary controls.

RNA was extracted from the sigmoid portion of the colon of human volunteers. The groups were: EX, exercised volunteers of average age 57 ± 10 years; age-matched sedentary controls (SED); young, volunteers of average age 24.3 ± 2 years. mRNA encoding p16 (A), p21 (B), IL6 (C), IL8 (D), and MMP3 (E) were quantified by qRT-PCR. mRNA encoding tubulin was used as internal control (N = 5-11 with each sample indicated by an individual dot). Panel F shows the relationship between p16 mRNA levels and the triglycerides to HDL ratio. All values are represented together with means and SEM. One-way Anova, *$p < 0.05$.

Our results are preliminary and limited, in particular in light of characterizing senescence-associated phenotypes and the type of cells more affected by these changes. Nevertheless, the findings shown here suggest that chronic high-volume high-intensity, unlike low-volume34, endurance exercise can play a major role in preventing the accumulation of senescent cells in cancer prone tissues like colon mucosa with age. This is important because data from transgenic mouse models, including our p16-3MR mouse19, have shown that ablation of senescent cells is sufficient to systemically reduce inflammation, rejuvenate tissue functions, alleviate various age-related conditions, improve health and extend longevity13. As senescent cells are likely contributor to dysregulated inflammatory responses, our data are in line with a previous report showing that the level of stress-induced (acute exercise) inflammatory markers is reduced in muscle and blood of lifelong aerobic exercising older men compared to old healthy nonexercisers35. In addition, prevention of cell senescence could partly explain the anti-cancer effect of lifelong aerobic exercise36. Future studies should be focusing on understanding which tissues are most affected, and what are the molecular and cellular mechanisms that mediate the senopreventative effect of endurance exercise training. Moreover, it will be key to analyze individuals following different physical exercise regimens, including resistance and high-intensity interval training.

METHODS

Patients and tissue collection

This study sample includes three groups of volunteers, named from hereafter EX, WD-o, and WD-y. The EX group consisted of 44 master athletes who ran at least 30 miles/week (range 30–90 miles/week) or expended similar amount of energy by cycling or swimming, for at least the previous three years (range 3–35 years). The control (WD-o) group comprised 44 age- and sex-matched individuals reporting less than 1 hour of physical activity per week, recruited from the St. Louis metropolitan area. A third control group with negligible numbers of senescent cells consisted of 6 very young (mean age 24.3 ± 2 years) sedentary controls (WD-y) consuming Western

diets. All the participants reported weight stability, defined as less than a 2-kg change in body weight in the preceding 6 months. Participants recorded all food and beverage intake for 7 consecutive days. Food records were analyzed by our dietitian by using the NDS-R pro-gram (v.4.03_31) and used to define the western diet consumers. None of the participants had evidence of chronic disease, smoked cigarettes, or took medications that could affect the outcome variables. The present study (HRPO #: 01-0804) was approved by the Human Studies Committee of Washington University School of Medicine, and all subjects gave written informed consent before their participation. Height and body weight were obtained in the morning after an overnight fast, with the participants wearing only underwear and a hospital gown. Total body fat mass and fat-free mass were determined by dual-energy X-ray absorptiometry (DXA; QDR 1000/w; Hologic). VO2max was determined by indirect calorimetry during an incremental exercise test to exhaustion37. Participants walked on a level treadmill at a pace that elicited 60–70% of age-predicted maximal heart rate for a 5-minute warm-up. The speed was then set at the fastest comfortable pace, and the grade was increased 1–2% every 1–2 minutes until volitional exhaustion, electrocardiographic changes, or other abnormalities that rendered it unsafe to continue. Blood pressure was measured with an oscillometric blood pressure monitor (DinamapProcare 200; GE Healthcare, Waukesha, WI) in the morning after a 12-h fast. In the EX group, blood pressure was measured at least 48 h after the last exercise session. A venous blood sample was taken to determine lipid and hormone concentrations after subjects had fasted overnight. In the EX group, blood samples were obtained ≥48 h after the last exercise session. Measurement of serum lipid and lipoprotein-cholesterol concentrations, glucose, insulin, C-reactive protein was performed in the Barnes-Jewish Hospital Laboratory by automated enzymatic, radioimmunoassay and ELISA commercial kits. Insulin resistance was calculated using homeostasis model assessment of insulin resistance 9HOMA-IR = [fasting glucose (mmol/l) × fasting insulin 59]/22.5).

RNA isolation and cDNA synthesis

Biopsy specimens of normal-appearing sigmoidal colon mucosa were collected from a subset of 11 EX, 11 WD-o, and 5 WD-y volunteers in the morning after an overnight fast and a preparation with an enema containing water. Colonic mucosal specimens were immediately washed in PBS and then

flash-frozen in liquid nitrogen and stored at −80 °C until processed. Tissues were homogenized in liquid nitrogen. For each sample, 20 mg of tissue powder was used to isolate total RNA using the Isolate II RNA Mini Kit (Bioline). In all, 250–500 ng of RNA was reverse transcribed into cDNA using a kit (Applied Biosystems).

Real time-qPCR

qRT-PCR reactions were performed with the LightCycler 480 Instrument II (Roche) using UPL system (Roche) with a SensiFast Probe kit (Bioline). The reactions were carried out in a total volume of 10 µl using a TaqMan assay. Tubulin was used for normalization of the CT values. List of primers/probe combination:

Tubulin: FW- cttcgtctccgccatcag; RV-cgtgttccaggcagtagagc; Probe #40

Cdkn2a (p16): FW-gagcagcatggagcctc; RV-cgtaactattcggtgcgttg; Probe #67

Cdkn1a (p21): FW-tcactgtcttgtacccttgtgc; RV-ggcgtttggagtggtagaaa; Probe #32

IL6: FW-caggagcccagctatgaact; RV-gaaggcagcaggcaacac; Probe #45

IL8: FW-gagcactccataaggcacaaa; RV-atggttccttccggtggt; Probe #72

MMP3: FW-caaaacatatttctttgtagaggacaa; RV-ttcagctatttgcttgggaaa; Probe #36

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare group variables, followed by Tukey post-hoc testing when indicated. One-way ANOVA with Games-Howell was performed for distributions where equal variances could not be assumed. Pearson correlation was used to assess associations between continuous variables. Statistical significance was set at $P < 0.05$ for all tests. All data were analyzed by using SPSS software, version 28.0 (SPSS Inc, Chicago). Data are expressed

as mean ± SEM or SD (indicated). A difference with P-values < 0.05 were considered statistically significant.

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3.2 Long-term calorie restriction and endurance exercise training are associated with decelerated biological and epigenetic aging in humans

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ABSTRACT

Long-term calorie restriction (CR) is widely accepted to be the most powerful non-genetic intervention to slow biological aging in multiple model organisms including Rhesus monkeys. However, little is known on the effects of chronic CR without malnutrition on biological age in humans. Recently developed indicators of biological aging based on blood biomarkers and DNA methylation (DNAm) epigenetic clocks provide a unique opportunity to investigate the long-term effects of CR on longevity and healthy aging in humans. Here, we performed a cross-sectional study comparing blood phenotypic age (Levine's PhenoAge) and multiple DNAm epigenetic biomarkers of aging in the colon mucosa of men and women practicing CR for an average of ~7 years (range, 3-15 years) with age-matched master athletes (EX) and sedentary controls consuming Western diets (WD). We show that candidate biomarkers of longevity and healthy aging like the blood PhenoAge, Horvath DNAmAge, and the epigenetic mutation load are consistently lower in individuals practicing long-term moderate CR and EX than in an age-matched sedentary control individuals consuming WD. In contrast, we show for the first time that excessive CR may accelerate biological aging in humans without impacting biomarkers of age-related disease risk. These findings provide evidence of the beneficial effect of long-term moderate (non-extreme) CR and regular endurance exercise training in decelerating biological aging in humans and suggest that these DNA-methylation biomarkers might be used to differentiate between optimal CR and starvation.

INTRODUCTION

Calorie restriction (CR) without malnutrition powerfully extends median and maximal lifespans in multiple model organisms. In humans, it causes metabolic and molecular adaptations that protect against some of the most common age-associated chronic diseases.[1] Data from long-lived rodents[2] and from Rhesus monkeys show that long-term CR markedly delays age-associated DNA methylation drift, resulting in estimated biological age of 7 years younger than their chronological age.[3] However, little is known about the effects of long-term CR with adequate vitamins and minerals intake on established and emerging biomarkers of biological aging in humans.

Aging is associated with progressive metabolic and hormonal alterations that translate into significant gene expression and epigenetic adaptations, including changes in DNA methylation (DNAm) of specific highly conserved CpG dinucleotides known to regulate the expression of critical developmental and aging genes.[4] Recently, robust 'epigenetic clocks' based on DNAm levels of multiple CpGs were developed and tested as potential biomarkers of epigenetic age, a proxy of a person's biological age, named DNAmAge.[5,6] Deviation of epigenetic from chronological age (i.e., epigenetic age acceleration) has been shown to strongly and robustly predict longevity and healthy aging.[7–9]

The epigenetic mutation load (EML), defined as the total number of whole-genome stochastic epigenetic mutations,[10] instead, has been proposed as a biomarker of biological aging alternative to the epigenetic clocks, as the number of epimutations increases exponentially with aging. Contrarily to DNAmAge, the EML is considered a biomarker of risk for age-related diseases rather than a proxy for longevity. In fact, a higher EML has been associated with an increased risk of several common cancers and neurodegenerative disorders.[11–13] Finally, Levine et al. have recently developed a novel indicator of aging based on specific clinical blood measurements, named 'phenotypic age' (PhenoAge), which was explicitly trained to predict mortality. An elevated PhenoAge has been associated with a higher risk of developing cardiovascular diseases, mental disease, and frailty, and it can be considered as a biomarker of health-span rather than lifespan.[6]

The purpose of the present study was to determine the effect of long-term CR without malnutrition and endurance exercise training on multiple biomarkers of biological age, age-related disease risk, and healthy aging. Blood PhenoAge, and colon mucosa Horvath DNAmAge and epigenetic mutation load together with other next-generation biomarkers of epigenetic aging (i.e.,

DNA-methylation GrimAge, DNA-methylation estimate of the Pace of Aging or DunedinPACE, DNA-methylation surrogate for telomere length or DNAmTL) were determined in lean men and women who had been practicing CR for an average of ∼7 years (range, 3-15 years), in age-matched endurance runners (EX) consuming a high-calorie diet, and in age-matched non-obese sedentary subjects consuming Western diets (WD).

RESULTS

Participants in this study were 41 men and women, with an average age of 56.1 years, standard deviation (s.d.) 10.6, consuming a ∼30% CR diet for an average of ∼7 years; 41 age- and sex-matched endurance master athletes (EX); and 35 age- and sex-matched sedentary individuals consuming Western diets (WD). Average calorie intake in the CR group was 1693.0 ± 293.3kcal/day, 40.5% lower than the EX group (2845.5 ± 509.0 kcal/day) and 26.7% lower than the WD group (2309.5 ± 319.4 kcal/day), respectively (p < 0.001 for CR vs EX; p < 0.01 for CR vs WD). The percentages of total energy intake derived from protein, carbohydrate, fat, and alcohol were 17%, 61%, 27%, and 1.8%, respectively, in CR; 15%, 50%, 36%, and 1.8% in EX; 14%, 48%, 36%, and 2.8% in WD.

In Table 1 we reported the study sample's summary statistics, including the distribution of age, sex, body mass index (BMI), body fat percentage, and of the nine blood biomarkers whose linear combination constitutes the Phenotypic Age (PhenoAge) according to Levine et al.[6] As shown in **Table 1**, BMI, Dual X-ray Absorptiometry (DXA) body fat, fasting glucose, C-reactive protein, and white blood cell count were significantly lower in the CR group than in the WD group (p < 0.0001) with intermediate values in the EX group. CR volunteers had significantly higher lymphocyte percentage and mean cell volume (MCV) of red blood cells than EX and WD (p = 0.03 and p = 0.04, respectively).

Blood-based 'phenotypic age' (PhenoAge)

The PhenoAge, computed as a linear combination of the nine blood-measured biomarkers listed in **Table 1**, underestimated the chronological age of this study sample. The average PhenoAge was 47.0 years (s.d. = 11.96) which is 9.1 years lower than the average chronological age (T-test p <

0.0001), but PhenoAge and chronological age were strongly correlated (Pearson R = 0.93, p < 0.0001, **Figure 1a**). To investigate differences among the WD, CR, and EXgroups, we computed the residuals of the linear regression of PhenoAge on chronological age, defined as the 'phenotypic age acceleration' (PhenoAA). The average (s.d.) PhenoAA were 1.53 (3.79), -1.43 (4.62), and 0.13 (4.56) in the WD, CR, and EX group, respectively. The pairwise T-test comparison indicated that the CR group is statistically different from the WD group (average mean difference = 2.96 years, p = 0.003). In contrast, the comparisons of WD vs EX, and CR vs EX, were not statistically significant (**Figure 1b**).

Colon mucosa DNA methylation aging biomarkers (Horvath DNAmAge and EML)

To investigate the impact of CR and EX on molecular biomarkers of biological age in humans, we carried out comprehensive DNA-methylation profiling (using Illumina HumanMethylation450 BeadChip) of colorectal mucosa biopsies of a subset of 35 participants who generously volunteered to undergo flexible sigmoidoscopy with collection of cold biopsies from the sigmoid colon: 12 individuals from the CR group, 11 from the EX group, and 12 from the WD group (**Supplementary Table S1**). We computed Horvath DNAmAge based on DNAm values of 353 CpG sites.[5] The average (s.d.) Horvath DNAmAge was 58.54 years (5.75), not statistically different from the average chronological age of the sub-group with DNAm data available (60.97 years, s.d. 8.30; T-test p = 0.16). The Pearson correlation coefficient for chronological age and Horvath DNAmAge was R = 0.82, p < 0.0001, **Figure 1c**. As described for the PhenoAge, we computed the DNAmAA as the residual of the regression of DNAmAge on chronological age. The average (s.d.) DNAmAA were 1.17 (2.77), -0.43 (4.09), and -1.08 (2.31) for WD, CR, and EX, respectively (**Figure 1d**). Pairwise T-tests indicated that DNAmAA in EX group was significantly lower than in the WD group (average difference = 2.25 years, p = 0.04), whereas DNAmAA in the CR group was not significantly different from the WD and EX groups (**Figure 1d**). Subsequently, we computed the EML, defined as the natural logarithm of the number of epigenetic mutations.[14] The Pearson correlation coefficient of chronological age with EML was R = 0.15 (p > 0.05). For consistency with the PhenoAA and DNAmAA biomarker analysis, we computed the residuals of the regression of EML on chronological age (named EML_AA), and we scaled EML_AA values to be interpreted in years of biological aging acceleration (see Methods, section 3.3). The average (s.d.) EML_AA were 1.83 (4.29), 0.98 (4.64),

and -3.06 (2.61) for the WD, CR, and EX groups, respectively (**Figure 1e**). Pairwise comparisons indicated that the EML_AA in the EX group was significantly lower than in the WD group (average difference = 4.91 years, p = 0.003) and in the CR group (average difference = 4.04 years, p = 0.02), whereas the EML_AA in the CR group was not significantly different from the WD group (**Figure 1f**).

Associations of BMI with PhenoAA, DNAmAA, and EML_AA

Because we have observed a higher variability in DNAmAA and EML_AA in the CR group than in the WD and EX groups (**Figure 1d,f**), we decided to perform additional analysis to explore the associations of BMI with the three above-mentioned biomarkers of biological aging stratified by group. Interestingly, the correlation between BMI and DNAmAA was significantly different in the CR group than in the WD group (p for differential effect = 0.03). The Pearson correlation coefficients for BMI and DNAmAA in the CR and WD groups were R = -0.55 (p = 0.05) and R = 0.05 (p = 0.88), respectively (**Figure 2c**). A similar pattern was observed for the EML_AA biomarker, with an inverse correlation between BMI and EML_AA in the CR group, and a positive correlation in the WD group (**Figure 2e**). Our results show for the first time that excessive CR seems to accelerate biological aging in humans. Indeed, unlike normal weight people practicing moderate CR, underweight CR individuals (BMI < 18.5 kg/m^2) experienced a marked acceleration of epigenetic aging (**Figure 2c,e**). When we excluded the four CR individuals with a BMI less than 18.5 from the analysis, the average (s.d.) DNAmAA of the normal weight CR group was -2.21 years (3.43) and statistically significant different from the WD group (average difference = 3.38 years, p = 0.04).

Next-generation epigenetic aging biomarkers: DNAmGrimAge, DunedinPACE, and DNAmTL

To further investigate the molecular effect of chronic CR and endurance exercise training, we carried out additional sensitivity analyses using the so-called next-generation epigenetic clocks: DNA-methylation GrimAge,[15] Pace of Aging Calculated from the Epigenome (DunedinPACE),[16] and DNAmTL (a DNA-methylation surrogate for telomere length).[17] However, because the DNAmGrimAge was trained on blood-derived DNAmCpGs, an overestimation of chronological age was found in our study using DNAm data from the colon mucosa. The average (s.d.) DNAmGrimAge was 82.70 years (6.10), 21.73 years higher than the average chronological age (T-test p < 0.0001),

but the Pearson correlation coefficient for chronological age and DNAmGrimAge was 0.96 (p <0.0001, **Figure 3a**). As shown in **Figure 3b,** residuals of the regression of DNAmGrimAge on chronological age (i.e., DNAmGrimAge_AA) were not significantly different between groups. The DNAm surrogate for telomere length (DNAmTL), instead, had an inverse correlation with chronological age (Pearson R = -0.53, p = 0.001, **Figure 3c**), and the DNAmTL_AA (residuals of the regression of DNAmTL on chronological age) was significantly higher in the EX group that in the WD group (p = 0.006) and in the CR group (p = 0.04, **Figure 3d**). Unlike the epigenetic clocks, the DunedinPACE provides a measure that can be interpreted as a ratio of the rate of increasing biological aging per year of chronological aging. A DunedinPACE value equal to 1 indicates that a person biological age is equal to the chronological age, whereas values higher or lower than 1 indicate accelerated or decelerated biological age compared with chronological aging. In our study, the DunedinPACE was higher than 1 for all the study participants (average = 1.13, s.d. 0.03), suggesting that both DunedinPACE and DNAmGrimAge overestimate biological aging in colon mucosa samples because they were trained on blood-derivedDNAm data. By construction, the DunedinPACE biomarker was not correlated with chronological age (**Figure 3e**), and despite we observed a trend for lower values in the CR and EX groups than in the WD group, these differences were not statistically significant (**Figure 3f**).

DISCUSSION

Calorie restriction without malnutrition but not endurance exercise training extend maximal lifespan in rodents,[18,19] suggesting that CR delays aging independently of adiposity in small mammals. However, in this cross-sectional study, we found that both people practicing long-term moderate CR and master athletes have lower epigenetic biomarkers of biological aging (Horvath DNA-methylation clock and epigenetic mutation load) in their colon mucosa than sedentary people consuming unrestricted Western diets. We also found that excessive CR is associated with accelerated DNAmbiological aging in humans without impacting biomarkers of age-related disease risk. These findings provide evidence of the beneficial effect of prolonged moderate (non-extreme) CR and regular, high-volume endurance exercise training in decelerating biological aging in humans, at least in a cancer-prone tissue such as sigmoidal colon. They also suggest that these DNA-methylation biomarkers might be used to differentiate between optimal CR and starvation.

The Levine's clinical chemistry PhenoAge algorithm accurately predicts mortality even after adjusting for chronological age in healthy and unhealthy populations.[6] Although the composite blood-based PhenoAge index is not as mechanistic as the DNAm clocks, it may play an important role in assessing the effects of anti-aging interventions such as CR and EX on multi-organ functions altering the risk of disease and death.[6] Our study show that age-adjusted PhenoAge was around 3 years lower in people who have been practicing CR with optimal nutrition for an average of 7 years than in age-matched sedentary controls consuming usual Western diets. These results are consistent with findings from the 2-year CALERIE randomized trial[20], and it is likely mediated, at least in part, by the powerful anti-inflammatory and insulin-sensitizing effects of CR that have been consistently shown in multiple model organisms and human trials.[21–24] Interestingly, in our study the Levine's PhenoAge biomarker of healthy aging, was not significantly different in high-volume endurance exercise training group than in the WD control.

Several methods to estimate the individual's biological age based on composite sets of omic-biomarkers have been developed in recent years. Among those, DNAm-based epigenetic clocks have been validated to identify epigenetic patterns that predict biological age in different tissues.[25] Rapidly renewing tissues such as colon mucosa show high age-related epigenetic variability, affecting cell senescence, mitochondrial dysfunction, and genomic instability, potentially resulting in accelerated timing of aging systemically.[26] Consistently with rodent data,[2] we found lower epigenetic age in humans practicing CR and master athletes than in sedentary WD controls. Strikingly, we observed accelerated epigenetic aging in people practicing extreme CR who were underweight (BMI<18 kg/m^2), confirming preclinical data showing that lifespan increases to a maximum as food intake is reduced, but then rapidly declines when the restriction becomes excessive, suggesting a pro-aging effect of extreme CR.[1] Results in mice model indicate that calorie restriction has a non-linear dose-dependent impact on lifespan, that is strain, age and sex specific.[27,28]

In this study, epigenetic age of the highly proliferating colon mucosa as assessed by DNAmAge was approximately 3 and 2 years higher in sedentary controls consuming unrestricted Western dietsthan in normal weight (BMI > 18.5) people practicing moderate CR and in master athletes, respectively. In previous studies, such differences were associated with lower longevity and a higher risk for aging-related diseases. For example, a systematic review and meta-analysis on epigenetic clocks by Oblak et al.[7] reported that one year increase in epigenetic age acceleration is

significantly associated with a higher risk of cancer, cardiovascular diseases, chronic obstructive pulmonary disease, diabetes, and mortality. In addition, another review by Fransquet et al.[29] estimated that a 5-year increase in epigenetic age is associated with an 8-15% increased mortality risk. Among the determinants of accelerated biological aging, cigarette smoking has been associated with increased epigenetic aging, with effect sizes comparable to those observed in our study. Xu et al. found that the epigenetic age of lung tissue and airway epithelial cells was 4 and 5 years higher in current smokers than in never smokers, respectively.[30]

Previous intervention studies do not show larger effect sizes than ours. For example, Fahy et al.[31] reported a 2.5-year difference in mean epigenetic age of immune cells between controls and individuals treated with drugs for thymic regeneration. A 2-year dietary and exercise intervention decelerated epigenetic age by 0.66 years, and EML by 2 years, in women with high breast density.[32] It is important to note that in most of these studies, epigenetic age was derived from circulating immune cells that may attenuate the differences. The duration might also influence the effectiveness of the anti-aging interventions. In the CALERIE trial, 2-year of CR caused a reduction in the DunedinPACE clock but did not significantly change other biomarkers of biological age, including the next-generation clocks DNAmPhenoAge and DNAmGrimAge.[33]

Stochastic epigenetic mutations increase exponentially with chronological age, and unlike PhenoAge and DNAmAge, EML appears to be a more specific biomarker of genomic stability and DNA repair activity, which have been both associated with cancer and neurodegenerative diseases onset and progression.[11,13,34] In our study, EML was 4 and 5 years lower in the colon mucosa of master athletes than in people practicing CR and sedentary control consuming WD, respectively. These results suggest that endurance exercise training is more powerful than CR in counteracting the age-dependent deterioration of cellular epigenetic homeostasis and genomic stability, at least in a highly proliferating and cancer prone tissues such as the colon mucosa. On the contrary, CR is more effective than EX in reducing biomarkers of biological age and healthy aging such as the Horvath DNAmAge and the blood Levine's PhenoAge.

Strengths and limitations

One limitation of this study is the reliance on cross-sectional samples, and ideally, randomized clinical trials should be conducted to confirm our observations. However, long-term (>2 years) CR

and endurance exercise trials are difficult to implement, due to lifestyle and adherence complications, in addition to cost. In this regard, our study is unique considering that our research volunteers practiced CR with optimal nutrition and engaged in high-volume exercise for a very long time, conditions almost impossible to reproduce in intervention trials. Another limitation is the use of whole colon mucosa tissue comprising multiple cell types. Single-cell methylome studies could provide invaluable information on the cell-specific effects of CR and EX on methylation and gene expression adaptations. On the other hand, unlike in other studies that investigated DNA methylation changes in whole blood, we have analyzed epigenetic data in the sigmoidal colon mucosa, a highly proliferating and tumor prone tissue which may be more informative in elucidating biomolecular mechanisms of aging and cancer such as genomic stability, cell senescence, and mitochondrial dysfunction. The relatively low sample size limits the power of this study. However, low statistical power leads to the missing identification of significant signals (type II errors) rather than false-positive associations (type I error). The differential effect of CR on epigenetic aging biomarkers has been observed on a small sample size (four underweight CR individuals), and it need further investigation. Finally, this study sample size does not allow us to discriminate the effects of CR and exercise from those driven by possible confounders, for example, differences in dietary quality or environmental exposures.

CONCLUSIONS

In conclusion, the results of this cross-sectional study suggest that long-term moderate CR and endurance exercise training can decelerate biological aging of colon mucosa via multiple and potentially complementary epigenetic mechanisms that affect both the inflammatory processes leading to mucosal injury and the regenerative capacity of the epithelium. Our findings help formulate novel hypotheses about the molecular mechanisms behind the beneficial effect of moderate (non-extreme) calorie restriction and regular endurance exercise training in humans. Additional mechanistic studies are needed to investigate whether these effects can be additive or synergistic. Nevertheless, our findings represent an important step forward in advancing the development of personalized and precision medicine to test the effectiveness and safety of geroprotective therapies in humans.

METHODS

Section 1: Study population

Forty-one well-characterized middle-aged, weight-stable, very lean men and women who have been practicing calorie restriction (CR) with adequate nutrition (at least 100% of RDI for each nutrient) for 3-15 years were recruited through the Calorie Restriction Society; four were from the St. Louis area and the others came to the Washington University Medical Center from other cities in the USA, Canada and UK. Forty-one men and women, who are long-term endurance runners (EX), matched with the CR group in terms of age, and sex were used as a lean comparison group. These master athletes ran an average 48 miles/week (range 20 to 90 miles/week), and had been training regularly for an average of 19 years (range 3–30 years). Thirty-five untrained (regular exercise < 1 h per week) men and women eating typical Western diets matched with the CR and EX groups in terms of age, and sex, served as a sedentary comparison group. All the volunteers in the control group (except three) had a BMI < 30 kg/m^2. All the participants reported weight stability, defined as less than a 2-kg change in body weight in the preceding 6 months. None of the participants had evidence of chronic disease, smoked cigarettes, or took medications that could affect the outcome variables. Individuals in the three groups were comparable for educational attainment as reported previously.[35] The characteristics of the study participants are summarized in **Table 1**. The present study was approved by the Human Studies Committee of Washington University School of Medicine, and all subjects gave informed consent before their participation.

Section 2: Study protocol

Subjects were admitted to the outpatient facilities of Washington University School of Medicine General Clinical Research Center in the morning after they had fasted for 12 h overnight. Height was measured without shoes to the nearest 0.1 cm. Body weight was obtained on a balance scale in the morning after a 12-h fast with the participants wearing only underwear and a hospital gown. Body mass index (BMI) was calculated by dividing body weight (in kilograms) by the square of height (in meters). Total body fat mass and lean body mass were determined by dual-energy X-ray absorptiometry (DEXA) (QDR 1000 w)1; Hologic, Waltham, MA, USA). Participants recorded all

food and beverage intake for 7 consecutive days, and food records were analyzed by our dietitian by using the NDS-R pro-gram (v.4.03_31). A venous blood sample was taken to determine the concentrations of the nine Levine's biochemical biomarkers after subjects had fasted for at least 12 hr; these measurements were performed in the Core Laboratory for Clinical Studies at Washington University in St.Louis.

Section 3: Colon mucosa biopsy and DNA methylation analysis

Endoscopic cold biopsy specimens were obtained from macroscopically normal sigmoid mucosa (Flexible Video Sigmoidoscope, Olympus OSF V60) after an overnight fast, and preparation with enema containing water, in a subgroup of 35 volunteers (12 individuals from the CR group, 11 from the EX group, and 12 from the WD group). Colonic mucosal specimens were immediately washed in PBS and then flash-frozen in liquid nitrogen and stored at -80°C until processed. Genomic DNA was isolated as per the standard DNA extraction procedure using the DNeasy Tissue Kit (Qiagen, Hilden Germany). DNA methylome profiling was carried out using Illumina HumanMethylation450 BeadChip. Data pre-processing was carried out using in-house software written for the R statistical computing environment as described previously.[11] For each sample and probe, measurements were set to missing if obtained by averaging intensities over less than three beads, or if averaged intensities were below detection thresholds estimated from negative control probes. Background subtraction and dye bias correction (for probes using the Infinium II design) were also performed. The probes close to SNPs with minor allele frequency greater than 0.05 in Europeans and potentially cross-reactive probes were removed.[36] Additionally, probes detected in < 20% of the samples were excluded from the analyses leaving us with N = 346,699 for the analyses. Methylation levels at each CpG locus were expressed as the ratios of intensities arising from methylated CpGs over those arising from the sum of methylated and unmethylated CpGs (beta values).

Section 4: Statistical analyses

Section 4.1: Computation of the blood phenotypic age (PhenoAge) biomarker

Blood phenotypic age (PhenoAge) was calculated as previously described by Levine et al.[6] PhenoAge is a composite score including age, and nine blood measured biomarkers: albumin, creatinine, fasting glucose, c-reactive protein (CRP), lymphocyte percentage, mean cell volume (MCV), red cell distribution width (RDW), alkaline phosphatase, and white blood cell (WBC) count.

Following the instruction by Levine et al., [6] first, we computed a 10-years Mortality Score according with the following formula:

$$MortalityScore = 1 - e^{-e^{xb}\left(exp\frac{(120\gamma-1)}{\gamma}\right)}$$

Where xb is the linear combination of age and the nine biomarkers, with weights listed in the Suppl Table 1 of Levine et al. original paper, and γ is a constant value equal to 0.076927. Then, the PhenoAge was computed according to the following formula:

$$PhenoAge = 141.50225 + \frac{ln(-0.00553 ln(1-MortalityScore))}{0.090164}$$

Phenotypic age acceleration (PhenoAA) was defined as the residuals of PhenoAge on chronological age. Positive values of PhenoAA (i.e., epigenetic age is higher than the chronological age) indicate accelerated aging and vice versa.

Section 4.2: Computation of the epigenetic clocks (DNAm colon mucosa)

DNA methylation age (DNAmAge) was computed as a linear combination of DNAm values of 353 CpGs, with weights (and intercept value) listed in the Additional File 3 of Horvath original publication.[5] Epigenetic age acceleration (DNAmAA) was defined as the residuals of DNAmAge on chronological age. Positive values of DNAmAA (i.e., epigenetic age is higher than the chronological age) indicate accelerated aging and *vice versa*. For sensitivity analyses, we computed two next-generation epigenetic clocks, namely DNAmGrimAge and DunedinPACE,[15,16] and a DNAm surrogate of telomere length.[17] Like the Horvath first-generation epigenetic clock, next-generation clocks are defined as a weighted average of CpGs, with weights listed in the original publications.[15–17] In this study, we computed an updated version of the epigenetic clocks and DNAm surrogate based on Principal Component Analysis (PCA), according to Higgins-Chen et al.[37], which has been shown to improve reliability and reproducibility of estimates across technical replicates.

Section 4.3: Computation of the epigenetic mutation load (EML, DNAm from colon mucosa)

For each CpG, we have examined the distribution of DNA methylation values across all the samples, and we computed the interquartile range (IQR) defined as the difference between the third quartile (Q3) and the first quartile (Q1). Then, we calculated the extreme values as Q1-(3×IQR) and Q3+(3×IQR). We defined individuals carrying a stochastic epigenetic mutation (SEM) for a specific *locus* as those with a DNA methylation level falling outside the interval identified by the boundaries previously defined.[10] Finally, we computed the epigenetic mutation load (EML) as the natural logarithm of the total number of epimutated*loci* across the epigenome for each sample. For comparison with PhenoAge and DNAmAge, EML data were rescaled to be expressed in years as described in Fiorito et al. (2019).[38] Consistently, we computed EML acceleration (EML_AA) as the residual of EML regression on chronological age.

Section 4.4: Association of biological aging biomarkers with CR and EX

The correlation of biological aging biomarkers with chronological age was investigated through Pearson correlation coefficient (R), and the inference test was based on the null hypothesis H0: R = 0, at alpha = 0.05. We investigated differences of the biological aging biomarkers (PhenoAA, DNAmAA, EML_AA, DNAmGrimAgeAA, DunedinPACE, and DNAmTL_AA) in the three groups (CR, EX, and WD) through pairwise Student T-test (alpha = 0.05). Since the distribution of PhenoAA, and EML_AA slightly deviate from the assumption of normality according to the Shapiro-Wilk test (*shapiro.test* R function, $p < 0.05$), dependent variables were Box-Cox transformed before performing the association test, and finally the results re-scaled to be expressed in years via inverse transformation.[39]

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4.   Secondary and tertiary prevention of breast cancer by Mediterranean Diet

Healthy lifestyle including both healthy diet and physical activity engagement improve body composition and nutritional status at different levels and can be considered as pivotal for cancer and metabolic disease prevention and treatment, as promoted by the third expert report on "Diet, nutrition, physical activity and breast cancer" of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) [dietandcancerreport.org] for cancer prevention [1].

Mediterranean lifestyle emphasises together with healthy dietary pattern, a regular physical exercise, which constitutes another key pillar for health promotion with healthy diet (2).

Obesity and overweight are characterised by excessive body fat deposition which promotes a pro-oxidative microenvironment with chronic low-grade systemic inflammation, increasing the risk of metabolic and cardiovascular diseases, and ultimately all-cause mortality. Healthy dietary patterns, weight loss intervention, and reduction of abdominal adiposity, instead, are related to a lower risk of cancer recurrence and all-cause mortality [3], as reported in breast cancer survivors (BCS).

Excessive deposition of adipose tissue exerts local and systemic alterations with elevated levels of free fatty acids (FFA) and triglycerides, increased blood glucose, and insulin resistance, and induces production of adipokines, especially leptin, and inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), TGF-β, that could provide metabolical *stimuli* to the tumour environment [4-5].

Among all types of cancer, breast cancer (BC) represents the most common cancer in women, while overweight or obesity are the second preventable cause of cancer, related to 33% of postmenopausal breast cancers that could be prevented by lifestyle modification [1]. Moreover, breast cancer survivors (BCS) are a group at high risk of second primary cancer or BC recurrence, and all-cause mortality [**3**]. Therefore, body weight management is crucial not only for primary prevention of cancer in general, but also for secondary cancer prevention in breast-specific sites. All women after a BC diagnosis undergo treatments for such a life-changing diagnosis and should be counselled to prevent excessive fat mass deposition, that is often a common event after the diagnosis [6].

To date, there is still a lack of evidence to suggest which dietary strategy is the best among those available after BC diagnosis.Therefore, the purpose of our experimental study was to investigate among BCS the effects of a Mediterranean diet on a long term period.

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4.1 Breast Cancer Diet "BCD": A Review of Healthy Dietary Patterns to Prevent Breast Cancer Recurrence and Reduce Mortality

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ABSTRACT

Breast cancer (BC) represents the most common cancer in women, while overweight and obesity are the second preventable cause of cancer. Weight gain and fat accumulation are common after BC diagnosis; moreover, weight gain during the treatment decreases the survival rate and increases the risk of recurrence in breast cancer survivors (BCS). To reduce the risk of second primary cancer or BC recurrence, and all-cause mortality in BCS, multiple interventions have been investigated to obtain reduction in weight, BMI and/or waist circumference. The aim of this narrative review is to analyze evidence on BCS for their risk of recurrence or mortality related to increased weight or fat deposition, and the effects of interventions with healthy dietary patterns to achieve a proper weight and to reduce fat-related risk. The primary focus was on dietary patterns instead of single nutrients and supplements, as the purpose was to investigate on secondary prevention in women free from disease at the end of their cancer treatment. In addition, BC relation with insulin resistance, dietary carbohydrate, and glycemic index/glycemic load is discussed. In conclusion, obesity and overweight, low rates of physical activity, and hormone receptor-status are associated with poorer BC-treatment outcomes. To date, there is a lack of evidence to suggest which dietary pattern is the best approach for weight management in BCS. In the future, multimodal lifestyle interventions with dietary, physical activity and psychological support after BC diagnosis should be studied with the aim of reducing the risk of BC recurrence or mortality.

INTRODUCTION

Breast cancer (BC) represents the most common cancer in women, while overweight or obesity are the second preventable cause of cancer, related to 33% of postmenopausal breast cancers that could be prevented by lifestyle modification [1].

Excessive body fat deposition and weight gain promote a pro-oxidative microenvironment with chronic low-grade systemic inflammation, posing breast cancer survivors (BCS) at high risk of second primary cancer or BC recurrence, and all-cause mortality [2]. Healthy dietary patterns, weight loss intervention, and reduction of abdominal adiposity, instead, are related to a lower risk of BC recurrence and low-risk BC and all-cause mortality.

The third expert report on "Diet, nutrition, physical activity and breast cancer" of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) [dietandcancerreport.org] for preventing cancer generally recommends maintaining a healthy lifestyle by being physically active and eating a healthy diet for proper weight management [1].

Therefore, body weight management is crucial not only for primary prevention of cancer in general, but also for secondary cancer prevention in breast-specific sites. All women after a BC diagnosis undergo treatments for such a life-changing diagnosis and should be counselled to prevent excessive fat mass deposition, that is often a common event [3].

The relation between overweight/obesity and cancer is still not fully clarified; the tumor environment could be metabolically stimulated by the excess of adipose tissue, with elevated levels of free fatty acids (FFA) and triglycerides, increased blood glucose, and insulin resistance. Increased production of adipokines, especially leptin, and inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), TGF-β could exert local and systemic functions [4]. Breast adipose inflammation, elevated aromatase expression, dysregulated insulin signals, and particularly, increased levels of circulating leptin, raising in proportion to BMI and the total amount of body fat, have been identified as a possible key driver of this intricate network between excessive fat mass and breast cancer [5]. These factors interfering with cell signaling, namely the PI3K-AKT-mTOR pathway, which regulates cell-cycle progression, apoptosis, and protein synthesis can, at least in part, explain in women with high body fat mass the higher risk of cancer progression and metastasis, as well as other site-recurrence (**Figure 1**).

Figure 1. Visual Abstract, the complex interplay between lifestyle modification and healthy weight maintenance/achievement for primary and secondary cancer prevention; healthy dietary patterns together with physical activity and psychological support can affect multiple, and not yet fully known, physiological and pathological pathways related to the excess of adipose tissue (immune system, inflammation, adipokines and inflammatory cytokines, insulin resistance and metabolic homeostasis, cell cycle signaling). Legend: BC breast cancer, DASH dietary approach to stop hypertension, DII dietary inflammatory index, CHFP Chinese food pagoda, MiRNA micro-RNA, IGFs insulin-like growth factors, * further research needed.

With this purpose, multiple interventions on breast cancer survivors (BCS) have been investigated especially in patients with overweight or obesity to obtain weight loss and reduce BMI and/or waist circumference as a proxy for proinflammatory fat accumulation. To date, there is still a lack of evidence to suggest which dietary strategy is the best among those available after BC diagnosis.

The aim of this review is to collect evidence on the risk of recurrence or mortality in BCS related to increased weight or fat deposition and the effects of interventions with healthy dietary patterns aiming to achieve a proper weight, improve waist circumference, or reduce obesity-related risk factors.

METHODS

A search was conducted using keywords: "breast cancer" OR "breast cancer survivors" OR "breast cancer prevention" AND "nutritional therapy" OR "dietary therapy" OR "diet", applying "humans" as a filter, and selecting articles published between 2015 and March 2021 on such databases as PubMed, Google Scholar, MEDLINE, EMBASE, and Scopus. Among 3640 results, 525 were included in the first selection after screening by title and abstract, 61 of which reported data on BCS. Among the latter, full texts of original studies and reviews were screened after exclusion of reports on "cancer" in general, thus only selecting those referring to site-specific "breast cancer". In this narrative review, we assess the effect of dietary patterns to investigate secondary preventions in women free from BC or at the end of treatment. Studies were excluded if carried out on a BC patient on chemotherapy-active treatment, if reporting only single nutrients and/or supplement treatments, and if reporting only adherence to dietary recommendations/guidelines (dietary quality) or healthy patterns but not data on recurrence or mortality risk from BC. When included, "healthy patterns" were specifically described or specified (e.g., Mediterranean, Dietary Approach to stop Hypertension). Studies reporting outcomes other than recurrence or mortality risk (e.g., quality of life or psychological outcomes) were excluded. Eventually, duplicates, abstract without the full text available, reports on mice or basic science, and study protocols were excluded (**Figure 2**).
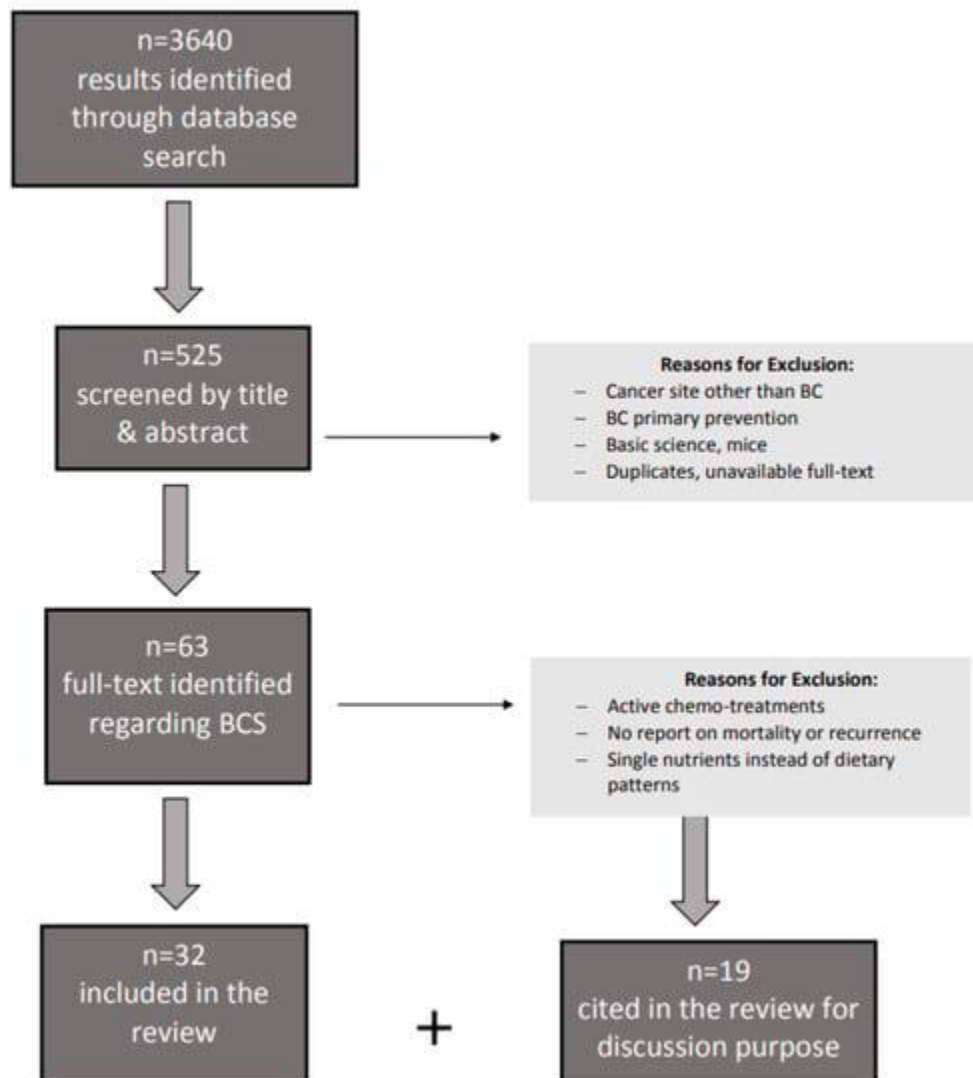
Figure 2. Flow diagram to describe paper identification and selection; Legend: BCS breast cancer survivors.

RESULTS

Among 61 selected papers, only 32 address a specific dietary pattern implemented in BCS not undergoing chemotherapy or any active treatment other than hormonal or immune therapy, aimed at the prevention of recurrence. In **Table 1**, studies analysing the relation between study outcomes and a specific food pattern or dietary index are listed (e.g., Mediterranean, DASH, DII) or a description of dietary characteristics of the intervention (e.g., fruit and vegetable intake). See also **Appendix A**.

Table 1. Trials analyzing outcomes of specific food patterns or dietary indexes in BCS.

REFERENCE	SUBJECTS (n)	METHODS	MAIN OUTCOME		DIETARY INDEX/PATTERN
Sun et al. 7	2295 postmenopausal women with invasive BC	HEI-2010 score assessed over 12-year follow-up in the WHI (Women's health initiative) study	Decreased diet quality after BC diagnosis was associated with higher risk of death from breast cancer		Healthy Eating Index (HEI)-2010
Wang et al. 8	3450 5-year BCS	Adherence scores to CHFP-2007, CHFP-2016, modified DASH, and HEI-2015	Higher adherence to CHFP and DASH dietary guidelines associated with reduced risk of overall death and BCr-specific recurrence or death among long-term BCS		Chinese Food Pagoda (CHFP) Healthy Eating Index (HEI) 2015 DASH diet
Farvid et al. 9	8927 women with stage I-III BC identified during follow-up of the	Assessing postdiagnostic fruit and vegetable consumption in the Nurses' Health Study (NHS; 1980-2010) and NHSII (1991-2011) with FFQ every 4 years	High fruit and vegetable consumption may be associated with better overall survival among BC patients,but not breast cancer-specific		

			mortality. High fruit juice consumption may be associated with poorer prognosis.		
Porciello et al. 10		309 women (stages I-III, mean age 52±1 yrs, BMI 27±7 kg/m2).	HRQoL was assessed with questionnaires measuring physical, mental, emotional and social factors: EQ-5D-3L, EORTC QLQ-C30, EORTC QLQ-BR23.	Higher adherence (PREDIMED score >7) to the MedDiet in BCS is associated with better QoL (physical functioning, sleep, pain, well-being)	Mediterranean Diet
Zucchetto et al. 11		1453 women	Retrospective cohort study FFQ over 12.6 years FU	No association between the inflammatory potential of diet and the survival of BC women.	Dietary Inflammatory Index (DII)
Jang et al. 12		511 women undergoing BC-surgery	213 months follow-up	Anti-inflammatory diets may decrease the risk of cancer recurrence and overall mortality in BCS particularly in younger age, premenopausal status, obesity, HR+, tumor size >2 cm, and lymph node metastasis.	Dietary Inflammatory Index (DII)
Lee et al. 13		364 BC patients and 364 age-matched controls	FFQ	Higher DII scores were related to an increased risk of BC for ER+/ PR+ tumors regardless of	Dietary Inflammatory Index (DII)

			menopausal status, but not for ER-/PR- status.		
Chlebowski et al. 14	48835 postmenopausal women, aged 50-79 years, with no prior breast cancer,	8.5 years FU in the WHI DM trial: Usual diet comparison group (60%) vs dietary intervention group (40%) with reduced fat intake to 20% of energy and increase vegetable, fruit, and grain intake.	Low fat dietary pattern may reduce the risk of death for breast cancer in postmenopausal women.		Low-Fat Diet
Andersen et al. 15	1965 women with BC	FFQ obtained up to three times, pre- and post-diagnostic, over a period of 18 years (median FU 7 years)	Pre-diagnostic intake of oatmeal/muesli was associated with lower all-cause mortality, and post-diagnostic intake of rye bread was associated with higher breast cancer specific mortality		
Castro-Quezada et al. 23	4010 women, aged 60-80 years, at high risk for CVD disease, initially free from BC	137-item FFQ obtained in the PREvención con DIetaMEDiterránea (PREDIMED) study, International Tables of Glycemic Index (GI) and Glycemic Load (GL) values	No associations were found between baseline dietary GI/GL and invasive breast cancer incidence in postmenopausal women		
McCullough et al. 24	4,452 women with locally and regionally staged breast cancer	A nine-point score reflecting concordance with ACS dietary recommendations was calculated pre and p[ost diagnosis	Diets consistent with ACS guidelines were not associated with breast cancer-specific mortality, but with other		ACS recommendations for cancer prevention

			causes of mortality.		
Zheng et al. 30	2,150 postmenopausal women with invasive BC, aged 50-79 years	FFQ on average 1.5 years after diagnosis a median 13.3 years of follow-up	Consuming a more anti-inflammatory diet after breast cancer diagnosis may be a means for reducing risk of death from CVD		Dietary Inflammatory Index (DII)
Wu et al. 34	3042 BCS	Cross-sectional study with dietary intake in the Women's Healthy Eating and Living (WHEL) Study	Positive associations between dietary acid load and CRP and HbA1c in BCS, as strong risk factors for BC recurrence and comorbidities		Dietary acid load (DAL)
Finocchiaro et al. 36	100 BCS	MD intervention with 6 months follow-up	MD is effective in reducing BMI and waist circumference, and enhancing healthy lifestyle in BCS		Mediterranean Diet (MD)
Thompson et al. 37	249 post-menopausal BCS	6-month non-randomized, controlled weight loss intervention with 2 dietary interventions, LFD and LCD	Loss of body weight and fat mass was effective irrespective of dietary approach on a structured program with monthly assessments		Low-fat (LFD) Low-carbohydrate (LCD)

Skouroliako u 38	70 BCS randomized to MD or control group for 6 months	Anthropometric and biochemical parameters (vitamin C, vitamin A, a-tocopherol and CoQ10 levels, dietary intake and adherence to MD	MD ameliorate serum antioxidant capacity, body composition and glycemic profile of postmenopaus al BCS		Mediterranean Diet (MD)
Parada et al. 39	1808 women with invasive BC	Interviews to assess lifestyle and dietary patterns in the Carolina Breast Cancer Study Phases I/II, 13-year FU	The unhealthy (vs. healthy) behavior and diet pattern was associated with all-cause mortality and with BC-specific mortality		
Dittus et al. 41	74 post-menopaus al BCS, age $\leq$ 65 years	A 24-week Internet-based behavioral weight loss (BWL) intervention	Behaviorally based weight loss interventions can result in improvements in biomarkers in BCS who achieved ≥5% weight loss and demonstrated significant improvements in insulin resistance		
Toledo 44	4282 women at high cardiovascular disease risk, aged 60-80 years	Randomized, single-blind,controlled triawithl a low-fat diet (control) vs 2 MD diet intervention 1:1:1 with 4.8 years FU	Beneficial effect of a Mediterranean diet supplemented with extra-virgin olive oil in the primary prevention of BC		Mediterranean Diet

The Role of Diet Quality in Breast Cancer Survivors

Women with BC show a 30% excess risk for second malignancies, even higher if considering contralateral breast cancers [25]. Weight gain during or after BC treatment increases the risk of recurrence and reduces the overall survival rate, while poorer BC survival has been associated with overweight and obesity [26]. While obesity and smoking are associated with cancer recurrence, physical activity represents the strongest driver to reduce BC recurrence and death. A report analyzing BCS stratified by age groups at diagnosis (<65 ≥ years), confirmed that all-cause mortality was significantly associated to BMI and physical activity, regardless if assessed pre- or post-diagnosis (in women ≥ 65 years, pre-diagnosis hazard ratio HR 1.27, 95% CI 1.14–1.41; post-diagnosis, HR 1.19, 95% confidence interval CI 1.04, 1.36); on the contrary, neither pre- nor post-diagnosis physical activity was associated with mortality [27].

In the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort the risk for second malignancies after invasive BC was particularly elevated for colorectal cancer (OR 1.71), lymphoma (OR 1.80), melanoma (OR 2.12), endometrium (OR 2.18), and kidney cancers (OR 2.40), and positively associated with age at first cancer, BMI, and smoking status [25].

Another review investigating the best approach to recommend to overweight or obese BCS found that multimodal weight-loss interventions, including diet, exercise, and psychosocial support, achieved greater reduction in body weight, BMI, and waist circumference and improved overall quality of life more than dietary change alone [2]. Unfortunately, the analyzed reports were highly heterogeneous with a high risk of bias due to the study designs, with low-quality evidence.

Interestingly, a review has analyzed the association between dietary patterns and/or main food groups with mortality and cancer recurrence in survivors of common cancers, including BC, prior to or after cancer diagnosis. Their findings suggest that reducing the amount of body fat after

diagnosis decreases the risk of breast cancer recurrence, and adherence to a high-quality diet, low-fat diet, or prudent diet after diagnosis is associated with a decreased risk of all-cause mortality in BCS. Conversely, adherence to a Western diet before and after diagnosis, is detrimental in terms of overall mortality risk and death from other causes among BCS [28]. Following a "prudent" dietary pattern is associated with a lower risk of overall death and death from causes other than breast cancer, while following a Western diet was a predictor of worse prognosis after breast cancer diagnosis [29].

Accordingly, a large prospective cohort study including 2295 postmenopausal women, the Women's Health Initiative (WHI), with over 12 years of follow-up showed that adherence to a lower quality diet after BC diagnosis, as assessed through the Healthy Eating Index (HEI)-2010 score, increased the risk of death from BC (adjusted HR 1.66, 95% CI 1.09 to 2.52). Instead, a better quality diet (≥15% increase in HEI-2010 score) was non-significantly associated with a lower risk of death, irrespective of change in BMI [6]. A Chinese report collected five-year dietary information in BCS and found that healthy dietary patterns, namely Chinese Food Pagoda (CHFP) and the Dietary Approach to Stop Hypertension (DASH) had 25% to 34% lower risk of total mortality, and 36% to 40% lower risk of BC-specific events for a high score of adherence, while the Healthy Eating Index 2015 was not associated with reduced risks of both overall death and BC-specific recurrence or death among long-term BCS [7].

Another large-scale study enrolled 8927 women with stage I–III breast cancer in the Nurses' Health Study (NHS; 1980–2010) and NHSII (1991–2011 follow-up) and assessed the associations of post-diagnostic fruit and vegetable consumption with BC-specific and all-cause mortality. The total fruit and vegetable and total vegetable consumption was related to lower all-cause [highest vs. lower quintile HR 0.82; 95% confidence interval (CI), 0.71–0.94; *p* for trend = 0.004, and HR 0.84; 95% CI, 0.72–0.97; *p* for trend = 0.001, respectively], but not related to BC-specific mortality. Total fruit consumption was not related to BC-specific or all-cause mortality. Greater intake of green leafy and cruciferous vegetables was associated with lower all-cause mortality, while higher fruit juice consumption, except for orange juice, which was associated with poorer BC-specific and all-cause survival [8].

In addition, higher adherence to healthy and anti-inflammatory dietary patterns, such as a Mediterranean diet, is associated to a higher quality of life [9]; however, an Italian retrospective

cohort study with 1453 BCS did not show any association between the inflammatory potential of diets and women's survival rates for both all-cause and BC-specific mortality after a median follow-up of 12.6 years [10].

To provide insight into the dietary inflammatory index (DII), a study assessed the risk for cancer recurrence and overall mortality after surgery for invasive BC finding significantly higher recurrence (HR 2.347, CI 1.17–4.71) and higher overall mortality (HR 3.049, CI 1.08–8.83) in patients with higher DII scores after adjusting for confounding factors, such as age (<50 years), premenopausal status, BMI (≥25 kg/m²), hormone receptor HR+, tumor size (>2 cm), and presence of lymph node metastasis [11]. A Korean report found a positive relation between DII and receptor positive BC for estrogen receptor (ER)+/progesterone receptor (PR)+ tumors regardless of menopausal status. The breast cancer odds ratio (OR) was higher in the highest DII tertile (OR = 3.68, 95% CI 2.34–5.80, p for trend <0.0001) than in the lowest tertile. Higher DII scores increased risk of (ER)+/(PR)+ breast cancer regardless of menopausal status (OR = 2.59 for premenopausal women; OR = 11.00 for postmenopausal women), but the risk was not increased for ER-/PR- status [12].

A low-fat dietary pattern (≤20% of total energy intake from fat) implemented on a long-term follow-up of 16.1 years was also able to reduce the incidence of deaths after BC in the intervention group compared to the usual diet [13].

A Danish study following up 1965 women for 7 years after BC diagnosis found that pre-diagnostic wholegrain intake of oatmeal/muesli was associated with lower all-cause mortality, while post-diagnostic intake of rye bread was associated with higher breast cancer specific mortality; moreover, a high intake of cheese was associated with a higher recurrence rate [14].

Another study observed the food intake trajectory for 2 years after BC diagnosis. Findings showed only a slight increase in fruit and vegetables and decreased alcohol intake, while total fat intake did not change post-diagnosis, with 45% of survivors maintaining a high-fat diet (fat > 40% of total calories) [30]. Dietary factors were not the only factors influencing diet after diagnosis, but sociodemographic, psychosocial, and other clinical factors, such as education, income, optimism, social support, physical well-being, and neuropathy symptoms also influenced dietary changes after BC diagnosis. The evidence supports the importance of proper psychological and nutrition counseling even after a BC diagnosis is made.

Breast Cancer Relation with Insulin Resistance, Dietary Carbohydrate, and Glycemic Index/Glycemic Load

Dietary patterns characterized by lower post-prandial glucose and insulin responses, those with low glycemic index (GI) and low glycemic load (GL), reflecting quality and quantity of carbohydrate intake, could help explain the reduction of mortality and recurrence risk in BCS. Mechanisms influencing BC development and recurrence include hyperglycemia, hyperinsulinemia, high insulin-like growth factor (IGF)-1, high circulating estrogen, inflammation, and impaired cellular differentiation/apoptosis [31]. Visceral adiposity and hyperinsulinemia have been pointed out as contributors that could promote and/or accelerate carcinogenesis, angiogenesis, and impair apoptosis. Metabolic syndrome and insulin resistance in obesity are characterized by an overexpression of insulin receptors, while insulin-related substrate (IRS), insulin, and IGFs are well-known influential factors for cell proliferation, differentiation, and regulation of the cell metabolism. Most breast cancer cells express IGF1 receptors, and IGF1 levels might be associated with an increased BC risk. Moreover, adipokines such as leptin, adiponectin, and other proinflammatory cytokines like tumor necrosis factor (TNF), interleukin (IL)-6, vascular endothelial growth factor (VEGF), and the hepatocyte growth factor are involved in tumor cell growth, apoptosis regulation, and neoangiogenesis. In addition, TNF-a, IL-6, and leptin can also increase aromatase levels and thus produce more estrogen, while insulin downregulates circulating levels of sex-hormone-binding globulin, thus elevating bioactive estradiol [32].

One meta-analysis of prospective cohort studies showed only a modest association between a dietary pattern with high GI or GL and the risk of breast cancer, even after adjustment for BMI, physical activity, and other lifestyle factors and for menopausal status and estrogen receptor status of the tumor [33]. Another meta-analysis found that GL and carbohydrate intake were positively associated with breast cancer among postmenopausal women with estrogen-negative tumors (relative risk RR for GL 1.28; 95% CI, 1.08–1.52; and summary RR for carbohydrates, 1.13; 95% CI, 1.02–1.25), independent of BMI [34].

A study on Mexican women concluded that total carbohydrate intake was associated with an increased risk of breast cancer among premenopausal women (OR 1.3, 95% CI 1.0–1.7; *p* trend = 0.03) with a positive association only among overweight women (OR 1.9, 95% CI 1.2–3.0; *p* trend = 0.01), but no association in women with BMI < 25 kg/m^2 [35]. On the contrary, a similar analysis in

more than 4000 postmenopausal women with obesity or diabetes at high CVD risk enrolled in the PREDIMED study, and yielded no significant associations of dietary GI and GL with an increased risk for invasive breast cancer in women [15].

DISCUSSION

The association between dietary factors and breast cancer is weak, and etiologic mechanisms are still unclear. Preclinical and clinical data support the evidence that obesity may worsen the incidence, severity, and mortality of breast cancer. The dietary guidelines used as a reference is the WCRF [1], but evidence and guidelines for cancer prevention, are not specific to the BC population, and tend to overlap with DASH or Mediterranean dietary patterns (rich in vegetables, fruits, fish, whole grains, and unsaturated fats (especially coming from nuts and extra-virgin olive oil), with moderate red wine intake and limited intake of red meat and simple carbohydrates) [16,36], or the so-called "prudent" diet (high in fruits, vegetables, whole grains, and chicken) (Table 1).

These healthy patterns rich in fruit and vegetables allow for a high intake of phytochemicals, such as polyphenols, which might reduce the risk of breast cancer incidence and recurrence [37]. In the "prudent" dietary patterns, major sources of polyphenols are olive oil, for phenolic acids (e.g., oleic acid, caffeic, oleuropein, hydroxytyrosol, oleocanthal) fruit and vegetables rich in flavonols (e.g., quercetin, kaempferol, myricetin, isorhamnetin), and isoflavones can be found in soy food. Isoflavones (genistein, daidzein, and glycitein) are considered natural estrogen receptor modulators, and defined as phytoestrogen, which can possibly protect against BC development, recurrence, and mortality. Unfortunately to date, further research in this field must be warranted, but more importantly, nutrients should be seen under a synergic point of view with different foods and different bioactive components interacting in a dietary pattern, and not only in separate analysis. The master regulator role of polyphenols is supported by recent evidence on the green-Mediterranean diet, an amplified version with green plant-based protein and polyphenols from Makai, green tea, and walnuts designed in the DIRECT PLUS randomized controlled trial. This modified version of the diet seems to increase the positive metabolic effects of the Mediterranean diet [38].

BC subtypes are mainly classified by hormonal receptor status: ER+ are BC with preponderance of tumor cells expressing estrogen receptors, and PR+ with cells expressing

progesterone receptors, where HER2 cells carry human epidermal growth factor receptors [1]. Hormone receptor positive cancers are the most common subtypes of BC, while about 10% are found to be hormone receptor negative and show poorer outcomes, being difficult to treat and often of higher pathological grade. Moreover, BC is also classified by menopausal status at the time of diagnosis as pre- or post- menopausal.

Being overweight or obese between the age of 18–30 years has shown an inverse association with the risk of developing pre-menopausal and post-menopausal BC (strong evidence), whereas in contrast, the risk of postmenopausal BC is increased for those with a body weight that is heavier than normal and those who gain weight in adulthood [1]. The mechanisms protecting against premenopausal BC women with greater body fatness are not yet well-known, but include implications of circulating levels of sexual hormones and binding globulin, as well as insulin-like growth factor 1 (IGF-1).

In postmenopausal women with obesity, the higher risk of estrogen receptor (ER)-positive BC is known, but similarly, increased adiposity in postmenopausal women with a normal body mass index also pose them at double risk of invasive BC (HR 2.21, 95% CI, 1.23–3.67). The group in the highest quartiles of trunk fat mass showed higher circulating levels of insulin, C-reactive protein, interleukin-6, leptin, and triglycerides, whereas levels of high-density lipoprotein cholesterol and sex hormone-binding globulin were lower [5]. Increased circulating levels of leptin with reduced levels of sex hormone-binding globulin and elevated expression of the estrogen synthesizing enzyme aromatase can increase the levels of free estradiol and possibly activate ERα stimulating cancer cell proliferation and survival in white adipose tissue accumulation and/or inflammation. Moreover, insulin resistance with dysregulated insulin signaling can activate the PI3K/Akt/mTOR and Ras/Raf/MAPK pathways [5,39,40,41,42].

Nevertheless, overweight and obesity are considered a risk factor for BC development, recurrence, and mortality, irrespective of hormone-receptor (HR) status. Women with obesity have a higher risk of developing triple-negative breast cancer (TNBC) anyway [42], a subtype of BC which, for growth and progression, is independent of estrogen, progesterone, and human epidermal growth factor 2 protein (HER2), and therefore is characterized by poorer prognosis with an increased risk of metastatic disease and lower survival rates. A review and meta-analysis also reported a 29% increased likelihood of death in overweight women with triple-negative breast

cancer (TNBC) compared with patients of normal weight, showing both shorter disease-free survival (HR 1.26, 95% CI 1.09–1.46) and lower overall survival rates (HR 1.29, 95% CI 1.11–1.51) [42]. The connection between TNBC and adiposity could explain the pro-inflammatory microenvironment with a cytokines shift promoting growth and neoangiogenesis, stimulated also by increased levels of circulating free fatty acids [43].

A critical review suggests that nutritional therapy in BC patients should be based on the patients' nutritional status, dietary habits, schedule, activities, and cultural preferences, to meet compliance and dietary adequacy for the improvement of overall health and prognosis [44]. Increased white adipose tissue in overweight and obesity, and increased levels of fat-specific cytokines, above all leptin, have been associated with proliferative signaling, inflammation, angiogenesis, that can sustain BC growth, tissue invasion, and metastasis [4]. High cancer recurrence risk among obese survivors can be driven by inflammatory cytokines, including C-reactive protein (CRP), Interleukins −3, −6, and −8 TNF-α [17]. Although diets with low inflammatory index showed non-BC-specific reduced cardiovascular mortality, compared to higher inflammatory scores, healthier dietary patterns can nonetheless improve overall survival rates in BC patients [45].

Circulating levels of IGFs and their binding proteins have been associated with BC risk, but evidence related to BC prognosis is still limited and inconsistent [46]. The IGF family is affected by fasting and the nutritional state; therefore, more research is needed to clarify the impact of these molecules in BC prognosis and how dietary patterns can affect it in the long term. The ER (+ or −) status of the women under study might also be another determinant in the relation between prognosis and circulating IGFs.

Although the presence of obesity increases the risk of cancer recurrence and death, its causative role is still not clear, and weight loss interventions deserve more research to clarify if benefits can be achieved through a combination of dietary intervention and aerobic exercise or by one intervention alone. A clinical study analyzingtumor markers (e.g., Ki67), gene expression on surgical specimens, blood cytokines, growth and metabolic factors found unclear benefits on tumor biology after pre-surgical caloric restriction in women undergoing a two-arm, single-blinded, randomized controlled and successful weight-loss trial [47]. The same study found some possible benefits of pre-surgical physical activity.

In order to shed some light on the link between BC and excess fat, in women with overweight or obesity, a study identified novel microRNAs associated to BMI and weight loss that could contribute to the development of cancer, identifying multiple pathways associated with cancer, and highlighting potential mechanisms explaining the link between BMI and increased cancer risk [48]. Among a pattern of 35 miRNAs, eight were associated with BMI, including miR-191-5p and miR-122-5p.

Another study worth mentioning is that which investigated the association between dietary acid load and inflammation as well as hyperglycemia in BCS, where a positive association was found in women with highest intakes of dietary acid load who showed a 30–33% increase in CRP and a 6–9% increase in HbA1c levels. This finding could provide a possible link explaining how dietary habits affect both systemic inflammation and hyperglycemia, a well-known risk factor for recurrence and reduction in both overall and disease-free survival in BCS [18]. The same group studied 3081 early-stage BCS enrolled in the Women's Healthy Eating and Living study spanning 7.3 years of follow-up, and reported a higher BC recurrence if baseline HbA1c levels were ≥5.6% (HR 2.15, 95% CI 1.34–3.48 for potential renal acid load PRAL, p-value 0.01, and 2.31 CI 1.42–3.74 for net endogenous acid production NEAP, p-value 0.05) [18].

Weight Gain and Weight Management during and after BC Diagnosis

Weight gain is common after BC diagnosis, and weight gain during treatment decreases the survival rate and increases the risk of recurrence [2,32]. Multiple mechanisms occur and contribute to weight gain and fat accumulation, including physical inactivity, decreased resting metabolic rate, overeating, hormonal changes, and chemotherapy. Obesity/overweight treatments should be provided in BC survivors. Large cohort studies with fairly long follow-up, like WHI [25], suggest the importance of diet quality even after BC diagnosis to decrease specific and overall risk of death. However, to date, evidence on pharmacological or surgical treatment for weight management in BCS needs to be integrated, as data are provided only for small sample sizes and require further investigation [49].

Weight management programs in BCS have been effective in reducing weight, body fat, waist, and hip circumference [2,19,20], and intervention based on Mediterranean Diet are also able to improve serum antioxidant capacity, cholesterol, and glycemic profile [21].

Although the purpose of this review was to focus on dietary patterns, WCRF underscores the importance of a healthy lifestyle comprised of behavioral components such as physical activity and smoking [1]. Accordingly, a study compared unhealthy vs. healthy behaviors and diet patterns in a 13-year follow-up, showing that unhealthy lifestyles, both dietary and behavioral, were associated with all-cause and breast-cancer-specific mortality (HR 1.4 and 1.2, respectively) [22].

Programs to induce weight loss in women after BC diagnosis have been proven to be efficient and feasible in improving anthropometric parameters, quality of life outcomes, and circulating biomarkers [19,20,50]. Significant improvement in insulin resistance biomarkers, measured through fasting insulin, area-under-the-curve insulin, and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index can be achieved with just ≥5% weight loss [23].

Together with metabolic risk and insulin resistance improvements, leptin and adiponectin decrease were correlated with a decrease in BMI and increase in cardiorespiratory fitness [51].

Interestingly, a longitudinal study followed BCS in five survivorship periods since diagnosis (years ≤ 3; 3 to ≤6 years; 6 to ≤9, 9 to ≤12, and 12 to 15 years) and found that non-drinker and non-smoker BCS slightly increased after diagnosis, and only in the recent survivorship period were BCS significantly more physically active and they consumed more fruit, but were less likely to be classified in the healthy weight range ($p < 0.01$) and have increased total energy [52]. Therefore, long-term support for behavioral change and nutrition counseling to maintain healthy lifestyle choices and healthy weight in BCS may be helpful after BC diagnosis.

In the end, many studies have reported an improvement in multiple quality of life parameters in BCS following healthy dietary patterns. Discussion of these studies goes beyond the purpose of our review, but this evidence represents further important support for multimodal and nutrition counseling for BCS.

The main limit is that this is not a systematic review, therefore some bias of selection and interpretation might be present, as it does not strictly follow the PRISMA checklist statement. Another limit is that we included in the discussion studies enrolling non-triple negative breast cancer (TNBC) patients undergoing hormonal or immune therapy to prevent cancer recurrence, while TNBC patients are not always eligible for such protocols. Therefore, this may cause another interpretation bias, and may explain, at least in part, the worse outcomes in patients with TNBC. In the end, the small amount of reports included in the review does not allow to clearly select a

specific diet to recommend in BCS and to respond to the main aim of the study. Nevertheless, this limit underlies the importance of the work itself as a trigger for more specific research on nutritional therapy for the prevention of recurrence in BCS, as well as in other cancer sites.

To date, studies on BCS are not enough to suggest which is the best diet to prevent recurrence of cancers of the breast and other sites after a cancer diagnosis. Nevertheless, Mediterranean [52] and other healthy and anti-inflammatory dietary patterns [11,27,29,36,37] have proven efficacy in primary prevention, and therefore might be taken as a reference to design future trials in BCS.

CONCLUSION

Obesity and overweight, lower rates of physical activity, and hormone receptor-status subtype are associated with poorer BC treatment outcomes. To date, there is a lack of evidence to suggest which dietary pattern is the best approach for weight management in BCS (**Figure 1**). In the future, multimodal lifestyle interventions with dietary, physical activity and psychological support after BC diagnosis are essential to meet the goal of reducing the risk of BC recurrence or mortality.

APPENDIX: Dietary index and dietary patterns description.

The Healthy Eating Index (HEI) measures the quality of diet following key recommendations of the Dietary Guidelines for Americans (ages 2 years and older). Developed in 1995, HEI has been periodically updated (HEI-2005, HEI-2010) with the latest version HEI-2015. A high total score reflects the overall diet quality with a maximum of 100 points. (https://www.fns.usda.gov/healthy-eating-index-hei)

The Dietary Inflammatory Index (DII) estimates the inflammatory potential of diet by filling a food frequency questionnaire. Proinflammatory diets are related to chronic inflammation, a risk for chronic diseases including cancer, diabetes, and cardiovascular disease.

The DietaryAcid Load (DAL) represents the food potential to produce acid or base in the body: foods rich in protein, such as meat, cheese, eggs..., increase the production of acid, whereas fruit and vegetables increase alkalis. It can be calculated by the Potential Renal Acid Load (PRAL) of food.

The Chinese Food Guide Pagoda (CFGP), proposed by the board of Chinese Nutrition Society, was first released to the public in 1989. It represents the Chinese Dietary Guidelines (CDGs) for general Chinese population aged 2 years and above. Its latest update was in 2016.

The Dietary Approaches to Stop Hypertension (DASH diet) is a healthy-eating plan mainly developed to help, treat or prevent hypertension and reduce the risk of cardiovascular disease. It is rich in fruits, vegetables and lean proteins, and restricts red meat, salt, added sugars and fat.

The Mediterranean Diet (MedDiet) was recognized by UNESCO as intagible cultural heritage of humanities in 2010. This dietary pattern is characterized by abundance of fruit, vegetables, legumes, cereals and nuts, noteworthy is the usage of olive oil, and the frequent consumption of fish, moderate consumption of dairy derivates and low consumption of red meat and simple sugars. (https://www.med-diet.eu/)

The term "Prudent Diet" has been in use since 1957 to describe a fat- and cholesterol-controlled diet. It is a generic term to identify healthy dietary patterns (including MedDiet and DASH diet) characterized by high intakes of whole grains, fruits, vegetables, legumes, nuts, fish, and low-fat dairy products, with low intake of processed foods, red meats, high sugar products and fats.

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4.2 Weight management in breast cancer survivors: effects of a Mediterranean diet intervention in real-life

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SUMMARY

Overweight and obesity are the second preventable cause of cancer, increasing the risk of its recurrence and poor outcomes, especially for breast cancer. When treated with a lifestyle intervention, breast cancer survivors may show different outcomes compared to generic women population and specific causes of drop-out need to be further investigated. The aim of this retrospective study was to investigate whether a Mediterranean diet contributed to weight management in BCS. The secondary aim was the identification of biological or anthropometrical predictors of dropout in this sample. We displayed that BCS overweight or with obesity treated with an hypocaloric Mediterranean diet that concluded the 12-months follow-up (34% of 182 women) significantly lost weight and improved their lipid profile. Moreover, lower age and higher diastolic blood pressure at baseline were found significant predictors of dropouts at 12 months. Understanding these predictors could help clinicians identify individuals who may be at a higher risk of discontinuing the intervention and design tailored strategies to support their adherence and engagement.

ABSTRACT

Background: Reducing obesity and weight gain, that often occurs during breast cancer (BC) treatment, may represent an efficient secondary and tertiary prevention against cancer. Purpose: This retrospective observational cohort study aimed to assess the impact of a Mediterranean diet on weight and anthropometric changes in women completing active breast cancer treatment. Additionally, we sought to identify factors associated with study dropout within one year.

Methods: A total of 182 female patients (20 normal weight, 59 overweight, 103 obese) received personalized Mediterranean diet interventions and underwent monthly outpatient visits.

Results: Dropout rates were 42.3% at 6 months and 64.1% at 12 months. Among the subgroup with obesity, BMI (p<0.001) and fat mass (p<0.05) decreased at 6 months. At 12 months, the subgroup with obese showed a borderline significant further reduction in BMI (p=0.062). BMI or weight loss did not predict dropout at any time point. However, age (OR=0.93) and diastolic blood pressure (OR=1.06) were significant predictors of dropout at 12 months.

Conclusion: Implementing a Mediterranean diet can lead to weight and anthropometric improvements in breast cancer survivors. Further research is necessary to explore the long-term effects of weight loss on these individuals and identify effective dietary approaches, also considering specific predictors of dropout.

INTRODUCTION

Overweight or obesity are the second preventable cause of cancer, being related to 20% of diagnosis. About 33% of postmenopausal breast cancers could be prevented by lifestyle modification[1-2]. Excessive body fat and weight gain in women, together with physical inactivity and alcohol consumption show the strongest evidence for the risk of breast cancer.

White adipose tissue (WAT) expansion and hypertrophy promote hypoxia, a pro-oxidative and pro-inflammatory microenvironment with immune cell infiltration leading to cell death and inflammation. A chronic low grade systemic inflammation, together with excessive expansion of visceral adipose tissue contribute to insulin resistance and alter hormonal pathways, promoting tumor cells growth and metastasis[3].

Adiposity per se may not be specific enough for the risk assessment of breast cancer, while metabolic parameters of health (e.g., insulin resistance index as HOMA-IR or fasting insulin) may be more biologically accurate for breast cancer risk stratification, reporting strong impact on the risk in postmenopausal women[1-5].

The dysfunction of visceral fat may be reversed by weight loss, improving those factors contributing to the development of cancer such as insulin resistance, pro-inflammatory molecules and metabolites, circulating growth factors hormones and adipokines[6]. Fighting obesity and weight gain, that often occurs during breast cancer treatment, may represent an efficient secondary and tertiary prevention against cancer. At the same time, it would help in primary prevention of chronic non-communicable diseases, including diabetes and cardiovascular disease, and it would contribute to healthy aging[7]. Metabolic syndrome, associated with central adiposity and insulin resistance, is highly prevalent in breast cancer survivors (BCS)[5,8-9], while long term trials on weight management are needed to investigate metabolic improvements, recurrence prevention and impact on mortality and quality of life in breast cancer treatments.

To maintain a healthy weight or achieve weight loss, several dietary patterns that are associated with a reduced intake of saturated fats and an increased intake of fibers, such as the Mediterranean diet, are associated with a reduced breast cancer risk, especially for the estrogen receptor negative (ER) subtype, the one at poorer prognosis among breast cancer[11].

The Mediterranean dietary pattern affects several important pathways improving metabolic health: (I) has a lipid-lowering effect, (II) confers protection against oxidative stress, inflammation, and platelet aggregation, (III) induces modification of hormones and growth factors involved in the pathogenesis of cancer, (IV) inhibits nutrient sensing pathways by specific amino acid restriction, (V) produces gut microbiota-mediated end-metabolites[12-13]. Diet composition influences not only systemic inflammation, but also diversity, enrichment and composition of gut microbiota and, at the same time, can promote metabolic health counteracting inflammation and cancer progression[13-15].

Although evidence suggest that BCS with overweight or obesity have higher all-cause mortality and increased risk of cancer recurrence with lower quality of life, there is still a lack of data to explain the impact of weight loss in this group of women and which dietary pattern would be the best approach for weight management in BCS [2,16].

While obesity interventions report high attrition rates, the need to maximise treatment retention in BCS with overweight or obesity requires the knowledge of factors associated with high risk of drop-out since the beginning of the weight loss to maintain the efforts[17]. Existing knowledge in this field, instead, reports a high variability of attrition rate with very little consistency of definitions and measurements of drop-out predictors across the studies[17-18]. Moreover, no data are available concerning BCS.

The primary aim of this study was therefore, to investigate whether in this particular cohort of women at the end of the active treatment for breast cancer (BC) the implementation of a Mediterranean diet achieves weight and anthropometric improvements. The secondary aim was to identify if any of the investigated parameters was linked to the dropout rate within the study period of one year.

MATERIALS AND METHODS

Patients

Between June 2014 and December 2020, patients diagnosed with BC by the Oncology Department were referred and enrolled by the Dietetics and Clinical Nutrition Unit at AOU "Maggiore della Carità" in Novara, Italy, in this single-center, retrospective, observational cohort study, and were followed up for 2 years.

The study was approved by the Internal Review Board and Ethics Committee of the hospital and performed in accordance with the current legislation on Observational Studies and the Declaration of Helsinki, to approve the retrospective utilization of the outpatient data (CE 124/2022), and to allow future contacts with the subjects for further investigations.

To be eligible for the study women were at their first diagnosis of BC, defined free-from disease (referring to the end of their cancer therapy after surgery, and after the end of chemo and/or radio therapy; oral hormonal therapy could be in place during the study). Included patients' age ranged between 30 and 80 years old. Exclusion criteria were a second cancer diagnosis, either breast or other site cancer, and any acute-state disease. Any psychiatric disorder that could impair the ability to freely consent or comply to the study requirements and dietary therapy. Subjects exercising $\geq$ 300 min per week were excluded to avoid potential confounding effects of high-level physical activity. Smoking, alcohol or drug abuse were also considered reasons for exclusion.

Drop-out patients were considered those not showing up at the main time point visits: T2 (6 months), T3 (12 months) and T4 (24 months) and for the rest of the follow-up visits. If a patient did not show up within the monthly visits, or at T2 and/or T3, but showed up later collecting data at T3 we did not consider that patient as a drop-out.

Patients were considered with normal weight if their BMI was lower than 25 Kg/m^2, or overweight if the BMI was $\geq$25 Kg/m^2 and < 30Kg/m^2; patients with obesity had BMI $\geq$30 Kg/m^2 divided in class I (BMI $\geq$30-34.9 Kg/m^2), class II (BMI $\geq$35-39.9 Kg/m^2), and class IIII (BMI $\geq$40 Kg/m^2).

Intervention & dietary characteristics of the traditional Mediterranean Diet

At baseline (T1) a physician and a dietitian conduced all the assessments and followed patients up with monthly visits. After a clinical screening and evaluation (see following), patients received a dietary intervention based on the Mediterranean Diet (MED) pattern. The amount of calories provided with the diet was eucaloric, for normal weight subjects, after a measurement through a 24hr recall. For women with overweight or obesity, the dietician formulated a hypocaloric diet aimed to obtain a target of 8-10% weight loss, applying a 500-1000 Kcal deficit from the estimated energy requirement, calculated as a mean between the 24hr recall and LARN 2014 formulas[19]. The MED diet was personalized and was constituted of 50-55% of total daily energy (TDE) from carbohydrates, <10% of TDE from sugars, 0.8-1.2 g/Kg/ideal body weight of proteins and about 30% of TDE from fats, with Extra Virgin Olive Oil (EVOO) as main source of fats and fish as source of omega-3 fatty acids—eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).

Qualitative characteristics of the MED were: 1) Minimally processed whole grains and legumes as the staple food; 2) plenty of a huge diversity of fresh and seasonal vegetables consumed on a daily basis and fresh fruits; 3) main fat used as condiment represented by cold-pressed extra virgin olive oil, seldom or never by butter and cream, and occasionally fat derives from nuts and seeds; 4) moderate consumption of fish (2-3 times weekly); 5) Low consumption of dairy products (mainly local cheese, milk and yogurt) ; 6) red and processed meat consumed in very low frequency (only once every week or two) and amounts; 7) alcohol mainly represented by wine, consumed in low to moderate amounts (one glass per day for women and two glasses per day for men), preferably during meals[20].

If the target weight loss was reached before the end of the 2-year follow up, the diet was recalculated in order to obtain a maintenance of the results, avoiding weight regain.

Anthropometric and biochemical measurements

At baseline (T1) data collected for analysis included clinical history, medications, anthropometrics (height without shoes was measured to the nearest 0.1 cm using a Harpenden stadiometerand body weight was measured with light clothing to the nearest 0.1 kg using an electronic scale;BMI was calculated in kg/m2 as body weight divided by squared height; waist circumference was measured with a non-elastic measuring tape in the mid-point between the lowest rib and the iliac crest during expiration and recorded to the nearest 0.1 cm), and blood pressure measured by

digital instrument in mmHg after participants had been sitting quietly for at least 15 minutes, with their right arm being supported at the level of the heart, and feet resting flat on the floor.

Every 1 or 2 months for a period of 24 months, a dietitian collected dietary records by a 24hr dietary recall, along with anthropometrics; medication modifications were recorded along the study, while subjects were instructed to keep physical activity at the same level during the study, to avoid any bias in the data interpretation, as its role is beneficial in cancer prevention and contributes to weight loss as well.

In addition, at the baseline (T1) and after 6-12 months (T2-T3) body composition assessment was performed through bioimpedance analysis to evaluate fat mass, fat-free mass and hydration status using a BIA 101 Akern, while blood samples obtained after 12-hour overnight fasting were tested, using standardized methods in the Hospital's Laboratory, including the following biochemical parameters: glucose, insulin, HbA1c, lipids (total cholesterol, HDL-cholesterol, and triglycerides), liver function test. Insulin-resistance at fasting was calculated using the formula of Homeostasis Model Assessment -Insulin Resistance HOMA-IR [Fasting Insulin (μUI/ml) x Fasting Glucose (mg/dL)]/22.5].

Statistical analysis

Descriptive characteristics of sample divided in BMI subgroups were expressed as mean ± standard deviation or as median with interquartile range depending whether the variables were having a normal or non-normal distribution. Non-normal variables were transformed into lognormal distributions accordingly. Statistical differences between subgroups were calculated with one-way ANOVA test and post-hoc analysis with Bonferroni. Wilcoxon signed-rank test was performed for calculating statistical differences between different time points.

To calculate the sample size, the main outcome considered for the analysis was the low-calorie Mediterranean diet induced weight loss, considered similar to other published literature[21]. Therefore, a sample of 59 subjects was sufficient to show a reduction of 2.2 Kg above the upper limit of the normal range (ULN) with a standard deviation (SD) of 15 Kg and a 90% power, 95% significance for a $p < 0.05$.

Since the drop-out rate was 85.4 % at 24 months and only 26 patients showed up at that follow-up visit,we analyzed only patients with data at 1 year. After investigating whether BMI class was associated with dropout probability at 6 and 12 months with Chi-squared test, dropout analysis was carried out by analyzing statistical differences (with independent measures T-test) among baseline parameters in patients that dropped out vs completers at 6 months or at 12 months. A logistic regression model for analysis of multivariate was used to identify predictors of attrition and results are presented as odds ratios. Results were considered significant at $p < 0.05$. Variables were included into the model given their availability, importance and clinical relevance. Statistical analysis was carried out with Statistical Packages for Social Sciences (SPSS, Chicago, IL, IBM) version 25.

RESULTS

During our study period, dietary intervention was provided to 182 female patients, out of which 20 were normal weight (NW), thus receiving a normocaloric MED diet, while 59 were overweight (OW) and 103 had obesity (OB), who were treated with a hypocaloric MED diet according to their needs. In the whole sample, mean age was 54 years and mean BMI was 31.6 Kg/m2. Table 1 illustrates the demographic characteristics of the participants at the beginning of the study and statistical differences between BMI subgroups.

After the first 6 months, 77 subjects (42.3%) had discontinued treatment and four patients deceased. Out of the 101 that continued, BMI was reduced in all subgroups (Table 2, Figure 1), in particular for OW (from median value 27.4 to median value 26.6 kg/m2; p<0.001) and OB (from median value 33.5 kg/m2 to median value 31.8 kg/m2; p<0.001). In the OB subgroup, it was also registered a reduction in FM% compared to baseline (Δ= -3.30 ± 3.22 %; p<0.05), an increase in HDL cholesterol (p<0.05), and a decrease in triglycerides (p<0.05).

No difference was seen in the drop-out rate at 6 months between different initial BMI subgroups (χ2 = 3.099; p = 0.212). Prevalence of NW/OW/OB and attrition rates are displayed in Table 3. Since BMI was not independently linked to attrition, after dividing the whole sample into completers and drop-outs, also the investigation of attrition differences showed no statistically significant results (Table 4).

At 12 months, 61 patients (33.9% of initial sample) had continued the study and 40 (21.9% of initial sample and 34.2% of all dropouts) were lost at follow-up. Patients that continued the study that were in NW and OW subgroup at start maintained the same BMI between T2 and T3 (p= ns), while subjects in the OB group, even if the difference between medians was not statistically significant, appeared to have additionally reduced their BMI (from T1 median value BMI-OB 33.7 to T3 median value BMI-OB 31.9 kg/m2; p = 0.062) (Figure 2).

Also in this situation, there were no observed differences in the dropout rate at 12 months among the BMI subgroups. (χ2 = 1.120; p = 0.571). Nevertheless, the analysis of differences at baseline according to T3 completers and drop-outs showed that age (p<0.05), HDL cholesterol (p<0.05) and diastolic blood pressure (DBP) (p<0.05) were statistically different (Table 5), while weight loss or BMI loss were not different in any BMI subgroup (p=ns). Finally, multivariate logistic analysis

evidenced that lower age (OR = 0.93; CI95%: 0.87-0.99; p<0.05) and higher DBP (OR = 1.06; CI95%: 1.02-1.11; p<0.05) at baseline were significant predictors of dropouts at 12 months.

At 24 months, only 26 patients (14.6% of initial sample) continued the study for the last visit. Only 2 patients were part of the NW group (7.7% of the whole sample and 10.5% of the NW original group), while OW and OB group had 7 and 17 women continuing till 24 months (26.9% and 65.4% respectively of the whole sample, and 12.3% and 16.7% of their original group). Due to thehigh attrition, we did not analyze data from these patientsas resultswould be underpowered.

DISCUSSION

Obesity is considered a risk factor for breast cancer (BC) occurrence and worse prognosis[22-24], while healthy dietary patterns have been associated to a decreased risk of BC, especially in postmenopausal, hormone-receptor negative women[11,25]. Nonetheless, there is still a lack of data to explain the impact of weight loss in BCS [2,16.]

Our study demonstrates that a Mediterranean diet in women after a breast cancer treatment is feasible, even with a long-term follow-up of 12 months, allowing a reduction in body weight, BMI and waist circumference. These results are fairly similar to other studies implementing lifestyle interventions[21].

While studies with samples larger than ourshave also showed the efficacy of weight loss programs delivered even remotely, we were able to demonstrate a significantly reduced fat mass (Δ= -3.30 ± 3.22 %; p<0.05) after six months of weight management in BCS with obesity. Moreover, improvements in HDL cholesterol and triglycerides were also evident, although we failed to demonstrate statistical significant variation in other metabolic parameter after 6 months. This can be considered very similar to what Campbell[27] showed in a small sample of 14 OW and OB BCS losing body weight (3.83 ±5.0, p< 0.02), fat mass (1.4 ±1.9, p< 0.02) and reducing waist circumference (4.2 ± 6.6, p< 0.05), although Campbell enrolled only women up to 35 Kg/m2 of BMI (class I obesity). In our results the baseline BMI group was not related to the drop out rate at 6 months, and no predictors could be related to the likelihood of dropout. This seems discordant to other reports that relate lower BMI to higher drop-out rate[28] or higher expected 1-year BMI loss to attrition rate[29-31]. These discordant results could be secondary to the setting, considering the psychological pressure of having a cancer history and different health expectations apart that of weight loss per se.

We also demonstrated that between 6 and 12 months the BMI decreased in the OB group, while the weight loss was maintained stable for NW and OW group. No difference among BMI groups was revealed in the dropout both at 6 and 12 months. It may be suggested that initial expectations and mental status at baseline are predictive factors to take care of, more than basal weight by itself.

Younger age in multiple studies is a determinant of drop out and our results are consistent with these findings[32]. A large randomized controlled trial in BCS seems in agreement with our finding that younger women are more likely to drop a weight management program, indeed authors demonstrated alsoa better weight loss in women aged 55 years or older than in younger age-groups[33].

In the end, the analysis of our sample on T3 completers (at 12 months) highlighted a difference in age (younger) and DBP (lower) for the drop out both at 6 and 12 months, while higher HDL was significant only at 6 months. Therefore, we could postulate that probably those younger and better "fit" subjects may be less engaged and motivated in a weight management program or may not see it as a treatment priority during or after the BC treatment, missing the prevention point of view.

Regardless uncertainties in mechanistic pathways, oncology societies still recommend weight-management to reduce body fat mass as a cancer prevention tool in clinical practice[7, 34]. Notably, overweight and obese state have been reported to influence treatment outcomes, in terms of cancer treatment regimens (dose cap chemotherapy, less radical surgery), differential pharmacokinetics and toxicities, surgical peri-operative complications[35-36]. The aim of our drop-out predictors analysis would be to promote retention in weight management program, in order to support BCS in the long-term management of the weight loss.

Importantly, positive effects of exercise on weight loss and improvements on insulin resistance and insulin signaling have been shown[37-40]. In our study we decided to keep the physical activity level (PAL) constant throughout the study with the exclusion criteria of more than 300 min per week of exercise. At the end of the enrollment, we did not exclude any subject as all women fell into the sedentary PAL (data not shown).

Notably at 12 months (T3) only about 34% of the initial sample showed up at the control visits. We noticed a high variability of attrition rate reported in published weight loss programs, ranging between 10% and 80% [41-43]. The wide heterogeneity of definitions and difference in measurements for pre-treatment predictors and post-treatment reasons of drop out, could explain the little consistency across the studies [44-45] and represents the main burden to compare studies, limiting the ability to improve retention by study design[18].

In addition, weight gain is common in our specific population of BCS, who underwent BC and related oncological treatments. Therefore, BCS bring a different background, medication prescriptions and higher motivation to engage in weight management compared to the general population. Motivation to follow a healthy and anti-inflammatory dietary pattern can be stronger and retention in dietary intervention can be higher in BCS [46], but at the same time they can experience more physical and psychological barriers to deal with, during and after BC treatment [47]. Within the general population it seems that women, but not men, with cancer history are less likely to drop from a weight management program[48]. Nonetheless, our sample attrition rate and weight loss fall within the range reported in other studies on general population living with obesity and related comorbidities[49-50].

Among the reports in the general population, the most common and consistent predictors of attrition were: younger age, lower age at first dieting, higher weight loss expectations with unrealistic goals[18,29-30] while non-smokers and those achieving a good weight loss at the beginning of the study (e.g. >5% within the first 6 months) less likely dropped out [42, 51-54]. Notably, the length of the trials may be related to the likelihood of dropping out, therefore some studies divided the analysis in short-term completers and full-length completers, confirming the previous findings and adding financial reasons and depressions as predictors of drop-out [55]. A particular study reported a very little attrition of only 8.8%, but it could not be compared to our sample since participants were provided with monetary incentives [56].

Commonly, the most frequent variables analyzed in the current literature to investigate on attrition reasons are dietary composition, psychological predictors and demographic information. Although our study is only exploratory with a post-hoc analysis of data collected at the end of a long-term follow-up, it can be can be considered a pilot study to design better and more detailed studies in the future, as it represents (to our knowledge) the only one in breast cancer patients analyzing body composition by bioimpedance, and reporting multiple metabolic parameters that could provide information on those characteristics to identify possible predictors of drop-out. All these data support the need of recall systems after 6-12 months in outpatients engaged in a lifestyle program also apart clinical trials to be able to reproduce their benefits into common clinical practice and different intervention settings.

Some limitations should be noted for our study.First, the major limit of our study is that follow-up span was too short to highlight any beneficial effect in terms of cancer prevention and/or mortality, with only a positive trend in some metabolic parameter that didn't reach statistical significance. Other limits of this study are the small sample size at T3 (1-year follow-up), as our subjects' retention halved at every time point, ending at 2 years with 26 BCS only (data not showed). Some missing information include the menopausal state and the measurement of inflammatory parameters, such as C-Reactive Protein, or inflammatory cytokines such as IL-6 or TNF- α. Another important limit of this study is that we did not record the level of physical activity (PAL) during the study and this can be considered a bias to analyze the metabolic improvement weather subjects increased their PAL after enrollment. Nonetheless, all the subject screened and included in the study were referring a sedentary lifestyle, therefore they were all included in the sample. To investigate on psychological determinant for distress, anxiety, or depression, specific psychometric testing could have been also collected. Similarly, although we have many information available in the patients' chart, we didn't include in the drop out analysis social determinants that may impact on the long-term follow-up [32]. In the end, quality of life represents another important field to be explored but we did not give any pre or post intervention questionnaires to gather information about it.

Concluding, only larger and longer (and expensive) epidemiological studies could substantially support in the future the importance of dietary intervention in cancer prevention, specifically showing the impact of Mediterranean diet to reduce BC risk [57].

CONCLUSION

In this retrospective study in women at the end of their active treatments for breast cancer the implementation of Mediterranean diet induced weight loss, BMI and waist circumference reduction at 12 months in a sample composed by different groups of BMI from normal weight to morbid obesity. Moreover, fat mass reduction and improvements in HDL cholesterol and triglycerides was showed in the group of women with obesity.

Drop out analysis revealed also that initial BMI was not independently linked to attrition, while younger age, higher HDL and lower DBP were related to the likelihood of future dropout.

In the future, our findings should be confirmed by randomized trials with appropriate sample size and follow-up length or multicentric real-life studies, to investigate lifestyle interventions including dietary modification, physical activity and psychological support after BC diagnosis in cancer primary prevention.

Table 1. Baseline characteristics of sample divided in BMI subgroups

	NW	OW	OB	P-value
Patients (%)	**20 (11%)**	**59 (32.4%)**	**103 (56.6%)**	
Age (y)	52 (15)	51 (10)	53 (17)	Ns
BW(Kg)	61.9 (7.2)	71.2 (7.8)	87.7 (15.8)	<0.001[1]
BMI (Kg/m2)	23.7 (1.1)	27.6 (2.4)	34.1 (5.5)	<0.001[1]
Waist (cm)	88.8 ± 6.4	98.5 ± 6.6	112.9 ± 9.8	<0.001[1]
PA (°)	5.1 ± 0.47	5.2 ± 0.4	5.2 ± 0.5	Ns
FM%	29.8 ± 3	34.4 ± 4.5	43.3 ± 4.6	<0.001[2]
ECW %	50.4 ± 2.6	49.6 ± 2.4	49.7 ± 2.8	Ns
Glucose (mg/dL)	85 (11.5)	88(13.5)	96 (17)	<0.001[3]
Insulin (µUI/mL)	8.7 ± 6.2	11.7 ± 11.6	15.5 ± 9.2	Ns
HOMA index	1.79 ± 1.26	2.59 ± 2.5	4.0 ± 3.0	<0.05[4]
Tot Chol (mg/dL)	204.8 ± 39.4	206.4 ± 35.6	207.9 ± 40.4	Ns
HDL(mg/dL)	55 (18)	56.5 (17.5)	50.5 (20)	Ns
LDL (mg/dL)	122.8 ± 31.7	124.0 ± 28.6	125.6 ± 39.3	Ns
Triglycerides (mg/dL)	91 (69.2)	106 (87)	130.5 (96)	Ns
SBP (mmHg)	120 ± 8.9	117.9 ± 15.7	129.1 ± 15.3	<0.01[5]
DBP (mmHg)	76.2 ± 6.9	72.9 ± 9.4	80.3 ± 11.9	<0.05[5]

p-value expresses statistical significance between the subgroups. [1]= p<0.001 between each couple; [2]= p<0.05 between NW and OW, p<0.001 between NW or OW and OB; [3]= p<05 between NW and OB, p<0.01 between OW and OB; [4]= p<0.05 between OW and OB; [5]= p<0.01 between OW and OB. Data expressed as mean ± standard deviation for normally distributed variables or as median (IQR) for non-normally distributed variables. NW = Normal weight; OW= Overweight; OB = Obese. BW = Body Weight; BMI = Body Mass Index; Waist = Waist circumference; PA = Phase Angle; FM% = Fat Mass %; ECW% = Extracellular Water % of Total Body Water; HOMA = Homeostatic Model Assessment; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; SPB = Systolic Blood Pressure; DBP = Diastolic Blood Pressure.

Table 2. Baseline descriptive characteristics in completers at 6 months and comparison with values at 6 months

	NW		OW		OB	
	T1	T2	T1	T2	T1	T2
BW(Kg)	61.9 (3.0)	58.8 (3.7)$^{\circ}$	71.0 (5.4)	66.5 (7.4)$^{\circ\circ\circ}$	85.2 (16.8)	79.0 (16.0)$^{\circ\circ\circ}$
BMI (Kg/m2)	24.0 (1.4)	23.0 (1.6)$^{\circ}$	27.4 (2.6)	26.6 (2.1)$^{\circ\circ\circ}$	33.5 (4.8)	31.8 (5.7)$^{\circ\circ\circ}$
Waist(cm)	87.6 ± 9.1	84.6 ± 7.2*	97.9 ± 5.7	94.2 ± 7.6***	111.8 ± 8.4	106.9 ± 9.2***
FM% (% of BW)	28.7 ± 0.5	28.4 ± 1.3	34.1 ± 7.1	33.2 ± 7.2	42.7 ± 4.4	39.4 ± 6.1*
ECW% (% of TBW)	51.1 ± 4.0	52.2 ± 2.2	49.5 ± 2.5	49.2 ± 1.3	48.8 ± 3.3	48.2 ± 5.8
PA (°)	5.0 ± 0.7	4.8 ± 0.4	5.2 ± 0.4	5.3 ± 0.2	5.4 ± 0.6	5.6 ± 1.3
Glucose (mg/dL)	82.0 (7.0)	83.0 (8.0)	88.5 (14.7)	90.0 (17.7)	96.0 (17.0)	97.0 (20.5)
Tot Chol(mg/dL)	222.0 ± 28.3	206.5 ± 6.4	212.9 ± 38.3	207.2 ± 35.7	207.5 ± 39.6	200.5 ± 31.4
HDL(mg/dL)	54.0 (5.7)	55.0 (4.8)	57.0 (19.0)	53.0 (24.0)	50.5 (18.5)	52.5 (13.7)$^{\circ}$
Triglycerides (mg/dL)	77.0 (132.2)	78.5 (68.4)	97.0 (86.0)	95.0 (110.0)	136.5 (94.0)	113.5 (73.5)$^{\circ}$

Data expressed as mean ± standard deviation for normally distributed variables or as median (IQR) for non-normally distributed variables. °,°°,°°° = difference between T2-T1 medians evaluated through Wilcoxon Signed-Rank Test. *,**,** = difference between T2-T1 means evaluated through T-test for paired samples. NW = Normal weight; OW= Overweight; OB = Obese ; BW = Body Weight; BMI = Body Mass Index; Waist = Waist circumference; PA = Phase Angle; FM% = Fat Mass %; ECW% = Extracellular Water % of Total Body Water; HOMA = Homeostatic Model Assessment; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; SPB = Systolic Blood Pressure; DBP = Diastolic Blood Pressure. Data expressed as mean ± standard deviation for normally distributed variables or as median (IQR) for non-normally distributed variables. °,°°,°°° = difference between T2-T1 medians evaluated through Wilcoxon Signed-Rank Test. *,**,** = difference between T2-T1 means evaluated through T-test for paired samples. NW = Normal weight; OW= Overweight; OB = Obese ; BW = Body Weight; BMI = Body Mass Index; Waist = Waist circumference; PA = Phase Angle; FM% = Fat Mass %; ECW% = Extracellular Water % of Total Body Water; HOMA = Homeostatic Model Assessment; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; SPB = Systolic Blood Pressure; DBP = Diastolic Blood Pressure.

Table 3. Attrition rate during the study time-points divided by BMI classes

	T1	T2	T3
Time	**Baseline**	**6 months**	**12 months**
N (% of first sample)	182	101 (55.5)	61 (33.9)
NW (% of total)	19 (10.4)	14 (14.5)	9 (24.6)
OW (% of total)	57 (31.3)	38 (37.6)	28 (45.9)
OB (% of total)	102 (57.3)	49 (47.5)	24 (39.3)
Class I-II-II obesity (% of OB)	50.0-30.4-19.6	60.8-21.4-17.8	58.3-25.1-16.6

* = p<0.05; **= p<0.01; ***=p<0.001. T1 = baseline; T2 = 6 months

N = number of patients; NW = Normal weight; OW= Overweight; OB = Obese

Tab 4. Baseline data according to dropout at 6 months

	Continuers	Drop-outs	p value
N	101	77	
Age (y)	53.0 (15.0)	52.0 (15.0)	Ns
Weight (Kg)	78.2 (17.8)	78.3 (18.6)	Ns
BMI (Kg/m2)	31.4 (6.6)	30.6 (9.5)	Ns
Waist(cm)	106.01 ± 11.5	105.5 ± 13.3	Ns
PA (°)	5.25 ± 0.46	5.18 ± 0.50	Ns
FM%	38.4 ± 7.5	36.8 ± 6.4	Ns
ECW %	49.6 ± 2.5	50.0 ± 2.7	Ns
Glucose (mg/dL)	90.0 (18.7)	92.0 (14.0)	Ns
Insulin (µUI/mL)	13.5 ± 10.3	13.4 ± 7.9	Ns
HOMA index	3.28 ± 2.66	3.23 ± 2.14	Ns
Tot Chol(mg/dL)	205.3 ± 35.9	211.7 ± 43.4	Ns
HDL(mg/dL)	52.5 (17.5)	52.5 (17.5)	Ns
LDL (mg/dL)	122.2 ± 32.4	132.2 ± 39.0	Ns
Triglycerides (mg/dL)	122.0 (100.0)	123.0 (88.5)	Ns
SBP (mmHg)	125.5 ± 14.2	124.6 ± 18.7	Ns
DBP (mmHg)	77.7 ± 10.9	78.4 ± 12.0	Ns

Data expressed as mean ± standard deviation for normally distributed variables or as median (IQR) for non-normally distributed variables. BW = Body Weight; BMI = Body Mass Index; Waist = Waist circumference; PA = Phase Angle; FM% = Fat Mass %; ECW% = Extracellular Water % of Total Body Water; HOMA = Homeostatic Model Assessment; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; SPB = Systolic Blood Pressure; DBP = Diastolic Blood Pressure.

Tab 5. Baseline data according to dropout at 12 months

	Continuers	Drop-outs	p value
Age (y)	55.0 (15.0)	51.0 (15.0)	<0.05
Weight (Kg)	77.5 (18.9)	78.8 (17.8)	ns
BMI (Kg/m^2)	30.8 (6.7)	30.8 (8.3)	Ns
Waist Circumference (cm)	106.1 ± 11.9	105.6 ± 12.6	Ns
PA (°)	5.14 ± 0.42	5.23 ± 0.50	Ns
FM%	34.9 ± 7.1	38.3 ± 6.7	Ns
ECW %	50.2 ± 2.5	49.7 ± 2.6	Ns
Glucose (mg/dL)	91.0 (19.8)	90.0 (16.0)	Ns
Insulin (μUI/mL)	13.9 ± 11.2	13.1 ± 8.4	Ns
HOMA index	3.35± 2.61	3.20± 2.47	Ns
Tot Chol (mg/dL)	205.0 ± 37.8	209.5 ± 39.6	Ns
HDL(mg/dL)	49.0 (15.5)	55.0 (16.2)	<0.05
LDL (mg/dL)	120.9 ± 31.7	130.0 ± 37.5	Ns
Triglycerides (mg/dL)	133.0 (105.0)	117.0 (82.0)	Ns
SBP (mmHg)	122.3 ± 13.2	126.8 ± 17.1	Ns
DBP (mmHg)	74.4 ± 11.3	79.9 ± 10.9	<0.05

Data expressed as mean ± standard deviation for normally distributed variables or as median (IQR) for non-normally distributed variables. BMI = Body Mass Index; Waist = Waist circumference; PA = Phase Angle; FM% = Fat Mass %; ECW% = Extracellular Water % of Total Body Water; HOMA = Homeostatic Model Assessment; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; SPB = Systolic Blood Pressure; DBP = Diastolic Blood Pressure.

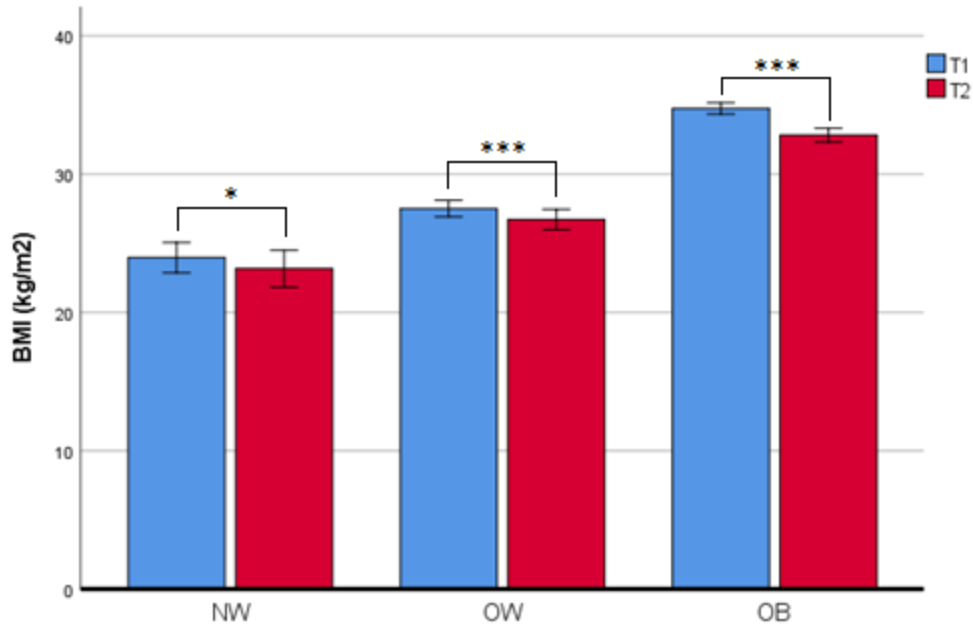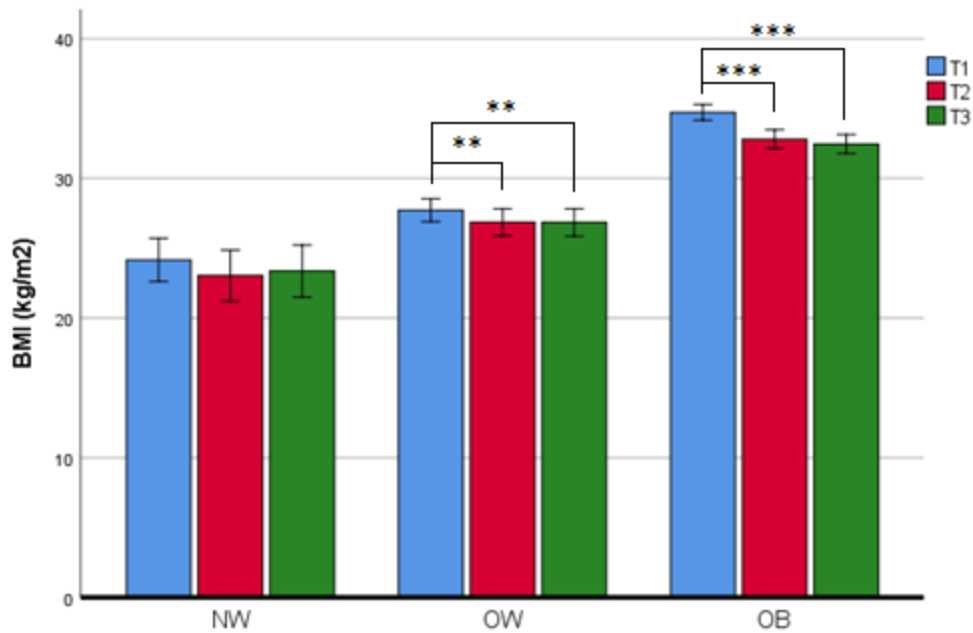**Figure 1.** Changes in BMI in completers at 6 months

Figure 2. Changes in BMI in completers at 12 months

* = p<0.05; **= p<0.01; ***=p<0.001. T1 = baseline; T2 = 6 months; T3 = 12 months

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