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Effects of nutritive antioxidants on bone during immobility

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List of abbreviations

aBMD	Areal bone mineral density
Aox	Antioxidant
BAP	Bone alkaline phosphatase
BDC	Baseline data collection period
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMR	Basal metabolic rate
Con	Control
CTX	C-telopeptide of type I collagen
CV	Coefficient of variation
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPD	Deoxypyridinolin
DRI	Dietary reference intake
DXA	Dual-energy X-ray absorptiometry
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FU	Follow Up
GC	(+)-gallocatechin
GPx	Glutathion peroxidase
GTE	Green tea extract
HCA	Hydroxycinnamic acid
HDBR	6° head-down tilt bed rest
LSC	Least significant change
HR-pQCT	High-resolution peripheral quantitive computed tomography

JNK	C-Jun Nterminal kinase
MAPK	Mitogen-activated protein kinase
MBDC	Mean values from baseline data collection days -4 to -1
MDA	Malondialdehyde
MEDES	Institute for Space Medicine and Physiology
NTX	N-telopeptide of type I collagen
OPG	Osteoprotegerin
P1CP	Carboxyterminal propeptide of type I collagen
P1NP	Aminoterminal propeptide of type I collagen
P38	P38 mitogen-activated protein kinases
PAL	Physical activity level
PGE2	Prostaglandin E2
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
PYD	Pyridinolin
R	Recovery
RAE	Retinol activity equivalents
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
ROS	Reactive oxygen species
SIRT	Sirtuin protein
SOD	Superoxid dismutase
TEE	Total energy expenditure
TRACP 5b	Tartrate-resistant acid phosphatase 5b
TRAP	Tartrate-resistant acid phosphatase
vBMD	volumetric bone mineral density
VO_{2max}	Maximal oxygen uptake
Wnt	Wingless related integration site
25(OH)D	Serum 25-hydroxyvitamin D
βCTX	β-crosslaps of C-telopeptide of type I collagen

General Introduction

Beside other factors, physical activity plays an important role in the maintenance of bone health. The relevance of biochemical strain to ensure bone modeling and remodeling was postulated by Frost's "mechanostat theory" [1]. This theory states that depending on the amount of load on the bone, bone mass and strength are either reduced, maintained or increased [1,2]. The mechanostat model is shown in **Figure 1**. According to this theory, for a person with an average load on bone, bone turnover shifts in favor of bone resorption and can result in a loss of bone mass when the mechanical load on bone is below 800 µStrain (e.g. because of reduced physical activity, disuse or weight loss) [3]. Mechanical strain between 800 µStrain and 1500 µStrain is considered to allow a balance between bone resorption and bone formation and bone mass and strength are maintained [3]. If the mechanical load is higher than 1500 µStrain bone modeling takes place and bone mass and strength are increased [3]. At a strain of 15000 µStrain the maximum elastic deformation is exceeded, resulting in bone fracture [3].

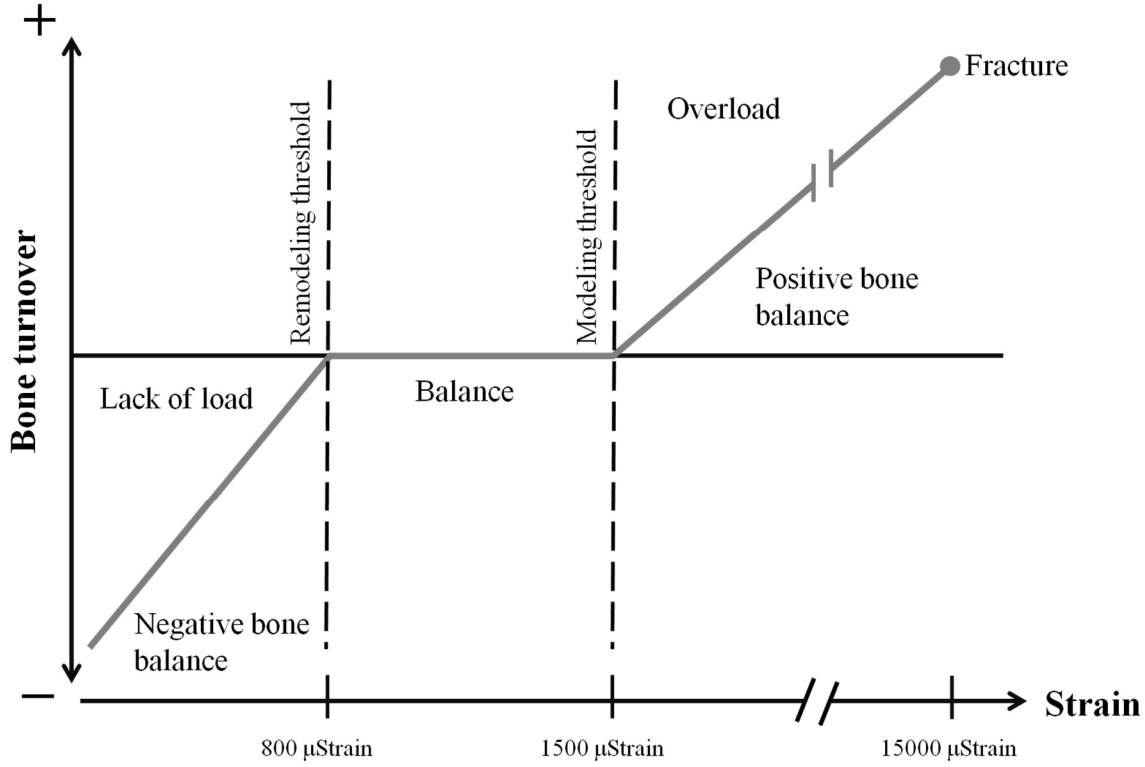

Figure 1 The mechanostat model by H. M. Frost (modified from Kerschan-Schindl, 2012 [3])

To maintain bone mass and quality, the normal bone remodeling process requires a tight coupling of bone resorption and formation [4]. Immobility decreases the mechanical loading of the skeleton. This alters bone remodeling, as mechanosensing pathways and related signal transduction pathways are no longer activated [4,5]. This can lead to a reduction in bone mass and strength and may elevate the risk of developing osteoporosis and finally the risk for fractures [4–6].

Osteoporosis causes more than 8.9 million fractures worldwide each year [7]. In the US alone, 1.5 million women and men suffer from fragility fractures every year [8] and it is hypothesized that one in five men and one in two women aged >50 years will suffer from an osteoporotic fracture at one point during their lifetime in the UK [8]. Forearm, hip and vertebrae are the most susceptible fracture sites and hip fractures are associated with the greatest morbidity and mortality [7,8]. Osteoporosis has a serious impact on individuals morbidity and mortality as well as on health care systems [8]. Thus, the prevention of this degenerative disease and its consequences gets extremely important and is essential for the maintenance of health, quality of life and independence of elderly people [7,9].

Aging, immobilization and osteoporosis are associated with an increase in oxidative stress that may result in oxidative damage [10–14] and several studies suggest that exceeding reactive oxygen species (ROS) production is associated with bone loss [15–18].

Aging is characterized by a decrease in physical activity [6] and a progressive decline in functional maintenance of tissue homeostasis [19]. Senescence is a process of irreversible growth stagnation after a finite number of cell divisions and contributes to organismal aging [20]. Senescent cells are associated with high levels of intracellular ROS and accumulated damage to deoxyribonucleic acid (DNA), proteins and lipids [14,20,21]. In this context, mitochondrial DNA seems to be more sensitive to oxidative damage than nuclear DNA, perhaps because of a limited DNA repair systems or its proximity to the main source of endogenous oxidant generation [21]. The increased damage of mitochondrial DNA can lead to an impaired mitochondrial function, characterized by disorder of electron transport, adenosine triphosphate generation and mitochondrial membrane potential which in turn lead to a higher release of ROS and result in augmented oxidative damage [21,22].

Bed rest studies also show an increase in oxidative stress [10,23,24]. Dedicated long-term bed rest increased erythrocyte glutathione peroxidase (GPx) activity at the end of bed rest. After 90 days of recovery, the activity decreased again significantly and returned to its initial value [23]. The oxidative damage marker of DNA, 8-oxo-7,8-dihydro-2 deoxyguanosine, and malondialdehyde (MDA), a product of lipid peroxidation, were significantly elevated after 60 days of bed rest. Serum and salivary concentrations of the antioxidant vitamins E and C were significantly attenuated [24]. Even during and after 10 days of bed rest, Debevec et al. found an increase in advanced oxidative protein products and MDA [10]. Stein et al. showed that oxidative damage increased after long-duration space flight [25,26]. Reasons for increased oxidative stress during space flight might be an elevated exposure to high-energy radiation, as well as increased free radical generation, due to an altered oxygen metabolism from impaired gas exchange with the lungs and changes in intermediary metabolism [25]. Markers for oxidative damage to membrane lipids [8-iso-prostaglandin F2 alpha (8-iso-$PGF_{2\alpha}$)] and DNA (8-oxo-7,8-dihydro-2 deoxyguanosine) increased post-flight, with a significant increase of 8-iso-$PGF_{2\alpha}$ excretion (35.5 ng/kg/d pre-flight vs. 87.1 ng/kg/d post-flight) during the duration of the post-flight measurement period (length of flight varied within subjects between four to nine months) [25]. This increase in post-flight excretion of products of oxidative damage was attributed to a combination of increased metabolic activity and loss of host oxidative defences during space flight [25].

Apart from mechanical loading, humoral factors (e.g. calcitriol, calcitonin, growth hormone, glucocorticoids and sex hormones) and local factors [osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B (RANK)], a balance between oxidants and antioxidants seems to be crucial to maintain a successful bone remodeling process [12,27]. Therefore, the supplementation of antioxidants for prevention and therapy of bone loss related diseases seems to be a promising approach [28–32].

Improvements in bone mineral density (BMD) and bone turnover markers were observed in men and women after consumption of polyphenol-rich fruits and vegetables, and/or omega-3-fatty acids [28,29,33–35]. Antioxidants seem to affect bone metabolism mainly by contributing to osteoblast differentiation and activity and osteogenesis [36]. On a cellular level they reduce osteoclast differentiation and activity and prevent apoptosis of osteoblasts and osteocytes [36]. On the molecular level they counteract osteoprotegerin (OPG) decrease and inhibit the increase of receptor activator of nuclear factor-kappa B ligand (RANKL), bone

acid phosphatase and protease activity (degradation of bone matrix) and promote bone alkaline phosphatase (bAP) and matrix protein synthesis [36]. Apart from that, their anti-inflammatory (inhibition of pro-inflammatory cytokines) and antioxidative capacities (activation of antioxidant enzymes) seem to play an important role in the maintenance of bone turnover [37–40].

There are different ways and techniques to assess bone turnover and bone loss. The gold standard method for BMD estimation and osteoporosis diagnosis is dual energy X-ray absorptiometry (DXA) [41–43]. The physical principle behind DXA is the transmission of x-rays with low and high-photon energy through the human body [44]. Due to their different attenuation coefficients for x-rays, soft tissue and bone tissue can be distinguished [44]. BMD is reported as areal BMD (aBMD) in g/cm² of the cortical and trabecular bone together [43]. High-resolution peripheral quantitative computed tomography (HR-pQCT) assesses bone microarchitecture and volumetric bone mineral density (vBMD) separately for the cortical and trabecular compartments of the distal radius and tibia and allows a direct quantification of bone microstructure [45,46]. Cortical bone builds the dense outer wall of all bones, is essential for weight bearing, body structure and fulfils mainly mechanical functions [47,48]. The cortical or compact bone makes up about 80% of the human skeleton [48]. The trabecular bone, also called cancellous bone makes up about 20% of the skeletal mass and is found in the ends of long bones and inside the flat bones [47,48]. Trabecular bone provides structural support and flexibility and its porous structure allows transmission of the mechanical load from the articular surface (joint) to the cortical bone, surrounding it [49]. Trabecular bone is more cellular and has a high level of metabolic activity [47]. The ratio of cortical to trabecular bone varies among the different skeletal sites with a cortical to trabecular ratio of 5:95 in the diaphysis of the radius, 25:75 in the vertebrae and 50:50 in the femur head [48]. Each year about 25% of trabecular bone and 4% of cortical bone are resorbed and replaced [47]. Due to their different structure and function trabecular and cortical bone are affected differently by chemical and mechanical signals which makes their separate evaluation important [49].

Bone biomarkers are a non-invasive approach to diagnose and monitor changes in bone remodelling, bone loss and associated diseases, such as osteoporosis [50]. Apart from BMD assessment they are widely used in clinical trials [50,51]. They can be divided into bone formation markers and bone resorption markers [50,52]. Markers of bone formation assess osteoblast activity or collagen formation. Markers of bone resorption represent the

degradation of typ I collagen and osteoclast activity [52]. Important, frequently used and validated bone markers are shown in **Table 1**.

Table 1 Markers of bone formation, bone resorption and bone turnover [52–54]

Bone formation markers	Bone resorption markers
Aminoterminal propeptide of type I collagen (P1NP)	C-telopeptide of type I collagen (CTX)
Carboxyterminal propeptide of type I collagen (P1CP)	N-telopeptide of type I collagen (NTX)
Bone alkaline phosphatase (bAP)	

Bone turnover marker
Osteocalcin

Bone formation and resorption markers are a good addition to BMD measurements, as changes can be detected earlier. Beside BMD they are good indicators for fracture risk and represent a valid method to predict subsequent BMD changes. [55,56].

For an integrated view of bone metabolism and osteoporosis prevention and therapy, a combined investigation of bone turnover markers, BMD and bone structure parameters is important, as they reflect bone changes on different levels, react differently in time and sometimes independently from each other [55].

Objectives

To assess the effects of antioxidants on bone metabolism, a systematic literature review summarized the results of human intervention studies investigating the effects of polyphenols – the most intensively studied group of dietary antioxidants – on BMD, bone biomarkers and bone structure parameters.

Based on the results of the literature search a human intervention study was conducted. The literature review revealed different influencing factors (e.g., habitual diet, lifestyle factors, and antioxidant bioavailability) that do not allow a clear conclusion regarding the effects of antioxidants on bone metabolism. Therefore, the human intervention study aimed to control these confounding factors by standardizing the habitual (study) diet with all bone active nutrients for both, the intervention and control group, as well as the physical activity (bed rest) of the participants. Beyond that, the human intervention study aimed an improvement in bioavailability by combining different antioxidants and to investigate their synergistic effects on BMD, bone markers and bone structure parameters.

We hypothesized that a combined antioxidant supplementation during 60 days of 6° head-down tilt bed rest (HDBR) would reduce bone resorption marker concentrations and improve bone formation marker concentrations, bone mineral content (BMC), BMD and bone structure parameters compared to non-supplemented controls.

1. Manuscript I

Putative effects of nutritive polyphenols on bone metabolism in vivo – evidence from human studies

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Abstract

For the prevention and treatment of bone loss related diseases, focus has been put on naturally derived substances such as polyphenols. Based on human intervention studies, this review gives an overview of the effects of dietary significant polyphenols (flavonoids, hydroxycinnamic acids, and stilbenes) on bone turnover. Literature research was conducted using PubMed database and articles published between 01/01/2008 and 31/12/2018 were included (last entry: 19/02/2019). Randomized controlled trials using oral polyphenol supplementation, either of isolated polyphenols or polyphenols-rich foods with healthy subjects or study populations with bone disorders were enclosed. Twenty articles fulfilled the inclusion criteria and the average study quality (mean Jadad score: 4.5) was above the pre-defined cut-off of 3.0. Evidence from these studies does not allow an explicit conclusion regarding the effects of dietary important polyphenols on bone mineral density and bone turnover markers. Differences in study population, habitual diet, lifestyle factors, applied polyphenols, used doses, and polyphenol bioavailability complicate the comparison of study outcomes.

Introduction

The human skeleton is continuously remodeled throughout life by osteoclast (bone resorbing cells) and osteoblast (bone forming cells) activities [1]. Bone remodeling ensures mineral homeostasis, maintains the integrity of the skeleton, and is responsible for removal and repair of damaged tissue [2]. The underlying close communication and interaction between osteoclasts and osteoblasts consist of four consecutive phases: activation, resorption, formation, and termination/resting [2,3]. In brief, during the activation phase, an initiating remodeling signal is detected by bone cell receptors supporting the migration of partially differentiated mononuclear preosteoclasts to the bone surface. Multinucleated osteoclasts are then formed promoting resorption of bone mass. In the third phase mononuclear cells prepare the bone surface for the osteoblast-mediated formation and initiate osteoblast differentiation and migration. Osteoblasts replace the removed bone with an equal quantity of new bone. Flattened lining cells cover the surface and mineralization occurs [2,3].

The main regulators of bone turnover are mechanical strain, systemic factors (e.g., calcitriol, calcitonin, growth hormone, insulin-like growth factor 1, glucocorticoids, and sex hormones), and local factors [e.g., the osteoprotegerin (OPG) - receptor activator of nuclear factor-kappa B ligand (RANKL) - receptor activator of nuclear factor-kappa B (RANK) system] [4–6].

Aside from these, the physiological balance between oxidants and antioxidants (redox status) also seems to be important for the maintenance of a balanced osteoclast- and osteoblast activity and therefore a successful bone remodeling process (Figure 1-1) [7,8]. Several in vitro and animal studies have shown that reactive oxygen species (ROS) production is involved in the regulation of bone status and in mineral tissue homeostasis mainly by promoting bone resorption [9–12]. Moreover, ROS act as signaling molecules in several signaling pathways in bone cells and enhance osteoclastogenesis (Figure 1-1) [8].

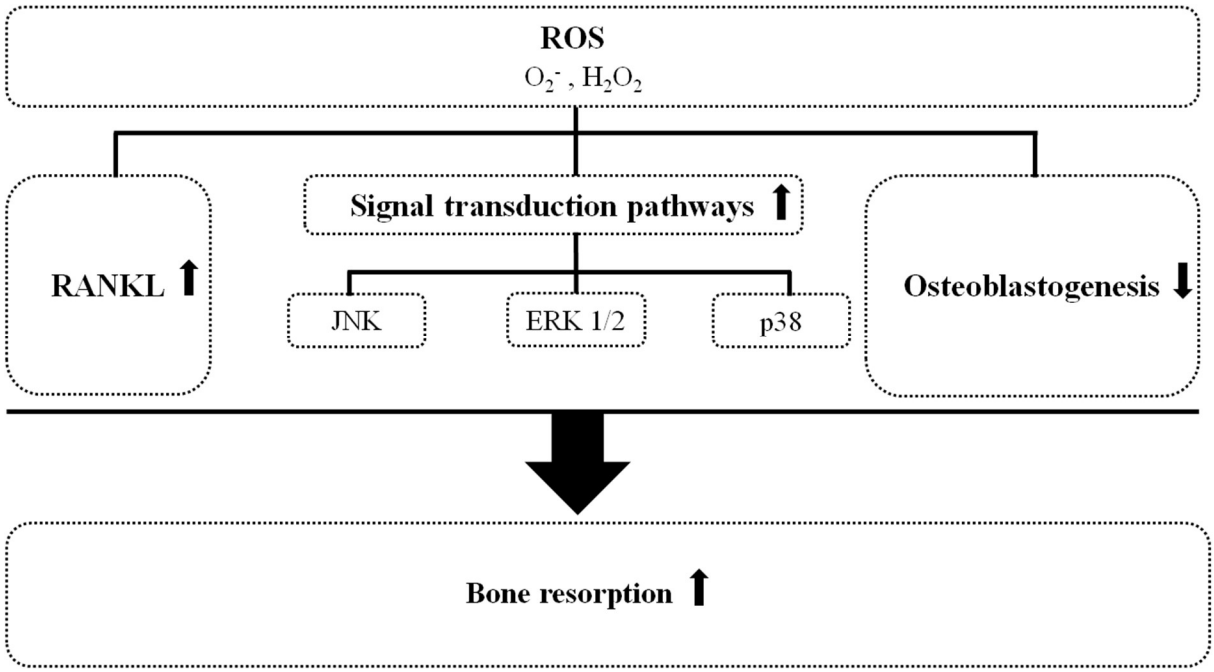

Figure 1-1 Impact of reactive oxygen species (ROS) on bone turnover [8–14].

ROS promote bone resorption by enhancing receptor activator of nuclear factor-kappa B ligand [RANKL]-induced osteoclast activity, by activation of osteoclastogenesis related signal transduction cascades (c-Jun Nterminal kinase (JNK), p38 mitogen-activated protein kinases (p38), extracellular signal-regulated kinase (ERK 1/2)), and by suppressing osteoblastogenesis. ↑, activation; ↑ inhibition

Under normal physiological conditions the ROS production by osteoclasts contributes to bone remodeling by stimulating the destruction of calcified tissue [13,14]. Exceeding ROS production and osteoclastic activity, however, were observed in different skeletal pathologies such as osteoporosis and bone fractures [15]. Several studies indicate a relation between oxidative stress and bone loss [16–19]. Oxidative stress associated with increased lipid peroxidation seems to enhance bone resorption resulting in reduced bone mineral density (BMD) [16,17]. A higher value of the superoxide dismutase (SOD)/glutathione peroxidase (GPx) ratio was observed in subjects with osteoporosis [18]. SOD generates H_2O_2 by removing superoxide and therefore has to collaborate with H_2O_2-removing enzymes like GPx or catalase to prevent oxidative stress [20]. The imbalance created by an altered SOD/GPx ratio leads to an increase in H_2O_2 levels [21]. High H_2O_2 levels promote osteoclastic differentiation and inhibit osteoblastic differentiation, which results in bone resorption [22,23].

Numerous observational studies have shown that intake of several portions of fruits and vegetables per day (~240—400 g) is associated with greater BMD and decreased fracture risk [24–26]. Recent reviews summarizing observational studies in Asia conclude that the consumption of soy isoflavonoids is inversely associated with the incidence of hip fractures

and osteoporosis risk in postmenopausal women [27,28]. Epidemiological studies focusing on tea drinking (green- and black tea) show adverse results with respect to bone health [29–33]. Observational studies evaluating the effects of habitual tea drinking on bone health showed, however, inconsistent results in both men and women [34]. The generally positive effects of fruit-, vegetable-, and tea consumption seem to be partly attributed to their content of alkaline-precursors which contribute to neutralizing acid loads from other components of the diet so that the skeleton is not used as a buffer to resorb and neutralize acid loads [35].

More important might be their content of active phytochemical compounds, such as polyphenols [36–39]. Due to their antioxidative potential, polyphenols may protect cells against oxidative damage induced by ROS and thereby attenuate the risk for the development of degenerative diseases such as cardiovascular diseases, cancer, diabetes, and osteoporosis [40,41]. In vitro- as well as animal studies suggest that polyphenols, apart from their antioxidative properties, affect bone metabolism by anti-inflammatory actions, suppression of osteoclastogenesis, and activation of osteoblastogenesis via different bone related pathways [42–50].

Polyphenols can be distinguished according to their chemical structure (number and arrangement of carbon atoms). Based on that, they can be classified into nine subgroups (Figure 1-2) [51]. Depending on the amount of vegetables and fruits consumed, the daily intake of polyphenols sums up to >500 mg/day (five portions of vegetables and fruits per day). The additional consumption of tea (green-, black-, white-, and Oolong tea), coffee, and cocoa can lead to intakes up to 1000 – 1500 mg [52].

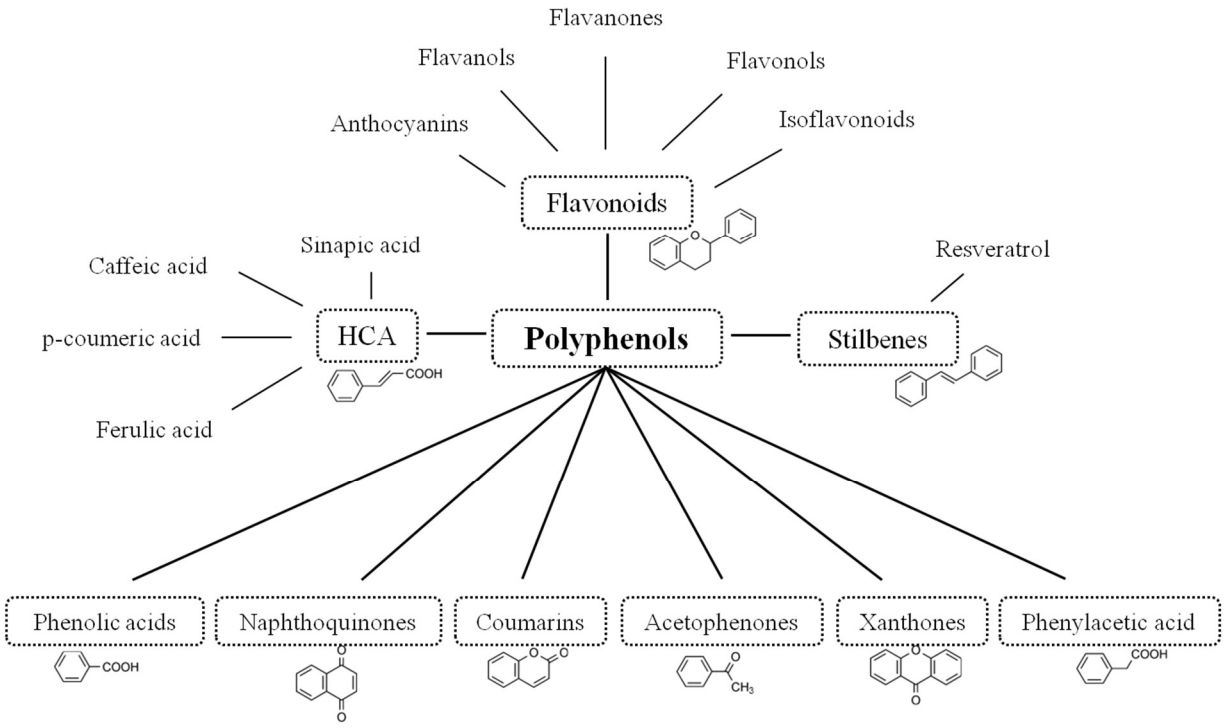

Figure 1-2 Polyphenol classification (modified from Crozier et al. [51])

The nine polyphenol subgroups are classified according to their chemical structure and are found throughout the plant kingdom. HCA, hydroxycinnamic acids

Relevant nutritive polyphenol subgroups are flavonoids, hydroxycinnamic acids, and stilbenes [51]. Flavonoids are found in a variety of fruits, vegetables, herbs, and beverages. [53]. The most abundant flavanols are (+)-catechin, (-)-epicatechin (EC), (+)-gallocatechin (GC), and (-)-epigallocatechin (EGC) and the gallic acid esters (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). Tea (camellia sinensis) is the most quantitative source of these compounds worldwide [53]. The predominating flavonols are quercetin, kaempferol, myricetin, and isorhamnetin [53]. They usually occur as glycosides and are mainly located in the flowers, leaves, and outer parts of the plant as peel or skin. Important dietary sources are onions, apples, and leafy vegetables [54,55]. Flavanones are mainly found in citrus fruits [56]. The dominant flavanone in lemon, mandarin, and sweet orange is the rutinoside hesperidin. Sour oranges and grapefruits are dominated by the neohesperidoside naringin [57]. Major flavones are luteolin and apigenin. They are usually present as O- and C-glycosides. Aglycons of flavones are not found in fresh plants but can occur after processing [53]. Luteolin and apigenin have been identified in several vegetables such as celery and artichoke and in different herbs such as rosemary, thyme, or parsley [58,59]. Isoflavonoids, such as genistein, daidzein, and glabridin are also referred to as phytoestrogens due to their estrogenic activity. Important dietary sources for genistein and daidzein are legumes such as soybeans [60].

Glabridin is an isoflavan found in the licorice root [61]. Anthocyanins are responsible for the red, blue, or purple color of several fruits and vegetables such as plums, cherries, raspberries, blackberries, blackcurrants, beetroot, and red cabbage [62]. Aglycons, such as cyanidin or delphinidin are rarely found in plants and most commonly bounded sugars are glucose, galactose, rhamnose, and arabinose, usually as 3-glycosides [63]. Hydroxycinnamic acids (HCA) are also widely found in the human diet and main derivates are caffeic, ferulic, ρ-coumaric, and sinapic acid. They usually occur as esters or glycosides of quinic acid. O-glycosylated ferulic, caffeic, and ρ-coumaric acids are present in tomatoes [64]. Other fruits containing hydroxycinnamic acids are plums, blueberries, cherries, and apples [65]. Stilbenes are present in vegetables and fruits such as spinach, berries, apples, and grapes. In plants, they are produced in response to stress, injury, or disease. The parent compound resveratrol can occur in cis- and trans configuration, as glucosides, aglycones, monomers, or polymers [66]. In higher concentrations resveratrol can be found in red grapes and, thus, in red wine, depending on the species [67].

The role of nutritive polyphenols in maintaining bone health is not finally resolved. Indeed, a final conclusion of the qualitative and quantitative role of nutritive polyphenols on bone metabolism and bone health can only be made on the basis of intervention studies. Thus, the aim of this literature review is to summarize and evaluate results of recently published human intervention studies investigating the effects of nutritive polyphenols, either as single substrates or as ingredients of foods, on bone metabolism.

Methods

The systematic literature search (U.S. National Library of Medicine National Institutes of Health online database PubMed) sought to identify all eligible English articles published between 2008 and 2018 in peer-review journals (last entry: 19/02/2019) with a clear focus on the major polyphenol subgroups. The following search terms were used and at least one of the terms in each of the following four lists had to be present in the title and/or abstract of the article: (1) clinical, experimental, human, in vivo, intervention; (2) bone, bone turnover, bone markers, bone loss; (3) nutrition, nutritional, supplementation, oral; (4) polyphenols, flavonoids, flavanols, flavonols, flavanones, flavones, isoflavonoids, isoflavones, anthocyanins, stilbenes, hydroxycinnamic acids. The following PubMed filters were applied: publication date (from 01/01/2008 to 31/12/2018) and species (humans). In addition,

reference lists of articles identified during the literature search have been checked for complete identification of eligible articles.

Article Selection

Studies meeting the following inclusion criteria were included in the evaluation: (a) randomized controlled trials; (b) oral polyphenol supplementation; (c) supplementation of isolated polyphenols or polyphenol-rich foods; (d) healthy subjects or study populations with bone loss related diseases (e) outcomes: BMD or bone turnover markers; (f) publication date: 2008—2018. As shown in Figure 1-3, 20 articles were finally included in this review. Two independent and experienced reviewers manually screened the title and/or the abstract of the articles that were flagged during the literature search for adherence to the above eligibility criteria. When the reviewers disagreed about the eligibility of a particular article the whole text of the article was read and a consensus decision was reached.

Data Presentation

Data extraction followed a predefined protocol. Human trials were categorized according to the polyphenol subclass (flavanols, flavonols, flavanones, flavones, isoflavonoids, anthocyanins, hydroxycinnamic acids, stilbenes) administered, or in the case of food consumption, according to the dominant polyphenol ingredient of the food items under investigation. To evaluate study quality the Jadad score was calculated for each study included [68]. In this score randomization, blinding, and dropout description are assessed. The scale ranges from 0 (low quality) to 5.0 (high quality) [68]. Scores above a defined cut-off of 3.0 indicate that reliable conclusions can be drawn.

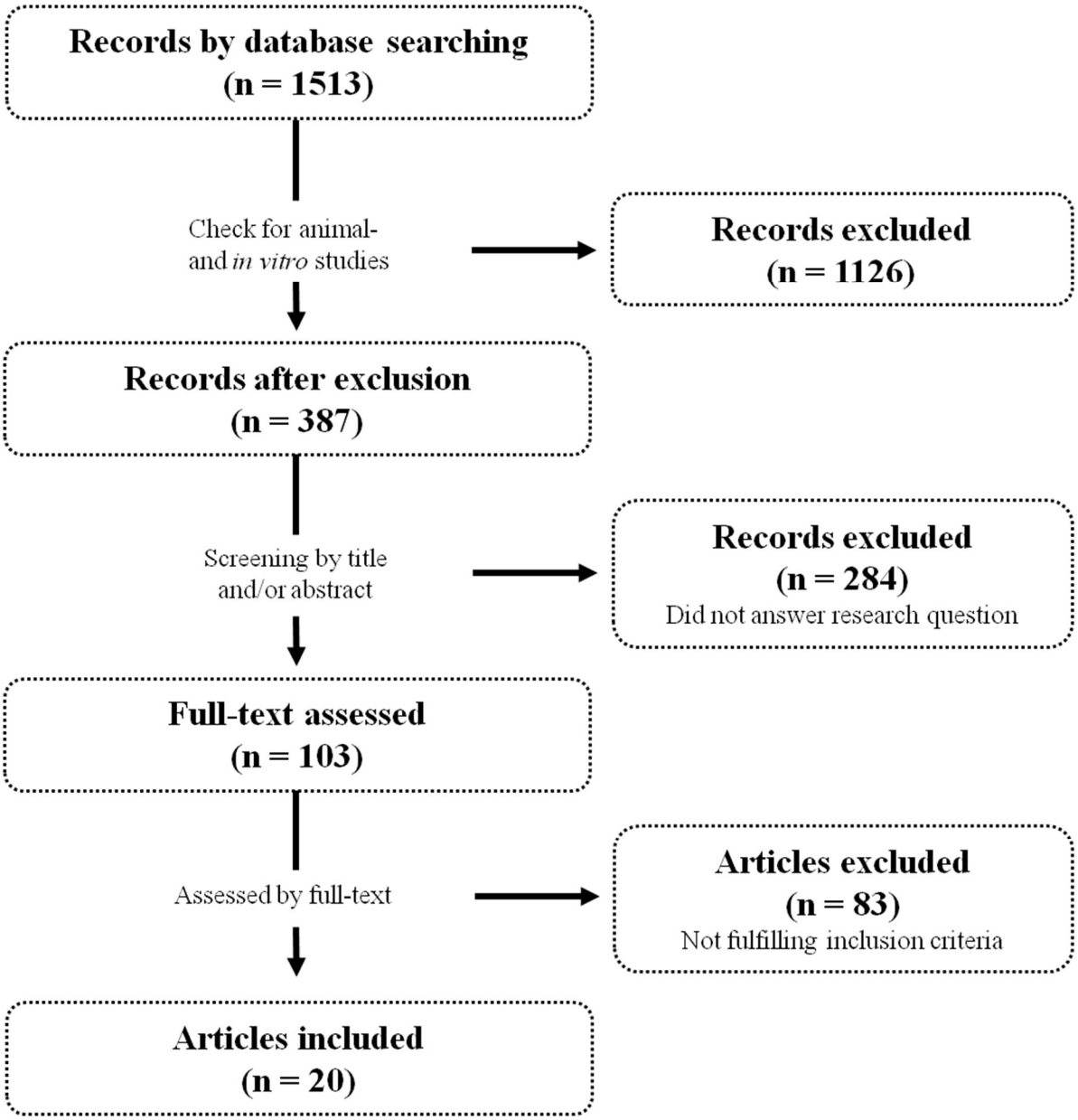

Figure 1-3 Study selection diagram.

The literature search revealed 1513 hits (PubMed filters: publication date (from 01/01/2008 to 31/12/2018) and species (humans). After removal of further animal- and in vitro studies 387 records were screened. Full-text was assessed for 103 records and 83 articles did not meet the inclusion criteria. Twenty articles were included.

Results and Discussion

Study details of the included studies are summarized in Table 1-1. The volunteer characteristics, intervention protocols, characterization of the control group, study duration, and observed effects on bone are shown. Most of the human trials were performed in postmenopausal women and participant numbers range from twelve to 431. Time of intervention varied between eight weeks and three years and health status of volunteer collectives differed.

Table 1-1 Overview of human intervention studies included.

	Participants				Intervention	Control group	Duration	Power analysis	Effects on bone	Jadad Score
	Number	Age (y)	Sex	Health status	(powder/food item)					
Flavanols										
Dostal et al. 2016 [69]	121	50–70	Female	Overweight/obese, postmenopausal, high breast cancer risk	GTE (843 mg EGCG/d)	Overweight/obese, postmenopausal women with high breast cancer risk	1 year	Yes (80%)	Total body BMD ↔	5
Shen et al. 2012 [70]	171	>50	Female	Postmenopausal, osteopenic	GTE (500 mg/d)	Postmenopausal, osteopenic women	6 months	Yes (85-90%)	bAP ↑ TRAP ↔ bAP/TRAP ratio ↑	5
Flavonols										
Law et al. 2016 [71]	30	40–80	Female, male	Healthy	Onion juice (100 ml/d)	Healthy men and women	8 weeks	No	Total body BMD ↔ bAP ↓ PTH ↔ Calcium ↔	5
Flavanones										
Martin et al. 2016 [72]	12	>50	Female	Postmenopausal, healthy	Hesperidin (500 mg)	Postmenopausal, healthy women	3 months	Yes (80%)	bAP ↔ DPD ↔	5
Isoflavonoids										
Alekel et al. 2010; Shedd-Wise et al. 2011 [73,74]	255	46–65	Female	Postmenopausal, healthy	Soy isoflavonoids (80 and 120 mg/d)	Postmenopausal, healthy women	3 years	Yes (94%)	Total body BMD ↔ spine BMD ↔ femur BMD ↔ neck BMD ↔	5

	Participants				Intervention	Control group	Duration	Power analysis	Effects on bone	Jadad Score
	Number	Age (y)	Sex	Health status	(powder/food item)					
Arcoraci et al. 2017; Marini et al. 2008 [75-77]	389	49–67	Female	Postmenopausal, osteopenic	Genistein (54 mg/d)	Postmenopausal, osteopenic women	2 years	Yes (80%)	Femur BMD ↑ spine BMD ↑ PYD ↓ DPD ↓ bAP ↑ RANKL ↓ OPG ↑	5
Brink et al. 2008 [78]	237	53±3	Female	Early postmenopausal, healthy	Isoflavonoid enriched foods (110 mg isoflavonoid aglycones/d)	Early postmenopausal, healthy women	1 year	Yes (84%)	Total body BMD ↔ bone markers ↔	5
Kenny et al. 2009 [79]	131	>60	Female	Postmenopausal, healthy	Isoflavonoids (105 mg/d)	Postmenopausal, healthy women	1 year	No	Total body BMD ↔ femur BMD ↔ spine BMD ↔ wrist BMD ↔	4
Sathyapalan et al. 2016 [80]	200	>50	Female	Early postmenopausal	Isoflavonoids (66 mg/d)	Early postmenopausal women	6 months	Yes (95%)	ßCTX ↓ P1NP ↓	5
Tai et al. 2012 [81]	431	45–65	Female	Postmenopausal with bone loss	Isoflavonoids (300 mg/d)	Postmenopausal women with bone loss	2 years	Yes (80%)	Femur BMD ↔ Bone markers ↔	5
Vupadhyayula et al. 2009 [82]	203	>50	Female	Postmenopausal, healthy	Isoflavonoids (90 mg/d)	Postmenopausal, healthy women	2 years	Yes (80%)	Spine BMD ↔ Femur BMD ↔	4
Wong et al. 2009 [83]	403	40–60	Female	Climacteric, healthy	Soy isoflavonoids (80 and 120 mg/d)	Climacteric, healthy women	2 years	Yes (80%)	Total Body BMD ↑ (120 mg/d) Bone markers ↔	5

	Participants				Intervention	Control group	Duration	Power analysis	Effects on bone	Jadad Score
	Number	Age (y)	Sex	Health status	(powder/food item)					
Anthocyanins										
Hooshmand et al. 2011 and 2014 [84, 85]	160	>50	Female	Postmenopausal, osteopenic	Dried plums (100 g/d)	Postmenopausal, osteopenic women	1 year	No	Ulna BMD ↑ Spine BMD ↑ OPG ↔ Sclerostin ↔	3
Hooshmand et al. 2016 [86]	48	65–79	Female	Postmenopausal, osteopenic	Dried plums (50 and 100 g/d)	Postmenopausal, osteopenic women	6 months	No	Total BMD ↑ TRAP ↓	3
Simonavice et al. 2014 [87]	27	64±7	Female	Postmenpausal, breast cancer survivors	Dried plums (90 g/d)	Postmenpausal women, breast cancer survivors	6 months	Yes (80%)	Spine BMD ↔ Femur BMD ↔ Forearm BMD ↔ Bone markers ↔	3
Stilbenes										
Ornstrup et al. 2014 [88]	74	49±6	Male	Obese, metabolic syndrome	Resveratrol (150 and 1000 mg/d)	Obese men with metabolic syndrome	16 weeks	Yes (80%)	Spine BMD ↑ (1000 mg/d) bAP ↑ (1000 mg/d) OPG ↔ P1NP ↔ CTX ↔ NTX ↔	5

↔, no changes; ↑, significant increase; ↓, significant reduction; GTE, green tea extract; BMD, bone mineral density; bAP, bone alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; PTH, parathyroid hormone; DPD, deoxypyridinolin; PYD, pyridinolin; RANKL, receptor activator of nuclear factor-kappa B ligand; ßCTX, ß C-telopeptide of type I collagen; P1NP, aminoterminal propeptide of type I collagen; OPG, osteoprotegerin; CTX, C-telopeptide of type I collagen; NTX, N-telopeptide of type I collagen.

The number of intervention studies conducted for the different polyphenol subgroups differ broadly (one study each for flavanols and stilbenes and ten studies for isoflavonoids). The main class of flavonoids investigated for their potential effects on bone metabolism is isoflavonoids because of their structural similarity to estrogen and their ability to bind to the estrogen receptor [89]. Another reason for the higher number of studies for this polyphenol subgroup might be their dietary significance particularly in Asian countries and for vegetarian- and vegan lifestyles.

The sample size of the included studies varies between twelve volunteers [72] and 431 subjects [73] and study durations range from two months [71] to two years [75]. Most studies (except four) conducted a power calculation prior to the beginning of the study.

Apart from that, outcome variables investigated differ broadly. Most studies examined BMD of volunteers [69,71,73,78], whereas other investigators analyzed different markers of bone turnover [70,72,80]. Bone turnover markers, such as bone alkaline phosphatase (bAP), aminoterminal propeptide of type I collagen (P1NP), C-telopeptide of type I collagen (CTX), and N-telopeptide of type I collagen (NTX) are beside the BMD good indicators for fracture risk. They are sometimes even stronger associated with this risk than BMD, as they predict fractures in two different ways: (1) the direct reduction of BMD via high bone turnover and (2) independently of BMD, by affecting bone microarchitecture and fragility [90]. Bone markers are also often used to monitor anti-resorptive therapies and provide a good method for the investigation of nutritional interventions, as changes can be observed more rapidly compared to BMD [90]. As summarized by Eastell et al. early changes in bone turnover markers may be predictive of BMD changes [90]. Reduction of CTX and NTX concentrations, for instance after six months predict an increase in lumbar spine BMD 2.5-4 years later and an increase of P1NP after three months is associated with changes in lumbar spine BMD after 18 months [90]. For shorter intervention periods (two to three months) it, therefore, might be reasonable to accompany the investigation of BMD with the examination of bone turnover markers as BMD changes might not be observed at this time point. Six of the nine studies that investigated the effects of BMD and bone turnover markers observed similar effects on these parameters (e.g., no changes for both outcomes) [75–78,81,86–88]. Three studies investigating both outcomes showed contradictory results [71,83–85]. Law et al. did not find any changes in total body BMD but observed a reduction in the bone formation marker bAP after consumption of 100 ml onion juice per day for two months [71]. The study

duration might not be long enough to already see changes in BMD. Hooshmand et al. found an increase of ulna and spine BMD after one year of dried plum consumption [84,85]. The changes for OPG and sclerostin they observed were not statistically significant but showed a trend in the same direction [84,85].

Studies examining the effects on bone metabolism in healthy volunteers (prevention of bone loss) did not find any beneficial effects [71–73,78,79,82]. Only one study investigating the effects in healthy women found a smaller reduction in whole-body BMD after 2 years of soy isoflavonoid supplementation (120 mg/d) compared to placebo [83]. However, the authors stated that the difference only translates to a minimal clinical effect and the supplementation did neither slow bone loss at key fracture sites nor affected bone marker concentrations [83]. Studies that investigated the effect of polyphenols as a treatment for osteopenic women (therapeutic effect) observed a positive impact on bone metabolism [70,75,84,86]. One might speculate that polyphenols may only have a therapeutic- but no preventive effect. However, further studies are needed to investigate and confirm this observation.

Doses applied show a high variation between the different studies (several mg up to 1 g per day). Results, however, do not indicate a dose-dependent effect, as 843 mg EGCG did not affect BMD [69], whereas 54 mg genistein improved BMD [75,76]. It has to be taken into account that we here compare different polyphenol subgroups. They might have a different potency and therefore different doses are needed.

Variations in study population (ethical background and age of participants), habitual diet (substituted polyphenols might not have an additional effect if volunteers already have a balanced diet), and lifestyle factors such as physical activity are other factors that might impact study results and lead to contrary findings.

A comparison between human- and animal studies shows that human intervention studies did not consistently confirm the beneficial effects found in animal models. The transferability of results from animal models to humans, however, is limited, because of differences in e.g., physiology, metabolism and bioavailability. It is likely that animals and humans metabolize polyphenols differently. This has to be considered in the evaluation of these results. Moreover, supra-nutritional doses are mostly used in animal studies and these amounts are not attainable within a plant-based diet by humans.

The bioavailability might be a further explanation of inconsistent study results. Bioavailability of polyphenols depends on external (e.g., food related factors and chemical structure) and internal factors (gender, age, colonic microflora, etc.) [91]. Interactions with other food components, such as fat, proteins, or other polyphenols, for instance, can affect the bioavailability of a single compound [92]. This is important for the valuation, particularly, of those studies investigating the effects of a single compound on bone metabolism. The presence of other polyphenols for example seems to increase the polyphenol bioavailability [92]. Therefore, it might be interesting to investigate whether the effective dose of single compounds can be reduced if they are applied with other polyphenols or as polyphenols-rich foods.

Conclusions

Obviously, recent intervention studies investigating the effects of nutritive polyphenols, either ingested via food or given as single compounds, on bone health showed inconsistent results. Consequently, final conclusions cannot be drawn. Differences in study population, habitual diet, lifestyle factors, and polyphenol bioavailability complicate the comparison of study outcomes. Future studies should take these confounding factors into account. Moreover, it might be of specific interest to evaluate whether the application of polyphenol mixtures (supplements) can lead to beneficial synergistic effects.

Author Contributions

K.A. conducted literature search and prepared the draft manuscript. M.H., N.B. and P.S. contributed to data interpretation; all authors reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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2. Manuscript II

Antioxidant supplementation does not affect bone turnover markers during 60 days of 6° head-down tilt bed rest: Results from an exploratory randomized controlled trial

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Abstract

Background: Immobilization and related oxidative stress are associated with bone loss. Antioxidants like polyphenols, omega-3 fatty acids, vitamins, and micronutrients may mitigate these negative effects on bone metabolism through scavenging of free radicals.

Objectives: We hypothesized that antioxidant supplementation during 60 days of 6° head-down tilt bed rest (HDBR) would reduce bone resorption and increase bone formation compared to nonsupplemented controls.

Methods: This exploratory randomized, controlled, single-blind intervention study conducted in a parallel design included 20 healthy male volunteers (age, 34 ± 8 years; weight, 74 ± 6 kg). The study consisted of a 14-day adaptation phase [baseline data collection (BDC)], followed by 60 days of HDBR and a 14-day recovery period (R). In the antioxidant group, volunteers received an antioxidant cocktail (741 mg/d polyphenols, 2.1 g/d omega-3 fatty acids, 168 mg/d vitamin E, and 80 μg/d selenium) with their daily meals. In the control group, volunteers received no supplement. Based on their body weight, all volunteers received an individually tailored and strictly controlled diet, consistent with DRIs. We analyzed biomarkers of calcium homeostasis, bone formation, and bone resorption during BDC, HDBR, and R, as well as for 30 days after the end of HDBR. Data were analyzed by linear mixed models.

Results: The antioxidant supplement did not affect serum calcium, parathyroid hormone, urinary C-telopeptide of type I collagen (CTX), urinary N-telopeptide of type I collagen, serum β–C-telopeptide of type I collagen (β-CTX), bone alkaline phosphatase, aminoterminal propeptide of type I collagen, osteocalcin, or urinary calcium excretion. In both groups, typical bed rest–related changes were observed.

Conclusions: Supplementation of an antioxidant cocktail to a diet matching the DRIs did not affect bone resorption or formation during 60 days of HDBR in healthy young men.

Introduction

Immobilization, as during extended bed rest, is associated with bone loss (1). Inactivity decreases mechanical loading of the skeleton; consequently, mechanosensing pathways and related signal transduction pathways involved in modulating bone remodeling are no longer activated. This adaptive process leads to a decline in bone mass and strength and, if continued, can increase the risk for developing osteoporosis (2, 3).

Apart from the reduced mechanical load, immobilization is also associated with an increase in oxidative stress (4, 5) resulting from either increased formation of reactive oxygen species (ROS) and/or dysfunction of antioxidant defense systems (6). During 6◦ head-down tilt bed rest (HDBR), a ground-based analog of space flight, there is an increase of the oxidative DNA damage marker 8-OH-deoxyguanosine, accompanied by increased excretion of bone resorption markers [e.g., N-telopeptide of type I collagen (NTX), pyridinium crosslinks, deoxypyridinoline] (5). Increased iron stores during spaceflight have also been associated with increased oxidative DNA damage and bone loss (7).

In skeletal pathologies such as osteoporosis and fractures, osteoclast activity exceeds that observed in healthy subjects (8, 9), and several studies have documented a connection between increased ROS production and bone loss (10–12). Apart from pharmaceuticals, recent focus has been put on antioxidants for prevention and treatment of bone loss–related diseases (13–17). Antioxidants reduce osteoclast activity and increase osteoblast activity via their anti-inflammatory effects, their antioxidative action, and/or by affecting bone cell receptors and activation or inhibition of bone-related pathways (Figure 2-1) (18–24). Polyphenols and omega-3-fatty acids increase NO and osteoprotegerin (OPG) production, and thus stimulate osteoblastogenesis. They decrease osteoclast activity via inhibition of receptor activator of NF-κB ligand (RANKL) production and reduction of proinflammatory cytokines (e.g., IL-1, IL-6, TNF-α) (18, 19). Moreover, they inhibit the osteoclastogenesis-related pathways of RANKL, mitogen-activated protein kinase, and NF-κB signaling (19, 24). Polyphenols promote bone morphogenetic protein, wingless related integration site, estrogen, and sirtuin protein (SIRT) transduction pathways, which are related to osteoblast differentiation, proliferation, and functioning (24). Polyphenols and antioxidative vitamins (e.g., vitamins E and C) also favor bone formation via their antioxidative action, either by directly scavenging ROS or by activating and restoring enzymes of the antioxidant defense system, like superoxide dismutase and glutathione peroxidase (18, 22, 24). Beneficial effects

of antioxidants on bone mineral density (BMD) and bone turnover markers were observed in men and women after consumption of polyphenol-rich fruits and vegetables and/or omega-3-fatty acids (13, 14, 25–27).

To date, intervention studies have focused on either polyphenols, omega-3 fatty acids, or antioxidant vitamins and their effects on bone metabolism in volunteers who maintained their habitual level of physical activity (26, 28, 29). To our knowledge, no previous human intervention study investigated the synergistic effects of an antioxidant (Aox) cocktail on bone turnover in healthy subjects during bed rest.

Thus, the aim of the present study was to determine the effects of an Aox cocktail on bone turnover markers in healthy men during long-duration bed rest. We hypothesized that an antioxidant supplementation during 60 days of HDBR exposure would positively affect bone metabolism.

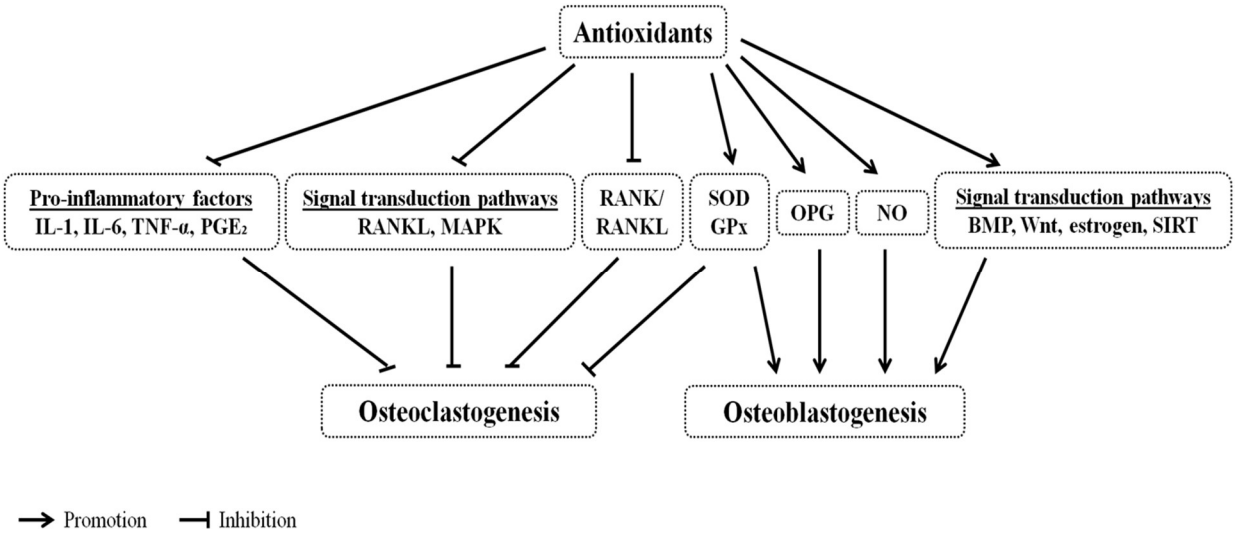

Figure 2-1 Effects of antioxidants on bone turnover.

This figure was designed based on the information in references 18–24. Antioxidants, like antioxidative vitamins, minerals, omega-3 fatty acids, and polyphenols, seem to reduce osteoclastogenesis and increase osteoblastogenesis via inhibition of inflammatory cytokines (like IL-1, IL-6, TNF-α, and PGE2), via activation of antioxidative enzymes (like SOD and GPx), via activation or inhibition of bone-related pathways (like estrogen signaling, BMP signaling, and MAPK signaling), and by affecting bone cell receptors like RANK/RANKL and OPG. BMP, bone morphogenetic protein; GPx, glutathione peroxidase; MAPK, mitogen-activated protein kinase; OPG, osteoprotegerin; PGE2, prostaglandin E2; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; SOD, superoxide dismutase; SIRT, sirtuin protein; Wnt, wingless related integration site.

Methods

This study (identifier: 375/15) was approved by the University Research Ethics Committee of the Rheinische Friedrich-Wilhelms University of Bonn, Germany. It was part of a comprehensive, long-duration HDBR study (Aox-cocktail study) sponsored by the European and French Space Agencies.It was approved by Comité de Protection des Personnes (CPP) Sud-Ouest Outre-Mer I and the French Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé) in accordance with the 1964 Declaration of Helsinki. In this Aox-cocktail study, 16 teams investigated the effects of an antioxidant supplement on multiple physiological systems, including effects on the cardiovascular and immunological systems, muscle metabolism, and neurosensory function. All subjects gave written informed consent before they started the study. All investigations took place at the Institute for Space Medicine and Physiology (MEDES), Toulouse, France.

Volunteers

We recruited 20 healthy, nonsmoking, active (10,000–15,000 steps/d) male volunteers via MEDES and European Space Agency websites and media. The 20 subjects (mean ± SD: age, 34 ± 8 years; body weight, 74 ± 6 kg; BMI, 24 ± 2 kg/m2) were included after a medical and psychological screening (Figure 2-2). The main exclusion criteria were orthopedic, musculoskeletal, and cardiovascular disorders; bone mineral density T-score ≤ -1.5; or a history of thyroid dysfunction, renal stones, diabetes, migraines, thrombophlebitis, orthostatic intolerance, substance abuse, and/or restricted diet (e.g., vegetarian, food allergies).

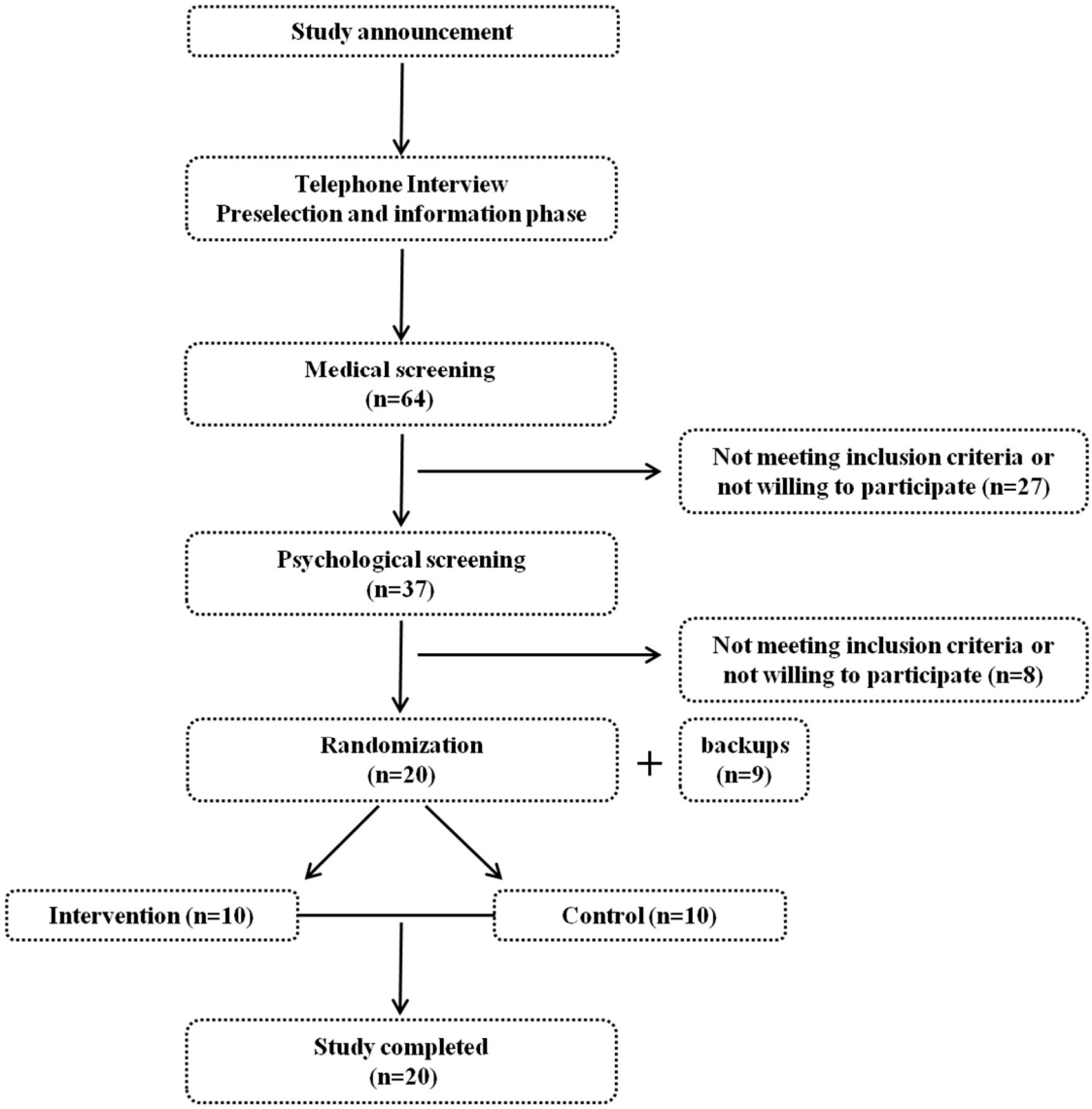

Figure 2-2 Flowchart of eligibility assessment, enrollment and allocation.

Study design

The study was performed in a randomized, controlled, single-blind parallel design and was divided into 2 campaigns (10 volunteers each). Each campaign consisted of 3 study phases: a 14-day adaption and baseline data collection (BDC) phase, a 60-day HDBR phase, and 14 days of recovery (R). During these 88 days participants lived in a metabolic ward at MEDES. Volunteers were matched in pairs according to their habitual physical activity level and then were randomly assigned either to the antioxidant (Aox) or control (Con) group. In each campaign, 5 volunteers were allocated to the Aox group and were provided with an Aox cocktail. The Con group received no placebo due to the characteristic taste of the fish oil capsules used in the antioxidant supplement. Thus, a single-blind study design was applied, as the investigators who took the samples and did the data analysis were blinded. During BDC,

volunteers followed a personalized training routine to avoid deconditioning before HDBR. During HDBR, all study protocols and activities were performed in the 6° head-down tilt position (30). Recovery included a physical rehabilitation program tailored for each volunteer. A follow-up (FU) sample collection occurred 30 days after reambulation.

Body weight assessment

The volunteers' body weight was measured every day in the morning after urination and before breakfast, according to bed rest standards (30).

Antioxidant intervention

The Aox cocktail was based on the results of previous studies (31, 32). In obese mice, the antioxidant supplementation reduced the oxidative stress marker malondialdehyde and increased blood antioxidative defenses (31). After fructose overfeeding and daily step reduction in healthy male participants, supplementation yielded an improvement in total antioxidative capacity. It consisted of a polyphenol supplement (XXS-2A-BR2, Spiral, part of Pole National de Compétitivité Vitagora Goût-Nutrition-Santé) equivalent to a daily dose of 741 mg bioactive polyphenols (flavanols, 323.4 mg; phenylpropanoides, 45.6 mg; oligostilbenes, 78 mg; hydroxycinnamic acids, 50.4 mg; flavanols, 135.6 mg; flavanones, 108 mg); an omega-3 fatty acid supplement containing 2.1 g of omega-3 fatty acids (1.0 g EPA and 1.1 g DHA; Omacor, Pierre Fabre); and a combined preparation of 168 mg vitamin E and 80 µg selenium (Solgar).

Polyphenol and omega-3 fatty acid supplements were administered 3 times per day (with breakfast, lunch, and dinner). Vitamin E and selenium were only consumed at breakfast. The polyphenol content was verified by HPLC on a reverse phase column with detection at 280 and 345 nm.

Diet

The energy intake differed between the study phases because of differences in physical activity. During the BDC period, energy intake was equal to 150% of the basal metabolic rate (BMR) + 10% of total energy expenditure (TEE; estimation of thermogenesis). During HDBR, it was equal to 130% of BMR + 10% of TEE, and in the recovery period the energy intake was 145% of BMR + 10% of TEE. Macro- and micronutrient intakes were according to bed rest standards (30) based on DRI values (33, 34). Protein intake was kept constant

throughout the study. Energy reduction during HDBR was achieved by reducing fat and carbohydrate intakes. All participants received 1 unique vitamin D dose (100,000 U Cholecalciferol) at the inclusion visit (BDC-15), with the exception of 1 volunteer. During the selection visit (approximately 3 months before study start), a slight vitamin D deficiency {i.e., serum 25-hydroxyvitamin D [25(OH)D] < 20 ng/mL} was observed in this volunteer. Therefore, he received a first dose of vitamin D just after the selection visit and a second dose was given to him 3 months later on HDBR day 3.

Fluid intake (beverages and water content of food) was 35–50 mL/kg/d. Volunteers were not allowed to consume coffee, tea, or alcohol. Participants received 3 meals (breakfast, lunch, dinner) and 1 snack in the afternoon each day. Menu plans and intake determinations were conducted using Nutrilog nutrition software (Nutrilog 3.11b, Nutrilog SAS). All meals were defined by a dietitian and prepared in the metabolic kitchen at MEDES. Main dishes were provided by an industrial manufacturer (Davigel). Nutrition values of the main dishes and processed foods were provided by Davigel and other industrial manufacturers and added to the nutrition software database to ensure accurate energy and nutrient intake monitoring. Each food item was weighed and served according to the energy needs of each volunteer. Leftovers were weighed and recorded, and dinner intake was adapted in accordance with breakfast and lunch leftovers, as necessary. A 10-day menu cycle was utilized.

Training/exercise

The habitual physical activity of the volunteers was assessed over a period of 10 consecutive days before arriving at MEDES for the study with an accelerometer (ActiGraph 3GTX) and by using the Monica Optional Study of Physical Activity questionnaire for measuring physical activity (35). The physical activity level (PAL) ranged from 1.5 to 2. The required fitness level for inclusion was a maximal oxygen uptake (VO_{2max}) level between 35 mL/(min·kg) and 60 mL/(min·kg) if aged <35 years and a VO_{2max} level between 30 mL/(min·kg) and 60 mL/(min·kg) if aged >35 years. To avoid deconditioning during BDC, volunteers performed 4 treadmill and 4 bicycle exercise sessions for 30–40 minutes each and walked in the facility according to their habitual exercise intensity. A detailed summary of the individual exercise intensity during BDC is shown in Supplemental Table 2-1. During HDBR, no exercise was allowed. To avoid muscular pain and thrombophlebitis, volunteers were massaged gently every day for 30 minutes and joints were mobilized passively. During R, according to their cardiovascular and muscular status, volunteers attended structured exercise

sessions of 60 minutes per day, with a focus on functional moves and daily life activities, such as walking, running, lifting, carrying, sitting, balance, and core strength.

Total body BMD and lean body mass assessment

Total BMD and total lean body mass were assessed as baseline values by DXA (QDR 4500 Elite, Hologic, software Apex version 3.3.0.1). Quality control was performed 3 times a week and every morning before the volunteer's measurements (the maximum CV of quality control was 1.5%).

Neutrophil counts

Neutrophils are crucial in innate immunity and express and release cytokines (36). Increased neutrophil counts are associated with oxidative stress and inflammation (37). Neutrophils were quantified from blood samples collected on EDTA using SYSMEX XN9100 (SYSMEX).

Sample processing and analysis

Blood samples were obtained at 07:00 after overnight fasting before getting out of bed in the recumbent (BDC and R) and head-down tilt positions (HDBR). Blood was collected according to the schedule shown in Figure 2-3A Blood was drawn in vacutainers (Becton Dickinson) from the antecubital vein. Plasma (EDTA) tubes were centrifuged at 1700 g for 10 minutes at 4◦C immediately after blood draw. Plasma was distributed in small tubes and frozen at −80◦C. For serum, after 30 minutes clotting at room temperature, tubes were centrifuged at 1700 g for 10 minutes and serum aliquots were frozen at −80◦C until analysis.

Urine was collected on a void-by-void basis. Urine samples were kept cold (+4◦C) in the dark, and were pooled in the laboratory for each 24-hour collection period. Pools of the urines collected over 24 hours were prepared, and aliquots were frozen at −20◦C until analysis. The 24-hour urine samples were collected on 4 consecutive days during BDC. For each time point during HDBR and R, samples were collected on 2 consecutive days. For the statistical analysis, the mean was calculated for consecutive collection days, resulting in samples from 1 baseline, 4 HDBR, 2 R, and 1 FU time points (Figure 2-3B).

Samples were held until after the completion of all study phases for analysis at the same time. All of each subject's samples were analyzed in the same assay, all testing was completed

using kits of the same production lot, and all testing was completed by the same operator. All analyses were performed in duplicate. Commercially available ELISA kits were used to analyze bone alkaline phosphatase (bAP; MicroVu BAP EIA, Quidel Corporation; inter-assay CV = 5.4%; intra-assay CV = 5.0%). Aminoterminal propeptide of type I collagen (P1NP) was analyzed using radioimmunoassay UniQ RIA kit (IDS, UK; interassay CV = 4%; intra-assay CV = 5.7%). Electrochemiluminescence immunoassay (Roche DeutschlandHolding GmbH) was used to analyze parathyroid hormone (PTH; inter-assay CV = 6.02%; intra-assay CV = 0.74%) levels. Osteocalcin and undercarboxylated osteocalcin were analyzed using RIA (Alpco; osteocalcin inter-assay CV = 6.5%; osteocalcin intra-assay CV = 2.5%; undercarboxylated osteocalcin inter-assay CV = 9.5%; undercarboxylated osteocalcin intra-assay CV = 4.2%). The serum calcium concentration was analyzed by a photometric calcium kit (Cobas 6000, Roche Deutschland Holding GmbH). Serum 25(OH)D was analyzed by eletrochemiluminescence (Atellica IM Vitamin D Total assay, Siemens Healthinners). The bone resorption marker β–C-telopeptide of type I collagen (β-CTX) was analyzed by ECLIA β-crosslaps (Roche Deutschland Holding GmbH; inter-assay CV = 5.5%; intra-assay CV = 1.9%). The urinary calcium concentration was measured via spectroscopy (Cobas 6000, Roche Deutschland Holding GmbH). Commercially available ELISA kits were used to analyze bone resorption markers C-telopeptide of type I collagen (CTX; urine crosslaps EIA, IBL International GmbH) and NTX (Osteomark ELISA, Ostex International Incorporated; inter-assay CV = 4.8%; intra-assay CV = 5.9%).

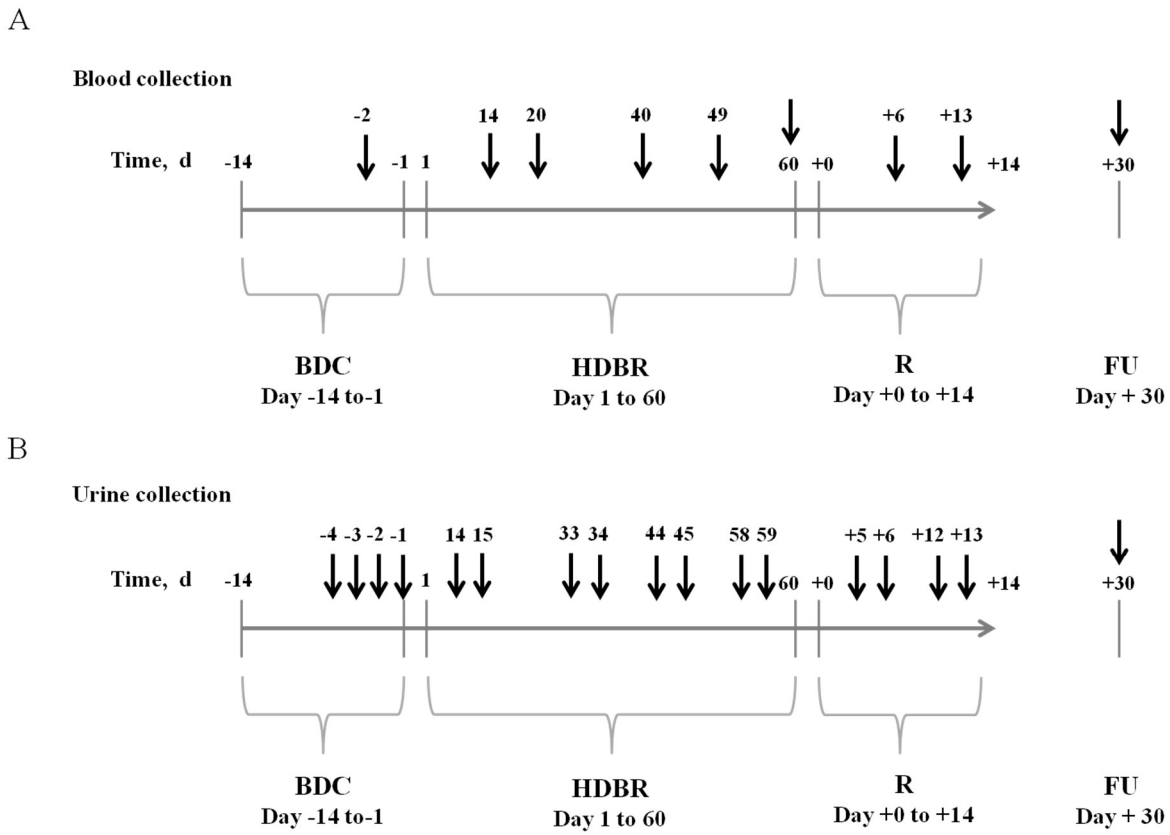

Figure 2-3 (A) Blood and (B) 24-hour urine collection time points.

Fasting blood samples were collected at different time points during BDC, HDBR, R, and FU. The 24-hour urine samples were collected on 4 consecutive days during BDC, on 2 consecutive days at different time points during HDBR and R, and at FU. BDC, baseline data collection; HDBR, 6◦ head-down tilt bed rest; FU, follow-up; R, recovery.

Sample size

The Aox-cocktail study is the first study to investigate the effects of antioxidants on different physiological systems in bed rest, and is therefore considered an exploratory study. Thus, no power analysis was performed.

Statistical analyses

All statistical analyses were performed using the IBM SPSS statistical software package (SPSS version 25, IBM Corporation).

Linear mixed-models procedure was used to test the effects of the intervention (Aox-cocktail), the time points (BDC, HDBR, R and FU), and the interaction between group and time on bone turnover markers. The fixed factors comprised the group (Aox and Con) and time (BDC, HDBR, R, and FU), and their interactions (group x time). The subject identifier was set as a random factor. Baseline values, baseline bone mineral density, VO_{2max}, and PAL were

included as covariates. Baseline values were not included in the outcome vector. In all tests, the residuals were checked for relevant deviations from a normal distribution. We calculated 95% CIs for mean group differences for bone formation and bone resorption markers for the difference between baseline and the end of HDBR.

To identify whether serum bone marker concentrations and urinary excretion rates were back to BDC levels at FU, paired t-tests were performed for intragroup comparisons and unpaired t-tests for intergroup comparisons.

Statistical significance was set as $P < 0.05$ and, unless otherwise stated, data are presented as the arithmetic mean ± SD.

Results

Baseline characteristics

Baseline characteristics of the study subjects are presented in Table 2-1. All volunteers completed the study, and the Aox and Con groups were similar for age, height, weight, BMI, lean body mass, BMD, and VO_{2max}.

Table 2-1 Baseline characteristics in healthy men who were or were not supplemented with antioxidants for 60 days of 6° head-down-tilt bed rest[1]

	Aox (*n*=10)	**Con (*n*=10)**
Age (y)	35 ± 7	34 ± 9
Height (kg)	1.76 ± 0.05	1.76 ± 0.05
Weight (kg)	73 ± 6	75 ±9
BMI (kg/m^2)	24 ± 2	24 ± 2
Lean body mass (kg)	52 ± 4	55 ± 6
Bone mineral density (g/cm^2)	1.056 ± 0.068	1.056 ± 0.158
VO_{2max}, mL/(min·kg)	42 ±5	40 ± 4

[1] Values are means ± SDs, n = 20. Aox, antioxidant group; Con, control group; VO_{2max}, maximal oxygen uptake

Body weight

Body weight was not different between Aox and Con groups (Figure 2-4). A significant time effect was found, with a drop in body weight during the first days of HBDR due to increased diuresis (38) and an increase in body weight during R. During the HBDR, the body weight was stable, except for 1 peak in body weight on HDBR day 50 due to a high-fat, high-energy meal test (39) performed by another scientific team on HDBR day 49.

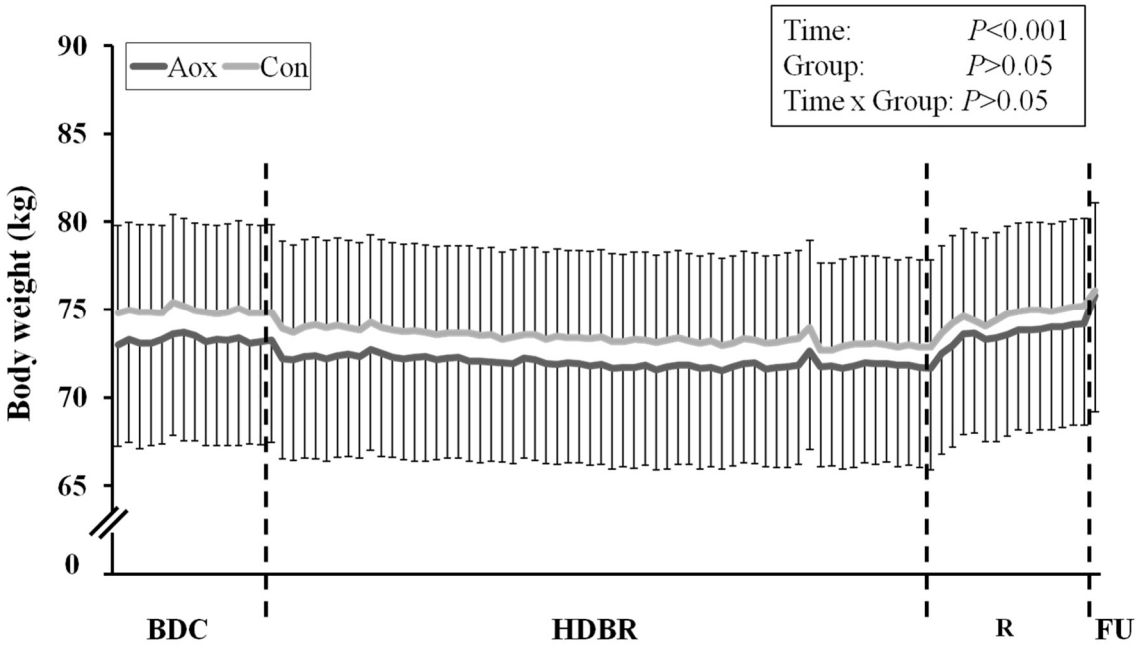

Figure 2-4 Body weights in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR.

The dotted lines show the division into the different study phases (BDC, HDBR, R, and FU). Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; FU, follow-up; HDBR, 6◦ head-down tilt bed rest; R, recovery.

Nutrient intake

Energy, macronutrient, and micronutrient intakes during the 3 study phases are illustrated in Table 2-2 for both study groups. Reduced energy intake during bed rest led to reductions of carbohydrate, fat, and micronutrient intakes during HDBR. Intakes of several micronutrients were above the DRI intake levels, according to bed rest standards (Supplemental Table 2-2) (30), but not above the tolerable upper intake level (40). During the whole study, the Aox and Con groups did not show statistically significant differences for energy, macronutrient, or micronutrient intakes.

Table 2-2 Energy- and nutrient intake in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR[1]

	BDC		HDBR		Recovery		*P*-values from linear mixed models[2]		
	Aox	*Con*	*Aox*	*Con*	*Aox*	*Con*	time	group	time x group
Energy (kcal/d)	2940 ± 180	2940 ± 250	2350 ± 130	2340 ± 180	2790 ± 170	2820 ± 230	<0.001	0.948	0.274
Carbohydrates (g/d)	393 ± 23	391 ± 33	293 ± 15	291 ± 22	369 ± 23	373 ± 30	<0.001	0.982	0.497
Protein (g/d)	87.5 ± 7.4	88.3 ± 10.7	86.9 ± 7.0	88.1 ± 9.9	88.1 ± 7.2	89.9 ± 10.4	0.057	0.782	0.445
Protein (g/kg)	1.19 ± 0.03	1.18 ± 0.03	1.21 ± 0.02	1.18 ± 0.06	1.20 ± 0.03	1.21 ± 0.03	0.325	0.270	0.252
Fat (g/d)	104 ± 7	104 ± 8	83 ± 5	83 ± 6	97 ± 5	99 ± 8	<0.001	0.958	0.187
Sodium (g/d)	3.95 ± 0.29	3.98 ± 0.37	3.62 ± 0.15	3.64 ± 0.19	3.81 ± 0.24	3.85 ± 0.20	<0.001	0.785	0.967
Calcium (g/d)	1.28 ± 0.08	1.28 ± 0.09	1.18 ± 0.04	1.18 ± 0.06	1.26 ± 0.06	1.28 ± 0.10	<0.001	0.840	0.675
Phosphorus (g/d)	1.40 ± 0.10	1.41 ± 0.13	1.34 ± 0.09	1.33 ± 0.11	1.37 ± 0.09	1.39 ± 0.14	<0.001	0.878	0.149
Vitamin K (µg/d)	320 ± 18	326 ± 26	340 ± 21	339 ± 24	365 ± 22	369 ± 39	<0.001	0.804	0.411
Vitamin A (mg RAE/d)	1.57 ± 0.08	1.58 ± 0.10	1.46 ± 0.07	1.44 ± 0.08	1.52 ± 0.06	1.52 ± 0.07	<0.001	0.999	0.457
Vitamin E (mg/d)	24.8 ± 1.4	24.7 ± 1.5	19.5 ± 1.0	19.4 ± 1.2	23.7 ± 1.2	23.9 ± 1.8	<0.001	0.955	0.637
Vitamin C (mg/d)	266 ± 14	270 ± 17	204 ± 8	203 ± 10	245 ± 17	243 ± 15	<0.001	0.965	0.573

[1] Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; HDBR, 6◦ head-down tilt bed rest.
[2] Statistical analyses were performed with linear mixed models

Neutrophils

Neutrophils showed a significant time effect ($P < 0.001$), due to an increase on R day 1 that was within normal limits (Figure 2-5). No differences were found between the Aox and Con groups throughout the study duration.

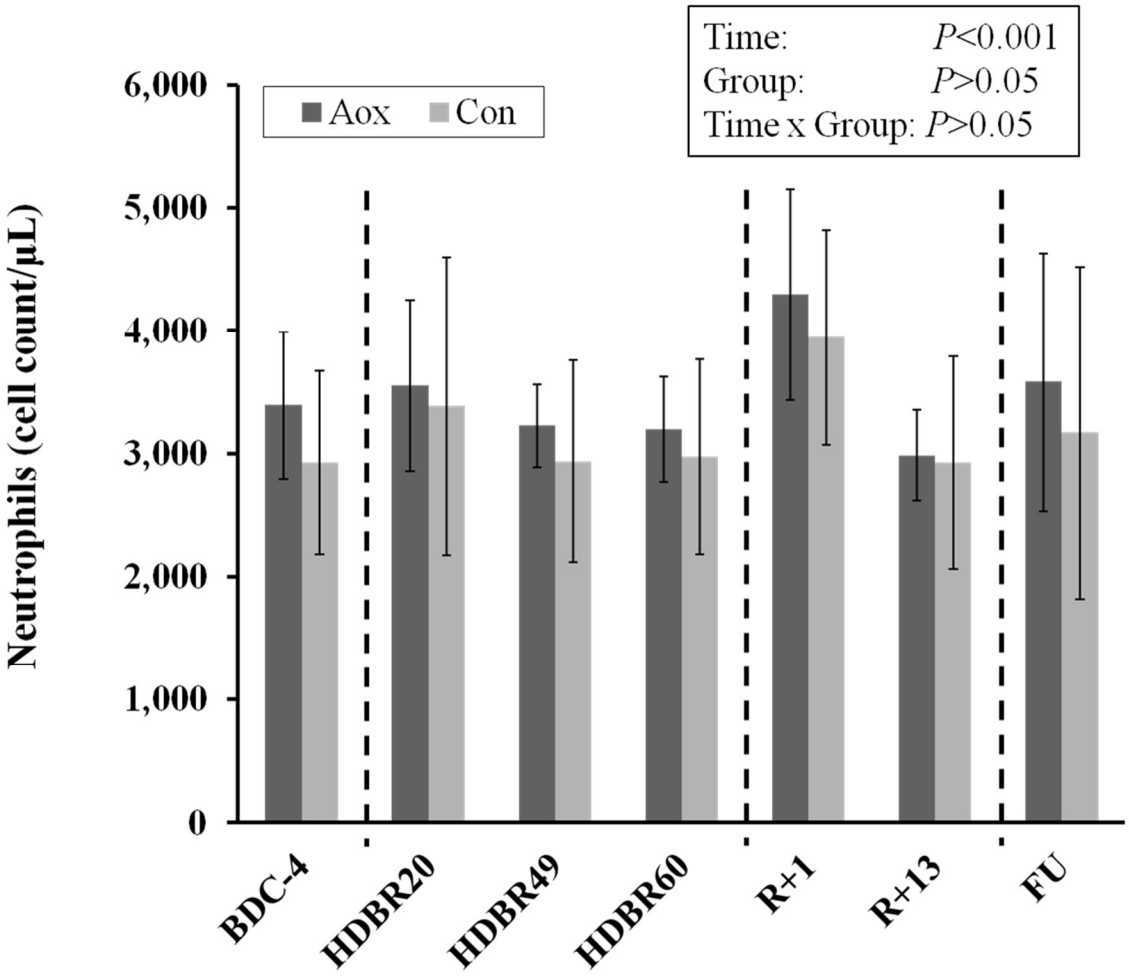

Figure 2-5 Neutrophil concentrations in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR.

The dotted lines show the division into the different study phases (BDC, HDBR, R, and FU). Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; FU, follow up; HDBR, 6◦ head-down tilt bed rest; R, recovery.

Calcium homeostasis and serum 25(OH)D concentrations

The Aox cocktail did not affect serum calcium or PTH concentrations ($P > 0.78$ and $P > 0.86$, respectively; Table 2-3). As expected, the serum calcium concentration increased and the PTH concentration decreased during HDBR ($P < 0.001$). At 30 days after the end of bed rest, the calcium concentrations were back to BDC values in both groups, while the PTH concentrations were higher compared to BDC values [Aox, +26 ± 24% ($P = 0.005$); Con, +33 ± 16% ($P < 0.001$)]. Serum 25(OH)D concentrations did not differ between the Aox and Con groups ($P > 0.86$) and were within normal limits (i.e., >20 ng/mL; Table 2-3).

Table 2-3 Calcium homeostasis and serum 25-hydroxyvitamin D concentrations in healthy men who were or were not supplemented with antioxidants for 60 days of 6◦ head-down tilt bed rest[1]

	BDC	HDBR					R		FU	*P*-values from linear mixed models		
		HDBR14	HDBR20	HDBR40	HDBR49	HDBR60	R+6	R+13		time	group	time x group
Serum Ca (mmol/l)[1]												
Aox	2.30 ± 0.05	2.40 ± 0.05	2.40 ± 0.07	2.41 ± 0.06	2.35 ± 0.06	2.38 ± 0.07	2.33 ± 0.05	2.33 ± 0.04	2.31 ± 0.03	<0.001	0.702	0.971
Con	2.32 ± 0.08	2.41 ± 0.08	2.41 ± 0.09	2.40 ± 0.08	2.37 ± 0.06	2.39 ± 0.05	2.33 ± 0.06	2.34 ± 0.06	2.32 ± 0.09			
Serum PTH (pg/ml)[1]												
Aox	26.9 ± 10.4	23.2 ± 7.9	21.6 ± 9.3	21.7 ± 9.9	21.7 ± 10.3	21.2 ± 8.5	23.9 ± 7.6	26.8 ± 7.8	33.3 ± 10.8	<0.001	0.378	0.230
Con	24.3 ± 5.0	21.8 ± 5.4	20.8 ± 5.4	19.9 ± 5.1	21.1 ± 4.2	21.3 ± 5.8	26.7 ± 5.1	25.6 ± 4.1	32.0 ± 6.1			
Serum 25(OH)D (ng/ml)[2]												
Aox	32.3 ± 8.7	__	32.1 ± 7.9	__	26.6 ± 7.8	__	__	21.4 ± 4.0	20.5 ± 4.5	<0.001	0.853	0.933
Con	33.8 ± 10.8	__	32.6 ± 8.8	__	27.1 ± 7.5	__	__	22.8 ± 3.2	22.4 ± 4.6			

[1] Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; FU, follow-up; HDBR, 6◦ head-down tilt bed rest; PTH, parathyroid hormone; R, recovery; serum 25(OH)D, serum 25-hydroxyvitamin D.
[2] Statistical analyses were performed with linear mixed models.
[3] For serum Ca and PTH during BDC, day BDC-2 was used for baseline measurements.
[4] For serum 25(OH)D during BDC, day BDC-4 was used for baseline measurements

Bone formation and bone turnover markers

The Aox cocktail did not affect the bone formation markers bAP and P1NP, or the bone turnover markers osteocalcin and undercarboxylated osteocalcin. Compared to Con group, the mean difference from baseline at HDBR day 60 was 1.83 U/L (95% CI [-4.24, 0.58]) lower in Aox group for bAP, <0.01 g/L (95% CI [-8.44, 8.44] lower in Aox group for P1NP; 0.25 ng/mL (95% CI [-2.82, 2.32]) lower in Aox group for osteocalcin and 0.67 ng/mL (95% CI [-1.21, 2.55]) higher in Aox group for undercarboxylated osteocalcin. All formation and turnover markers showed a significant time effect, with no changes during HDBR and an increase in R (P < 0.001; Figure 2-6). At FU, all formation markers were still elevated compared to BDC values.

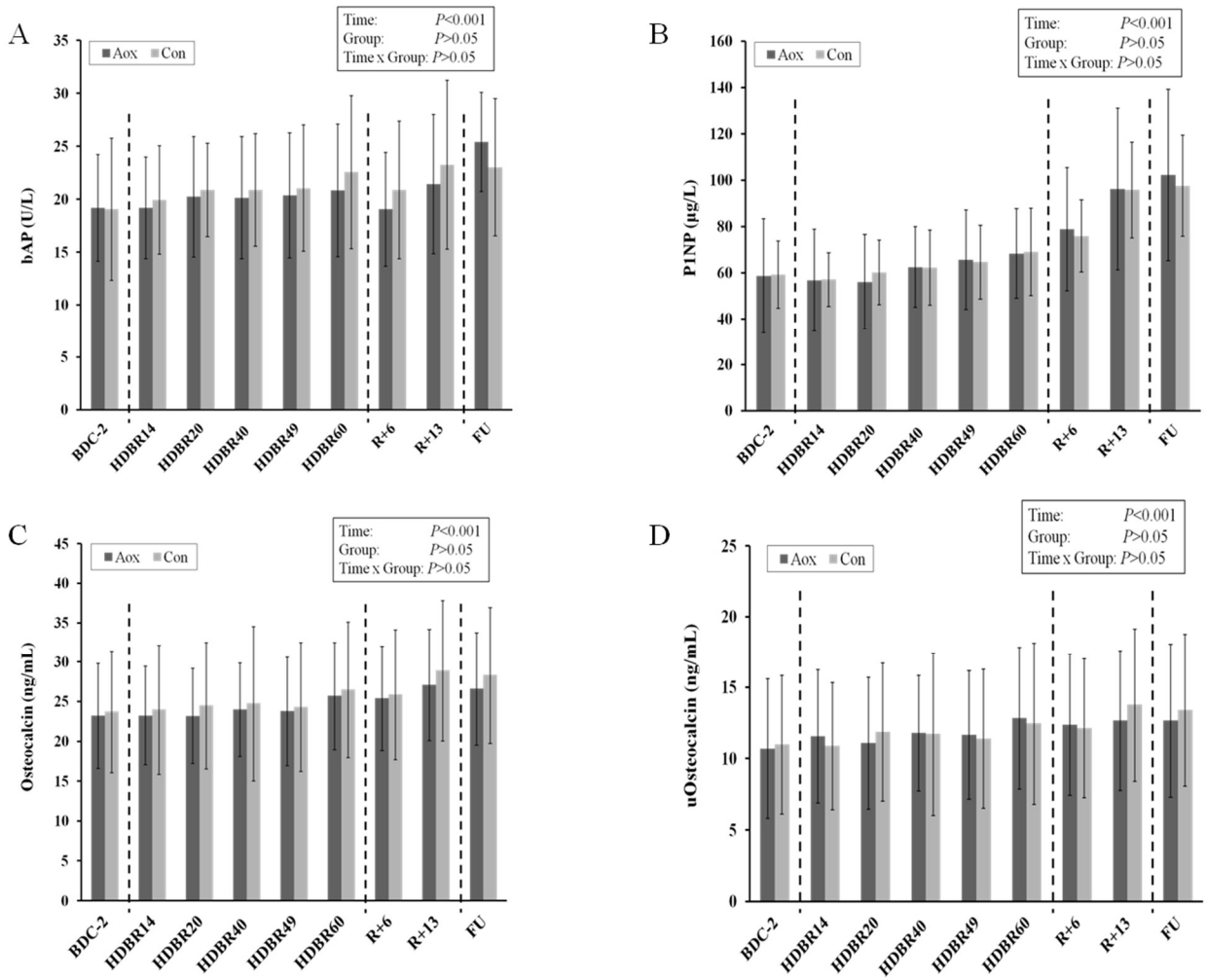

Figure 2-6 (A) Serum bAP, (B) P1NP, (C) osteocalcin, and (D) uOsteocalcin concentrations in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR.

The dotted lines show the division into the different study phases (BDC, HDBR, R, and FU). Values are means ± SDs, n = 20. Aox, antioxidant group; bAP, bone alkaline phosphatase; BDC, baseline data collection; Con, control group; FU, follow-up; HDBR,6◦ head-down tilt bed rest; P1NP, aminoterminal propeptide of type I collagen; R, recovery; uOsteocalcin, undercarboxylated osteocalcin.

Calcium excretion and bone resorption markers

Urinary calcium, CTX, and NTX excretions and serum β-CTX concentrations did not decrease with antioxidant supplementation (Figures 2-7 and 2-8), but were significantly *(P* < 0.001) higher during HDBR compared to BDC or R. Compared to Con group, the mean difference from baseline at end of HDBR was 337 µg/d (95% CI [-1150, 399]) lower in Aox group for CTX, 17.65 mmol/d (95% CI [-84.96, 120.26]) higher in Aox group for NTX and 0.05 µg/L (95% CI [-0.05, 0.14]) higher in the Aox group for β-CTX. Resorption markers were back to baseline values at FU.

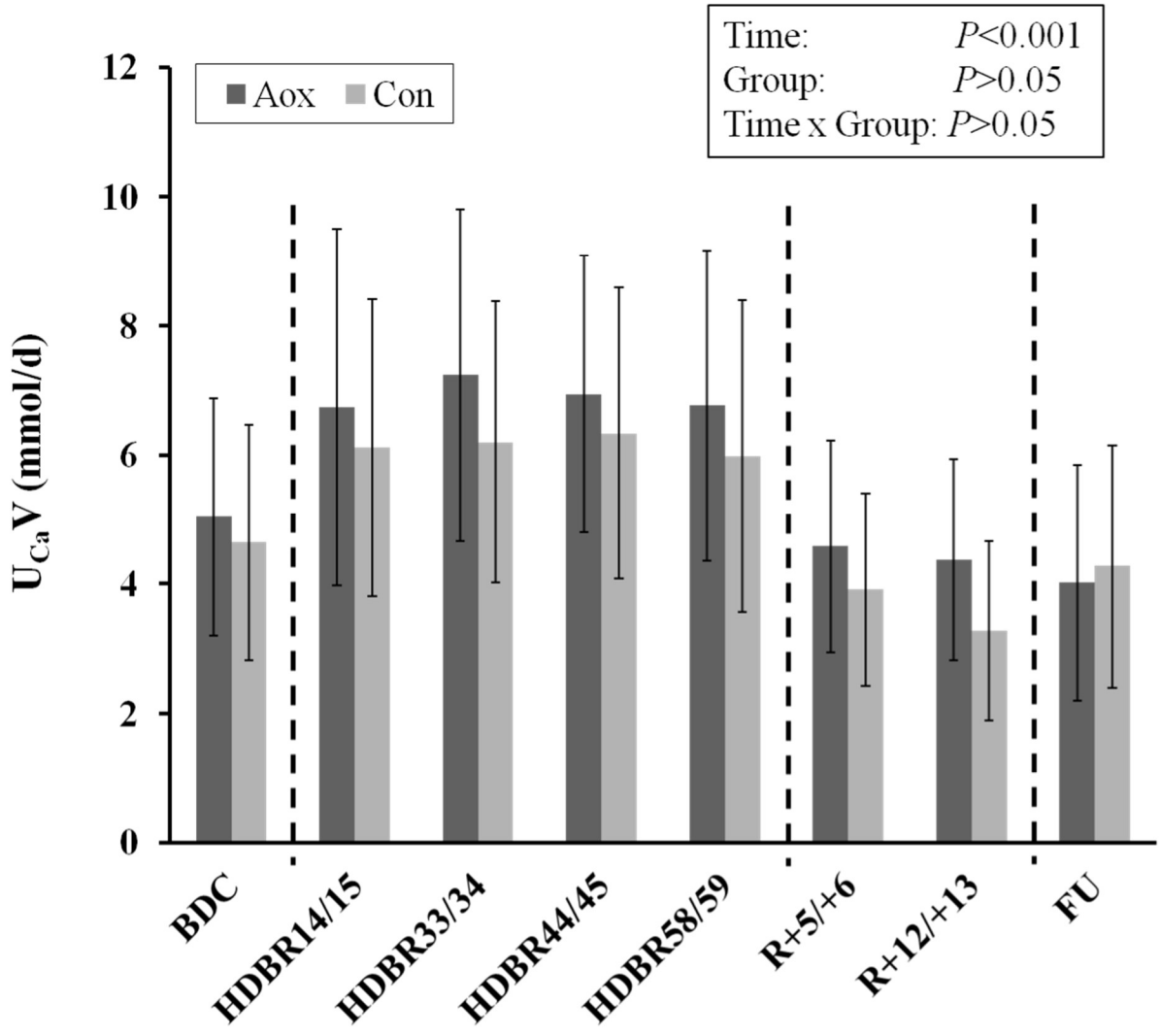

Figure 2-7 Calcium excretion in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR.

The dotted lines show the division into the different study phases (BDC, HDBR, R, and FU). Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; FU, follow-up; HDBR, 6◦ head-down tilt bed rest; MBDC, mean values from baseline data collection days -4 to -1; R, recovery; UcaV, calcium excretion.

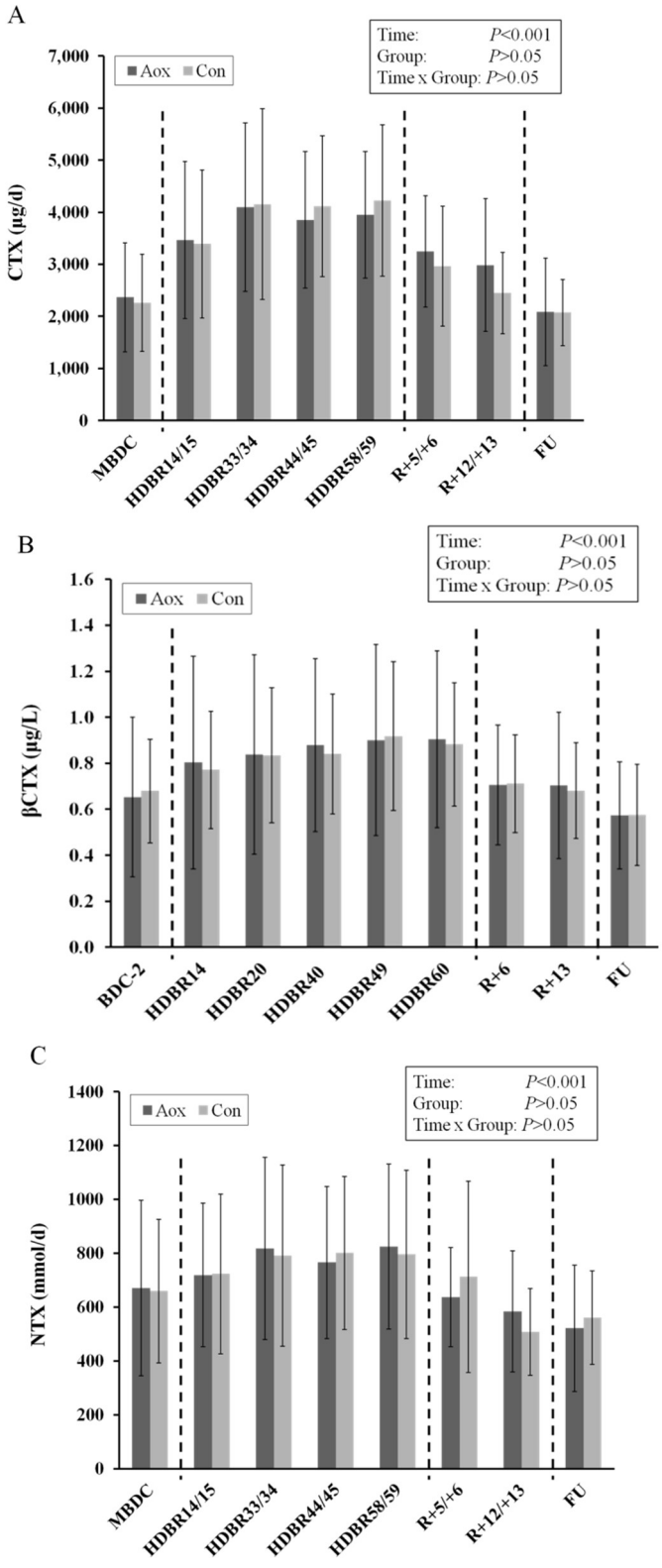

Figure 2-8 (A) Urinary CTX excretion, (B) βCTX serum concentration and (C) urinary NTX excretion in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR.

The dotted lines show the division into the different study phases (BDC, HDBR, R, and FU). Values are means ± SDs, n = 20. Aox, antioxidant group; BDC, baseline data collection; Con, control group; CTX, C-telopeptide of type I collagen; FU, follow-up; HDBR, 6◦ head-down-tilt bed rest; MBDC, mean values from baseline data collection days -4 to -1; NTX, N-telopeptide of type I collagen; R, recovery; βCTX, β-crosslaps of C-telopeptide of type I collagen.

Discussion

In the present study, we aimed to mitigate bone loss in HDBR by supplementing diet intake with an Aox cocktail. Our data demonstrate that supplementation of antioxidants to a wellbalanced diet did not affect bone resorption or formation in bed rest. The HDBR-related changes observed (e.g., increased bone resorption, unchanged bone formation, decreased PTH) are comparable to those from previous bed rest studies (1, 41, 42).

Evidence from in vitro, animal, human observational, and some human intervention studies suggests a positive effect of antioxidants on bone health, including reduction of bone resorption and an increase of bone formation processes; changed activity of bone cell receptors (like receptor activator of NF-κB, OPG, estrogen receptors, TNF receptor, and toll-like receptors 2 and 4) and bone turnover–related transcription factors; and improvements in BMD (18, 19, 43, 44). The fact that we could not find a beneficial effect of the antioxidant supplement containing polyphenols, omega-3 fatty acids, vitamin E, and selenium on bone markers may be due to several reasons.

Other data presented from this 60-day bed rest study report minimal effects of the supplement on oxidative stress parameters, including 4-hydroxynonenal, protein carbonyls, glutathione peroxidase, and catalase (45). The number of neutrophils did not change during HDBR, suggesting no oxidative stress or inflammation processes in HDBR, but the neutrophil count showed an increase right after the end of bed rest, before declining again (Figure 2-5). The instant increase at R + 1, although within the physiological limits (2000/μL to 7500/μL), might be due to a combination of the following aspects: 1) body fluid redistribution when volunteers switch from a prone to an upright position, which can be observed at landing in astronauts as well (46) and seems to be accompanied by an increase in the number of neutrophils in peripheral blood (47); 2) a transient increase in metabolic stress due to the sudden change in position, causing mobilization of neutrophils stored in the bone marrow; or 3) changes in expression of adhesion molecules on neutrophils, as found after short-duration spaceflight (48). The minimal effect on oxidative stress parameters and neutrophils, as well as the fact that proinflammatory cytokines did not change during HDBR (Jean-Pol Frippiat, Lorraine University, unpublished data) indicates that we could not reproduce the increase in oxidative stress observed in previous bed rest studies (4, 5). This might be a reason why we did not find any effect of the Aox cocktail on bone resorption and formation markers. The most likely explanation for the lack of increase in oxidative stress during HDBR and the

missing effect of the Aox cocktail is the fact that in this study, all volunteers were adequately supplied with energy, macronutrients, and micronutrients, based on DRIs (33, 34). A diet meeting DRIs already contains between 500 and 1500 mg/d of antioxidants (49). Thus, diets of both groups likely contained sufficient amounts of antioxidants, which counteracted the development of oxidative stress. The additional supplementation might not have induced a further benefit (reduction of bone resorption and increase of bone formation) if volunteers already received a nutritive, well-balanced diet.

Apart from that, differences in study duration, investigated outcome variables, subject demographics (sex, age, bone health status, genetics), applied antioxidant doses, bioavailability, and antioxidant supply (in study diet) in the control group can be further explanations for the contradictory results.

Previous studies showing beneficial effects of polyphenols on BMD and bone markers lasted at least 4 months (28, 50, 51), while the present study was 2 months. It is possible that effects on bone turnover markers would have been found with a longer study duration, but our hypothesis was that the impact of bed rest on bone health would make this a viable study design despite the shorter length. Similarly, other studies investigating the effects of soy isoflavonoids or green tea polyphenols (duration > 1 year) could not find an effect on bone turnover markers or BMD, either (52–54). A daily administration of 4 g EPA/DHA in postmenopausal breast cancer survivors for 3 months showed a significant reduction of the resorption marker deoxypyridinoline after 3 months compared to controls (26). When only fish-oil responders were included for analysis, deoxypyridinoline values were not significantly different between groups, but serum CTX concentrations showed a significant decrease in fish-oil responders compared to controls (−14% in fish-oil responders from baseline to 3 months after interventions vs. −3% in the control group) (26). That study duration was longer and the omega-3 fatty acid doses applied were higher than in the current Aox-cocktail study. Still, Hutchins-Wiese et al. (26) could not find an effect on bone formation markers bAP or P1NP (26), and an application of 1.48 g EPA/DHA per day for 12 weeks did not alter serum βCTX values in mild to moderately depressed men and women maintaining their habitual activity level (55). A recent meta-analysis of 8 human intervention trials investigating the effects of omega-3 fatty acids on bone turnover markers did not find an effect on bAP or CTX, but did find a decrease of osteocalcin. Study durations of included studies varied between 3 and 18 months (56). The lack of an effect with longer study

durations could indicate that the study duration is not the reason for the contradictory study results for the omega-3 fatty acids. Here, differences in study population (age, sex, and health status) and supplemented doses are more likely to explain variations.

The investigated outcome variables are another methodological difference that may account for observed differences. Some studies assessed the effect of antioxidants on BMD (52, 57), others on bone turnover markers only (58–60), and others on both (51, 61, 62). We examined biochemical markers only because they react rapidly, are good predictors of bone loss and fractures, and are sometimes even more strongly associated with the fracture risk than is the BMD (63, 64). The bone markers investigated in studies differ as well. Wong et al. (51), examining the same bone markers as in the Aox-cocktail study, could not find an effect of soy isoflavonoids on bAP, NTX, or osteocalcin after 2 years of supplementation. This is similar to our results (Figures 6 and 8). Hooshmand et al. (62), however, found a decrease of the resorption marker tartrate-resistant acid phosphatase 5b (TRAcP 5b) after 6 months of supplementation with 50 g/d dried plums; a marker we did not analyze. TRAcP5b is an osteoclast enzyme involved in the breakdown of the bone matrix, whereas NTX and CTX are degradation products of the mature type I collagen (65). However, TRAcP 5b, NTX, and CTX were all intensively studied and are good indicators for bone resorption (65). Therefore, the differences here are more likely to be explained by differences in the study population and the type of polyphenol supplementation.

Bone health status, sex, and age also differed between our study and previous trials. Participants included in the Aox-cocktail study were healthy men, while studies that observed a beneficial effect were conducted with osteopenic or osteoporotic women (26, 28, 66, 67). Our participants were aged between 20 and 45 years, whereas the age range in previous studies was mainly 50–80 years (26, 28, 66). Sex, age, and physiological conditions affect the bioavailability of antioxidants (68), and therefore differences in bioavailability between our study and previous studies could explain some of the contradictory outcomes. Study findings so far indicate that antioxidants might be more effective in postmenopausal women with risk factors for osteoporosis.

Differences in the applied antioxidant doses may also have contributed to differences in study findings. Omega-3 fatty acid consumption in previous studies ranged from 320–4200 mg EPA/DHA daily (56). With 2100 mg EPA/DHA per day, the supplement dose in the present

study was in the middle range. It is difficult to assess the effective dose because of inconsistent data. Some studies with a lower supplementation dose found effects, whereas studies using higher doses could not observe effects, and vice versa (56). Zwart et al. (27) showed a negative correlation between the mean daily intake of total omega-3 fatty acids and the percentage change in urinary NTX excretion during 60 days of bed rest. Although in our Aox-cocktail study the omega-3 fatty acid doses consumed were even higher, we did not see any effect on NTX excretion. While in the study by Zwart et al. (27) volunteers consumed omega-3 fatty acid–rich foods, in the presented study omega-3 fatty acids were administered in capsules. The different type of administration (whole food compared to supplement) might explain the contradictory results here. Polyphenol doses used in previous studies also show high variation, and the amount of polyphenol subclasses administered varied between several mg (62, 66) and 1 g per day (50). Both the lower and higher doses showed positive results. The 741 mg/d polyphenol mixture used in the Aox-cocktail study is in the upper range. Still, the bioavailability of polyphenol subgroups (classification according to chemical structure) is different, and this might explain why we did not find any effects while other studies using different polyphenols did. Apart from the abovementioned internal factors (age and sex), bioavailability also depends on food-related factors. An important determinant is the food matrix the polyphenols are in (the presence of nutrients like lipids, proteins, vitamins, and/or other polyphenols) and meal composition (compounds in other foods, affecting the absorption) (68, 69). Thus, differences in the type of antioxidant administration (extract vs. food) and the meals the antioxidant supplements are consumed with might be further reason for the inconsistent results.

As mentioned above, volunteers in the Aox-cocktail study were sufficiently supplied with all nutrients right from the beginning of the study. Other studies investigating the effects of antioxidants on bone resorption and formation did not provide any information about the initial nutrient status, the nutrient intakes and antioxidant intake during the study, or the habitual diet of study participants (28, 50–55). Based on our results, we assume that an effect can only be seen if test persons are inadequately supplied. This highlights how important it is to determine the initial nutrient status, as the antioxidant effect seems to depend on the initial condition of the volunteers. Administration of antioxidants in addition to an optimal nutrient supply seems to have no further improving effects.

The major strength and an outstanding characteristic of the presented study were the highly controlled study conditions. Volunteers stayed in a metabolic ward, with a strictly controlled environment and monitoring during the duration of the study. Energy and nutrient intakes were individually tailored and strictly controlled, with no differences between the study groups (Table 2-2). Participants received the antioxidant supplement with their meals, so high treatment compliance was ensured. The similarity of the study results with previous bed rest studies underlines the good methodological approach. Baseline characteristics of the Aox and Con groups were similar (Table 2-1), which emphasizes the successful randomization and methodology. A potential limitation was the absence of a placebo. This was due to the characteristic taste of the fish oil capsules used and difficulties in finding a matching placebo. Other studies used olive oil as a control (70, 71) or compared the effects of fish oil to other oils, such as primrose oil (71). However, evidence from in vitro and animal studies suggests that olive oil may have a positive impact on bone mass (72), and therefore might not be suitable as a placebo. Another limitation is the fact that the presented study is an exploratory study. Thus, no power analysis was performed, and the statistical analysis was not adjusted for multiple testing.

In conclusion, the tested Aox cocktail applied as a countermeasure in this study did not reduce the overall negative effects of HDBR on markers of bone formation and resorption. Based on our results, supplementation of an Aox cocktail in addition to a diet already rich in antioxidants may therefore not be recommended in the dose and form used here as a contribution to countermeasures to maintain BMD.

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Author Contributions

The authors' responsibilities were as follows – MH, NB: designed the study; KA, NB, J-PF, MH, conducted the study; KA, NB, J-PF, MH, SMS, SRZ: analyzed the data; KA, RF: performed the statistical analysis; KA, MH: wrote the manuscript and had primary responsibility for the final content; and all authors: revised drafts of the manuscript and read and approved the final manuscript.

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Supplemental Table 2-1 Structured exercise sessions during BDC period – individual data of healthy men who were or were not supplemented with antioxidants for 60 days of HDBR [1]

Volunteers	BDC-14, BDC-12, BDC-7, BDC-3	BDC-13, BDC-9, BDC-4, BDC-1
Aox		
A1	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 19' (9 km/h)	bicycle: 40' (104 W)
B1	bicycle: 40' (114 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 19' (9 km/h)
F1	bicycle: 40' (110 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 18' (9 km/h)
G1	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 18' (9 km/h)	bicycle: 40' (122 W)
I1	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 19' (9 km/h)	bicycle: 40' (85 W)
C2	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 14' (9 km/h)	bicycle: 40' (85 W)
E2	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 13' (9 km/h)	bicycle: 40' (85 W)
F2	bicycle: 40' (95 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 16' (9 km/h)
H2	bicycle: 40' (90 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 17' (9 km/h)
I2	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 19' (9 km/h)	bicycle: 40' (105 W)
Con		
C1	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 19' (9 km/h)	bicycle: 40' (113 W)
D1	bicycle: 40' (90 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 17' (9 km/h)
E1	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 16' (9 km/h)	bicycle: 40' (117 W)
H1	bicycle: 40' (109 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 20' (9 km/h)
J1	bicycle: 40' (106 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 18' (9 km/h)
A2	treadmill: 5' (5 km/h) + 5' (6 km/h) + 16' (9 km/h)	bicycle: 40' (100 W)
B2	bicycle: 40' (105 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 17' (9 km/h)
D2	bicycle: 40' (85 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 17' (9 km/h)
G2	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 16' (9 km/h)	bicycle: 40' (105 W)
J2	bicycle: 40' (115 W)	treadmill: 5' (5 km/h)+ 5' (6 km/h) + 16' (9 km/h)

[1] n=20. Aox, intervention group; BDC, baseline data collection; Con, control group; W, watt

Supplemental Table 2-2 Micronutrient intake in healthy men who were or were not supplemented with antioxidants for 60 days of HDBR in comparison with recommended dietary allowance[1]

	BDC		HDBR		R		RDA[2]
	Aox	*Con*	*Aox*	*Con*	*Aox*	*Con*	*BR-Standards*
Minerals							
Sodium (g/d)	3.95 ± 0.29	3.98 ± 0.37	3.62 ± 0.15	3.64 ± 0.19	3.81 ± 0.24	3.85 ± 0.20	3-4.5
Calcium (g/d)	1.28 ± 0.08	1.28 ± 0.09	1.18 ± 0.04	1.18 ± 0.06	1.26 ± 0.06	1.28 ± 0.10	1-1.2
Phosphorus (g/d)	1.40 ± 0.10	1.41 ± 0.13	1.34 ± 0.09	1.33 ± 0.11	1.37 ± 0.09	1.39 ± 0.14	0.7-1.5
Chloride (g/d)	7.09 ± 0.52	7.12 ± 0.67	6.52 ± 0.29	6.56 ± 0.37	6.86 ± 0.35	6.98 ± 0.45	6.-7.5
Potassium (g/d)	4.43 ± 0.24	4.48 ± 0.28	3.83 ± 0.17	3.82 ± 0.19	4.30 ± 0.17	4.35 ± 0.35	3.5-5
Magnesium (mg/d)	427 ± 30	430 ± 31	376 ± 22	373 ± 24	436 ± 42	343 ± 34	300
Iron (mg/d)	14.3± 0.9	14.5 ± 1.3	12.9 ± 0.8	12.9 ±1.0	14.1 ± 0.7	14.4 ± 1.3	10
Zinc (mg/d)	16.6 ± 1.3	16.7 ± 1.8	15.9 ± 1.1	16.1 ± 1.7	17.1 ± 1.1	17.5 ± 1.9	12-15
Fluoride (mg/d)	2.06 ± 0.23	2.13 ± 0.27	1.93 ±0.08	2.00 ± 0.17	2.19 ± 0.11	2.26 ± 0.23	1.5-4
Iodine (µg/d)	228 ± 18	232 ± 26	236 ± 14	239 ± 20	258 ± 14	262 ± 25	200

		BDC		HDBR		R		RDA[2]
		Aox	*Con*	*Aox*	*Con*	*Aox*	*Con*	*BR-Standards*
Vitamins								
Vitamin (mg RAE/d)	A	1.57 ± 0.08	1.58 ± 0.10	1.46 ± 0.07	1.44 ± 0.08	1.52 ± 0.06	1.52 ± 0.07	1
Vitamin (mg/d)	E	24.8 ± 1.4	24.7 ± 1.5	19.5 ± 1.0	19.4 ± 1.2	23.7 ± 1.2	23.9 ± 1.8	20
Vitamin (µg/d)	K	320 ± 18	326 ± 26	340 ± 21	339 ± 24	365 ± 22	369 ± 39	80
Vitamin (mg/d)	C	266 ± 14	270 ± 17	204 ± 8	203 ± 10	245 ± 17	243 ± 15	100
Thiamin (mg/d)		2.25 ± 0.15	2.25 ± 0.21	1.88 ± 0.11	1.88 ± 0.14	2.31 ± 0.12	2.34 ± 0.19	1.5
Riboflavin (mg/d)		2.33 ± 0.19	2.34 ± 0.22	2.10 ± 0.14	2.10 ± 0.18	2.23 ± 0.16	2.25 ± 0.21	1.5
Pantothenic (mg/d)	Acid	6.80 ± 0.47	6.86 ± 0.67	6.17 ± 0.37	6.17 ± 0.49	6.55 ± 0.42	6.65 ± 0.62	5
Vitamin (mg/d)	B6	2.72 ± 0.17	2.74 ± 0.24	2.47 ± 0.14	2.48 ± 0.19	2.71 ± 0.15	2.73 ± 0.25	2
Folate (µg/d)		500 ± 24	504 ± 30	453 ± 21	454 ± 20	496 ± 26	499 ± 30	400
Vitamin (µg/d)	B12	3.48 ± 0.36	3.54 ± 0.50	4.29 ± 0.34	4.33 ± 0.56	3.86 ± 0.39	3.94 ± 0.56	2
Niacin (mg/d)		36.1 ± 3.3	36.7 ± 4.9	37.6 ± 3.6	38.4 ± 5.1	37.7 ± 3.3	38.6 ± 5.2	20
Biotin (µg/d)		51.6 ± 2.8	51.6 ± 3.8	44.1 ± 2.0	43.7 ± 2.7	48.8 ± 2.3	49.4 ± 4.0	100

[1] Values are mean ± SD, *n*=20.
[2] Recommended dietary allowance according to bed rest standards [30]. Aox, intervention group; BDC, baseline data collection; BR, bed rest; Con, control group; FU, follow-up; HDBR, 6° head down tilt bed rest; R, recovery; RAE, retinol activity equivalents

3. Manuscript III

Effects of antioxidant supplementation on bone mineral density, bone mineral content and bone structure in healthy men during 60 days of 6° head-down tilt bed rest: Results from a randomised controlled trial

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Abstract

Dietary countermeasures to mitigate detrimental spaceflight-induced effects on bone health would alleviate the requirements and the consequences imposed by other types of countermeasures for this risk. We hypothesised that antioxidant supplementation during 60 days of 6° head-down tilt bed rest (HDBR), an analogue of spaceflight, would have a protective effect on bone mineral density (BMD), content (BMC) and bone structure parameters. An exploratory, randomised, controlled, single-blind intervention trial was conducted in a parallel design with 20 healthy male volunteers (age 34 ± 8 y, weight 74 ± 6 kg). The study included 14 days of baseline data collection (BDC) before bed rest, followed by 60 days of HDBR and a 14-day recovery period. Ten subjects in the antioxidant group received a supplement (741 mg/d polyphenols, 2.1 g/d omega-3 fatty acids, 168 mg/d vitamin E and 80 µg/d selenium) daily. Ten subjects in the control group received no supplement. The diet was consistent with dietary reference intakes, individually tailored based on the subject's bodyweight and strictly controlled. We measured whole-body, lumbar spine and femur BMD and BMC, as well as BMD of the cortical and trabecular compartments of the distal radius and tibia, and cortical and trabecular thickness during BDC, HDBR and recovery. Data were analysed using linear mixed models. The supplementation of an antioxidant cocktail did not mitigate the deteriorating effects of HDBR on BMD, BMC and bone structure parameters. Our findings do not support a recommendation for antioxidant supplementation for astronauts.

Introduction

Reduced mechanical loading of the skeleton and the related increase in oxidative stress (Zwart et al., 2013) induced during bed rest and spaceflight are associated with bone loss (Zwart et al., 2009, 2013), as observed by a decrease of whole-body and regional (e.g., spine, femoral head and tibia) areal bone mineral density (aBMD) and bone mineral content (BMC) (Hargens & Vico, 2016; Leblanc et al., 1990; Vico & Hargens, 2018). After long-duration bed rest, subjects had changes in the structure of their radius and tibia, as determined by peripheral quantitative computed tomography (pQCT) and by high-resolution peripheral quantitative computed tomography (HR-pQCT) (Armbrecht et al., 2011; Belavy et al., 2011; Beller et al., 2011; Cervinka et al., 2014; Hargens & Vico, 2016; Rittweger et al., 2005, 2009, 2010). Reductions in volumetric BMD (vBMD) and cortical thickness at the distal tibia were observed in both male and female bed rest subjects (Armbrecht et al., 2011; Belavy et al., 2011; Rittweger et al., 2009), whereas increases in the thickness of the tibial trabecular compartment were found only in men (Armbrecht et al., 2011). Men had reduced levels of cortical bone in the radius (Belavy et al., 2011), whereas women had reduced levels of trabecular bone in the radius (Armbrecht et al., 2011).

The pursuit of countermeasures to alleviate spaceflight-induced bone loss extends as far back as human spaceflight itself. Physical countermeasures such as exercise tend to be resource intensive, requiring mass and volume for equipment, and substantial crew time (Loehr et al., 2015). Pharmaceutical countermeasures offer promise (Sibonga et al., 2019), but typically this involves repurposing drugs that were developed to treat diseases such as osteoporosis and are typically targeted at elderly patients with the disease, not middle-aged, healthy individuals in an extreme environment, and any medication can be associated with potential side effects. For example, astronauts can develop hypocalcaemia as a side effect of drugs such as bone resorption-inhibiting bisphosphonates (Smith et al., 2014, 2015). Dietary and nutritional countermeasures require very little additional mass or volume for spaceflight and are generally free of side effects. Higher intakes of fish and omega-3 fatty acids were associated with less bone loss during exposure to real or to simulated microgravity (Zwart et al., 2010). Likewise, a lower dietary-induced net endogenous acid production protects bone during unloading (Frings-Meuthen et al., 2011; Zwart et al., 2004, 2005, 2018). Conversely, greater increases in iron stores in astronauts during flight were associated with oxidative stress and detrimental changes in bone health (Zwart et al., 2013). Given the evidence that oxidative

stress is associated with bone loss, an obvious question is whether supplementing with antioxidants can protect bone during spaceflight.

The antioxidative and anti-inflammatory actions of antioxidants support bone metabolism, affect bone cell receptors and suppress or activate bone signal transduction pathways (Đudarić et al., 2015; Torre, 2017; Wauquier et al., 2015; Wong et al., 2019). On the basis of the results of preliminary studies (Aires et al., 2019; Damiot et al., 2019), we selected an antioxidant cocktail containing omega-3 fatty acids, polyphenols, vitamin E and selenium to test in bed rest subjects. We hypothesised that this antioxidant supplement would decrease bone turnover and thus mitigate loss of bone mineral content and density. The effects of this supplemented antioxidant cocktail on bone biochemical markers in bed rest subjects have been published previously (Austermann et al., 2021), and no changes were detected between the subjects who received the supplement and those who did not. However, although markers of bone resorption and formation reflect bone homeostasis and provide insight into the dynamics of bone turnover (Shetty et al., 2016), these biochemical markers do not reflect the direct effects on the bone tissue. BMD measurements represent the inorganic mineral content in bone (Kranioti et al., 2019), and these measurements have been used to assess bone loss in clinical investigations and to evaluate and diagnose bone loss-related diseases such as osteoporosis (Blake & Fogelman, 2007; Kranioti et al., 2019). Thus, BMC, BMD and bone structure parameters might provide an integrated view of the bone and extend the understanding of changes in bone quality during bed rest. The data described in the present paper complement the previous bone biomarker data (Austermann et al., 2021) and demonstrate the effects of the antioxidant supplement on whole-body, lumbar spine and femur aBMD and BMC, as measured by dual-energy X-ray absorptiometry (DXA), and of vBMD, as measured by HR-pQCT.

Materials and Methods

We report here unpublished data regarding bone densitometry from a 60-day bed rest study. Other aspects of this study have been published, including bone biochemistry. As such, the details of the study design, subject recruitment, enrolment, randomisation, intervention, diet and study implementation have been described previously (Arc-Chagnaud et al., 2020; Austermann et al., 2021; Brauns et al., 2021a, 2021b; Liu et al., 2021; Mendt et al., 2021; Shur et al., 2022). The study design was reviewed and approved by the University Research Ethics Committee of the Rheinische Friedrich-Wilhelms University of Bonn, Germany

(identifier: 375/15). It was part of a comprehensive 6° head-down tilt bed rest study, referred to as the antioxidant cocktail (Aox cocktail) study, sponsored by the European and French Space Agencies. Sixteen scientific teams investigated the effects of an antioxidant cocktail on physiological systems, including the immune, cardiovascular, musculoskeletal and neurosensory systems, during 60 days of 6° head-down tilt bed rest (HDBR) (Arc-Chagnaud et al., 2020; Austermann et al., 2021; Brauns, Friedl-Werner, Gunga, & Stahn, 2021; Liu et al., 2021). This trial was approved by Comité de Protection des Personnes / Sud-Ouest Outre-Mer I and the French Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé) in accordance with the 1964 Declaration of Helsinki and its later amendments and was registered at https://clini caltr ials.gov/ under identifier NCT03594799 at 19/07/2018. All subjects gave written informed consent before participating in the study, and all investigations took place at the Institute for Space Medicine and Physiology (MEDES), Toulouse, France.

Subjects

Twenty healthy male volunteers (age: 34 ± 8 y, mean ± SD, bodyweight: 74 ± 6 kg; body mass index [BMI]: 24 ± 2 kg/m2) participated in the study after completing a medical and psychological screening. To be eligible for participation, individuals were required to meet a specific level of physical fitness as determined by their maximal oxygen uptake (VO_{2max}). For those under the age of 35 years, this was defined as a VO_{2max} level between 35 mL/(min·kg) and 60 mL/(min·kg), while for those over the age of 35 years, the required level was between 30 mL/(min·kg) and 60 mL/(min·kg). Primary exclusion criteria included any history of bone disease, knee problems, joint surgery or fractures, or presence of osteosynthesis, musculoskeletal and cardiovascular disorders, a whole-body bone mineral density T-score ≤−1.5, and a history of substance abuse, and/or a restricted diet (such as vegetarianism or food allergies).

Study design

A randomised, controlled, single-blind intervention study was performed in a parallel design and was implemented in 2 campaigns. Each campaign included 10 volunteers who resided in the metabolic ward at MEDES. The duration of each campaign was 88 days and consisted of 3 study phases: an adaption and baseline data collection phase (BDC) for 14 days, a 6° head-down tilt bed rest (HDBR) intervention period (60 days) and 14 days of recovery (R). Volunteers were first paired based on their usual level of physical activity and then were randomly –by flipping a coin– assigned to either the antioxidant (Aox) or control (Con)

group. In each campaign, five volunteers were designated to the Aox group and were given an antioxidant supplementation, while the Con group did not receive a placebo due to the distinct flavour of the fish oil capsules utilised in the antioxidant supplement. As such, a single-blind study design was implemented, with the investigators responsible for sampling and data analysis being unaware of the group assignments. To avoid deconditioning before HDBR, volunteers followed a personalised training routine during BDC. All study protocols and activities during HDBR were performed in 6° head-down tilt position (Orlov & Sundblad, 2014). Recovery included a physical rehabilitation program individually tailored for each participant, as described previously (Austermann et al., 2021).

Physical activity level (PAL) and maximal aerobic capacity assessment (VO_{2max})

Subjects' habitual physical activity was assessed over a period of 10 consecutive days before arrival at MEDES with an accelerometer (ActiGraph 3GTX) and using the Monica Optional Study of Physical Activity Questionnaire to measure physical activity (Pereira et al., 1997). Standard VO2max tests adapted for bed rest studies were applied (Orlov & Sundblad, 2014).

Antioxidant intervention

The Aox cocktail developed for this study was used because the results of previous studies showed that, after 20 days, it reduced oxidative stress markers and improved antioxidative capacity in mice and humans with reduced physical activity (Aires et al., 2019; Damiot et al., 2019). The supplement consisted of 741 mg/d polyphenols (XXS-2A-BR2 Spiral, part of Pole National de Compétitivité Vitagora Goût-Nutrition-Santé), 2.1 g of omega-3 fatty acids (Omacor®, Pierre Fabre), and a combined preparation of 168 mg vitamin E and 80 µg selenium (Solgar). Polyphenol and omega-3 fatty acid supplements were administered with breakfast, lunch and dinner. Vitamin E and selenium supplements were consumed once a day at breakfast (Table 3-1).

Table 3-1 Composition of the Aox-cocktail composition, daily intake amount and time of administration

Constituent	Daily intake (mg)	Administration time
XXS-2A-BR2(total polyphenols)	***741***	3 times a day (breakfast, lunch and dinner)
Flavonols	323	
Flavanols	136	
Flavanones	108	
Oligostilbenes	78	
Hydroxycinnamic acids	50	
Phenylpropanoids	46	
Omacor® (*total omega-3 fatty acids)*	***2100***	3 times a day (breakfast, lunch and dinner)
Eicosapentaenoic acid (EPA)	1000	
Docosahexaenoic acid (DHA)	1100	
Vitamin E	***168***	Once a day (breakfast)
Selenium	***0.08***	Once a day (breakfast)

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

Diet

Because of differences in physical activity, energy intake differed between study phases with lower intakes during bed rest. The macro-and micronutrient intakes followed international bed rest standards (Orlov & Sundblad, 2014), largely based on US and German dietary reference intake (DRI) values (Food and Nutrition Board of the Institute of Medicine, National Academies, 2011; German Nutrition Society, Austrian Nutrition Society, Swiss Society for Nutrition Research, Swiss Nutrition Association, 2013). To avoid any impact of energy intake on bone turnover, each subject's diet (energy and nutrient intake) was individually tailored based on the participant's basal metabolic rate and activity, with energy intake being equal to 150% of the basal metabolic rate (BMR) + 10% of total energy expenditure (TEE; estimation of thermogenesis) in BDC, 130% of BMR + 10% of TEE in HDBR and 145% of BMR + 10% of TEE in R (Austermann et al., 2021; Orlov & Sundblad, 2014). During the entire study, the diet was monitored, and energy and nutrient intake were traced. Meals were defined by a nutritionist and meal plans were created with nutrition software (Nutrilog 3.11b, Nutrilog SAS). Each day volunteers received three meals (breakfast, lunch and dinner) and one snack in the afternoon. A detailed description is presented by Austermann et al. (Austermann et al., 2021).

Bone mineral density and bone mineral content analysis via dual-energy X-ray absorptiometry

As part of the bed rest core data, which are analysed for each of the ESA-sponsored bed rest studies (Orlov & Sundblad, 2014), whole-body, lumbar spine and femur BMD and BMC were assessed at BDC-4, HDBR30, HDBR58 and R + 12 days using DXA (QDR 4500 Elite, Hologic, Zaventem, Belgium, software Apex version 3.3.0.1) according to manufacturer's guidelines. Quality control was performed 3 times a week and every morning before the subjects' measurements were taken (the maximum coefficient of variation [CV] of quality control was 1.5%). All measurements were performed by the same operator. To assess whether changes in aBMD were clinically relevant, the least significant change (LSC) was calculated as 2.77*CV% (Shepherd & Lu, 2007), and changes >LSC were considered to have a clinical effect.

HR-pQCT

To assess the density and thickness of the bone tissue, HR-pQCT was performed as part of the bed rest core data (Orlov & Sundblad, 2014) on the left distal radius and tibia on BDC-4, HDBR20, HDBR40, HDBR60, R + 13, and R + 30 using XtremeCT (Scanco Medical AG, Wangen-Brüttisellen, Switzerland) according to manufacturer's guidelines. If any conditions (e.g. previous fracture) were found that exclude a valid measurement on the left side of the body, the right radius or tibia was measured for the duration of the study. Quality control was performed weekly and every morning before the subjects' measurements were taken (the maximum CV of quality control was 2%). The standard measurement protocol uses the following settings: an X-ray tube potential of 60 kVp, X-ray tube current of 95 mA, matrix size of 1536 × 1536 and slice thickness and in-plane voxel size of 82 μm. At each site, a total scan length of 9.02 mm in axial direction divided into 110 computerised tomography slices was simultaneously measured. Single-scan effective dose was 3 μSv. To assure accuracy and high reproducibility, the subjects' scanned limb was immobilised in a padded, anatomically formed carbon fibre cast and secured in the gantry. The subjects were instructed to refrain from any kind of movement during the measurement (2.8 min), and the measurements were always performed by the same operator. To account for motion artefacts, scans were inspected and validated according to the following scale (grades 1–5) and only images with grades of 3 or less were included in the analysis:

1. No motion artefacts visible.
2. Very slight artefacts (horizontal streaks are visible at the upper and lower ends).

3. Some artefacts (horizontal streaks are visible, cortex is intact).
4. Large horizontal streaks are visible (continuity of cortex is moderately disrupted and trabeculae are smeared).
5. Major horizontal streaks are visible (complete disruption of cortex continuity and trabecular structure).

To assess whether changes in vBMD and structure parameters were clinically relevant, LSC was calculated as 2.77*CV% (Shepherd & Lu, 2007), and changes >LSC were considered to have a clinical effect.

Sample size

The Aox-cocktail study is considered an exploratory study as it is the first study that investigates the effects of antioxidants on different physiological systems in bed rest. Therefore, no power analysis was performed.

Statistical analyses

All statistical analyses were performed using a statistical software package (SPSS version 25, IBM Corporation). The linear mixed-models procedure was used to test the effects of intervention (Aox-cocktail), time points (BDC, HDBR and R), and the interaction of intervention and time on whole-body, lumbar spine and femur BMD, BMC, local BMD on radius and tibia, and cortical and trabecular thickness. Fixed factors were group (Aox and Con groups), time (BDC, HDBR and R) and interaction (group x time). Subject identifier was set as a random factor. Baseline values, VO2max and PAL were included as covariates. Baseline values were not included in the outcome vector. In all tests, the residuals were checked for relevant deviations from a normal distribution.

To identify whether local BMD and bone structure parameters had returned to BDC at R + 30, paired t-test was performed for intra-group comparison and an unpaired t-test for the inter-group comparison. To assess whether bone metabolism (measured as bone biomarkers) differed between recovered and non-recovered subjects, linear mixed model procedure was used. Fixed factors were group (recovered and non-recovered), time (BDC, HDBR and R) and interaction (group x time). Subject identifier was set as a random factor. VO2max and PAL were included as covariates. Bone was considered recovered when total BMD at the tibia and the radius at R + 30, as measured by HR-pQCT, and whole-body aBMD at R + 12, as measured by DXA, were within the LSC.

Statistical significance was assigned to $p < 0.05$, and unless otherwise stated, data are presented as the arithmetic mean ± standard deviation.

Results

All subjects completed the study and the Aox and Con groups were similar in age, height, weight, BMI, lean body mass and VO2max (Austermann et al., 2021) (Table 3-2).

Table 3-2 Baseline characteristics of study participants (adapted from Austermann et al. [57])[1]

	Aox (*n* 10)	**Con (*n* 10)**
Age (y)	35 ± 7	34 ± 9
Height (m)	1.76 ± 0.05	1.76 ± 0.05
Weight (kg)	73 ± 6	75 ± 9
BMI (kg/m^2)	24 ± 2	24 ± 2
Whole-body aBMD (g/cm^2)	1.056 ± 0.068	1.056 ± 1.158
VO_{2max} (mL/(min·kg))	42 ± 5	40 ± 4
PAL	1.7 ± 0.1	1.7 ± 0.1

[1] Values are mean ± SD. aBMD, areal bone mineral density; Aox, antioxidant group; BMI, body mass index; Con, control group; PAL, physical activity level; VO_{2max}, maximal oxygen uptake.

Body weight, energy, macronutrient and micronutrient intakes did not differ between the Aox and Con groups (Austermann et al., 2021).

Whole-body, lumbar spine and femur bone mineral density and bone mineral count

Whole-body and femur BMD and BMC showed a significant time effect (BMD, $p < 0.001$; BMC, $p = 0.03$) with a decrease during the recovery phase (Table 3-3). Lumbar spine BMD and BMC did not change throughout the study duration. No difference was detected between the Aox and Con groups for whole-body, lumbar spine and femur BMD and BMC.

At R + 12, total aBMD had not recovered for 5 of the 20 participants, as shown by BMD changes from baseline >LSC. We have previously reported that bone biomarkers did not differ significantly between recovered and non-recovered subjects at any time during the study (Austermann et al., 2021).

Table 3-3 Whole-body, lumbar spine and femur BMD and BMC during the different study phases in Aox and Con groups[1]

			BDC-2	HDBR		R+12	*P*-values from linear mixed models		
				HDBR30	HDBR58		time	group	time x group
Whole-body (g/m^2)	BMD	Aox	1.056 ± 0.068	1.046 ± 0.060	1.050 ± 0.071	1.024 ± 0.076	<0.001	0.44	0.06
		Con	1.056 ± 0.158	1.047 ± 0.154	1.046 ± 0.147	1.018 ± 0.149			
Whole-body (g)	BMC	Aox	2177.88 ± 213.07	2130.21 ± 199.72	2145.02 ± 218.80	2100.02 ± 217.26	0.003	0.45	0.50
		Con	2191.49 ± 448.62	2196.34 ± 467.25	2204.62 ± 427.73	2136.59 ± 445.60			
Lumbar spine (g/m^2)	BMD	Aox	1.072 ± 0.078	1.048 ± 0.083	1.066 ± 0.094	1.058 ± 0.093	008.	0.46	0.14
		Con	1.039 ± 0.142	1.028 ± 0.146	1.029 ± 0.144	1.033 ± 0.140			
Lumbar spine (g)	BMC	Aox	75.94 ± 8.41	74.13 ± 9.01	75.33 ± 9.29	74.90 ± 9.65	0.08	0.43	0.65
		Con	71.28 ± 14.74	70.79 ± 13.57	71.30 ± 13.49	71.21 ± 14.19			
Femur (g/m^2)	BMD	Aox	1.062 ± 0.081	1.061 ± 0.079	1.051 ± 0.082	1.041 ± 0.080	<0.001	0.43	0.06
		Con	1.046 ± 0.130	1.042 ± 0.123	1.038 ± 0.118	1.008 ± 0.131			
Femur (g)	BMC	Aox	42.87 ± 5.26	42.41 ± 5.18	41.57 ± 4.88	41.32 ± 4.90	0.003	0.51	0.13
		Con	42.58 ± 7.48	41.91 ± 7.33	41.64 ± 6.83	39.34 ± 8.34			

[1]Data are mean ± SD, n =20.
Statistical analysis was performed with linear mixed model.
Aox, antioxidant group; BDC, baseline data collection; BMC, bone mineral content; BMD, bone mineral density; Con, control group; HDBR,6° head down tilt bed rest; R, recovery

Local bone mineral density and bone structure parameters

The Aox cocktail did not affect HR-pQCT parameters (Table 3-4). Radius average BMD, cortical BMD and cortical thickness decreased during HDBR and increased during the recovery phase. R + 30 values had returned to BDC levels. The trabecular BMD of the radius did not change during the different study phases. In the Aox group, radial trabecular thickness increased significantly throughout HDBR, whereas, in the Con group, radial trabecular thickness decreased at HDBR20, then increased towards the end of HDBR. In Con group, radial trabecular thickness levels dropped again during the recovery phase, whereas levels were still elevated in Aox group. For both groups, average and cortical BMD at the tibia increased during HDBR and decreased during the recovery phase. Tibial cortical thickness and trabecular BMD decreased during the recovery phase. Tibial trabecular thickness increased during HDBR and decreased during the recovery phase, but values were not below BDC levels. For all parameters, the change from baseline at R + 30 was not different between the Aox and Con groups. Total BMD at the tibia and radius were within the LSC at R + 30 for all subjects; thus, BMD was considered recovered at these skeletal sites.

Table 3-4 HR-pQCT parameters during the different study phases in Aox and Con groups[1]

		BDC-4	HDBR			R+13	R+30	*p*-values from linear mixed models		
			HDBR20	HDBR40	HDBR60			time	group	time x group
Radius total BMD (mgHA/cm^3)	Aox	379 ± 55	374 ± 54	374 ± 57	375 ± 57	380 ± 54	380 ± 56	<0.001	0.12	0.62
	Con	357 ± 47	350 ± 46	349 ± 47	347 ± 48.2	355 ± 51	355 ± 49			
Radius cortical BMD (mgHA/cm^3)	Aox	877 ± 41	873 ± 38	874 ± 46	877 ± 44	880 ± 39	881 ± 43	<0.001	0.18	0.98
	Con	858 ± 51	850 ± 51	851 ± 52	852 ± 53	856 ± 52	858 ± 52			
Radius cortical thickness (mm)	Aox	0.95 ± 0.18	0.91 ± 0.18	0.91 ± 0.19	0.92 ± 0.19	0.95 ± 0.18	0.95 ± 0.18	<0.001	0.46	0.61
	Con	0.89 ± 0.18	0.85 ± 0.17	0.84 ± 0.18	0.83 ± 0.18	0.88 ± 0.19	0.88 ± 0.18			
Radius trabecular BMD (mgHA/cm^3)	Aox	219± 36	219 ± 34	218 ± 35	217 ± 35	218 ± 36	219 ± 35	0.06	0.25	0.65
	Con	212 ± 20	210 ± 21	210 ± 22	208 ± 22	210 ± 21	210 ± 21			
Radius trabecular thickness (mm)	Aox	0.086 ± 0.007	0.087 ± 0.015	0.089 ± 0.013	0.091 ± 0.012	0.088 ± 0.010	0.092 ± 0.014	0.03	0.24	0.66
	Con	0.086 ± 0.012	0.081 ± 0.007	0.084 ± 0.010	0.088 ± 0.010	0.087 ± 0.014	0.086 ± 0.009			
Tibia total BMD (mgHA/cm^3)	Aox	373 ± 48	375 ± 50	375 ± 51	374 ± 51	369 ± 50	371 ± 52	<0.001	0.50	0.91
	Con	341 ± 63	341 ± 62	341 ± 63	340 ± 63	335 ± 64	336 ± 63			
Tibia	Aox	880 ± 35	884 ± 33	885 ± 35	883 ± 33	877 ± 34	878 ± 33	<0.001	0.15	0.67

		BDC-4	**HDBR**			**R+13**	**R+30**	***p*-values from linear mixed models**		
			HDBR20	HDBR40	HDBR60			time	group	time x group
cortical BMD ($mgHA/cm^3$)	Con	872 ± 49	875 ± 47	875 ± 49	875 ± 48	867 ± 49	867 ± 50			
Tibia cortical thickness (mm)	Aox	1.56 ± 0.20	1.57 ± 0.21	1.57 ± 0.21	1.55 ± 0.23	1.54 ± 0.22	1.54 ± 0.22	<0.001	0.85	0.96
	Con	1.38 ± 0.40	1.38 ± 0.40	1.37 ± 0.40	1.36 ± 0.40	1.34 ± 0.40	1.35 ± 0.40			
Tibia trabecular BMD ($mgHA/cm^3$)	Aox	225 ± 35	226 ± 36	226 ± 36	225 ± 36	224 ± 36	224 ± 36	<0.001	0.26	0.98
	Con	210 ± 26	210 ± 26	210 ± 26	210 ± 27	208 ± 28	209 ± 27			
Tibia trabecular thickness (mm)	Aox	0.093 ± 0.007	0.095 ± 0.007	0.098 ± 0.006	0.095 ± 0.004	0.094 ± 0.006	0.095 ± 0.008	0.02	0.36	0.83
	Con	0.087 ± 0.012	0.090 ± 0.015	0.091 ± 0.013	0.088 ± 0.010	0.087 ± 0.013	0.086 ± 0.015			

[1]Data are mean ± SD, *n* 20.
Statistical analysis was performed with linear mixed model.
Aox, antioxidant group; BDC, baseline data collection; BMD, bone mineral density; Con, control group; HA, hydroxyapatite; HDBR, 6° head down tilt bed rest; R, recovery

Discussion

The purpose of this study was to assess whether loss of bone mass during HDBR could be mitigated by supplementing test subjects' diets with an antioxidant cocktail. We found that whole-body, lumbar spine and femur aBMD and BMC, vBMD of the cortical and trabecular compartments of the distal radius and tibia, and radial and tibial cortical and trabecular thickness were not affected by the administration of the Aox-cocktail. These results support our previous findings that the antioxidant supplements did not affect markers of bone formation and resorption (Austermann et al., 2021). Only a minimal effect of the supplement on oxidative stress and inflammation parameters was found (Arc-Chagnaud et al., 2020; Austermann et al., 2021), although the data from the preliminary studies in ambulatory mice and humans (Aires et al., 2019; Damiot et al., 2019) suggested a larger impact over a shorter time period.

The findings from human studies investigating the effects of polyphenols on aBMD are inconsistent (Austermann et al., 2019). Some studies found increases in aBMD in subjects who received polyphenol supplements or consumed polyphenol-rich foods, whereas other studies found polyphenol interventions had no effect on aBMD (Austermann et al., 2019). Few nutritional intervention studies in humans have investigated the effects of antioxidants on vBMD and bone structure parameters. In healthy post-menopausal women, 120 mg/d isoflavonoid supplementation over a period of 3 years increased trabecular vBMD at the distal tibia, and cortical vBMD at the midshaft femur, as measured by pQCT (Shedd-Wise et al., 2011). A 1000 mg/d resveratrol supplementation for 16 weeks did not affect vBMD and bone structure parameters, as measured by HR-pQCT at the distal radius and tibia in ambulatory obese men (Ornstrup et al., 2014), which is consistent with our findings. However, Ornstrup et al. (Ornstrup et al., 2014) report that subjects who took the resveratrol supplement had more trabecular vBMD at the lumbar spine, as measured using quantitative CT, than a control group who did not take the supplement.

There are several reasons that might explain the differences between the outcomes of nutritional intervention studies on humans such as the ones reported previously (Austermann et al., 2021), including differences in the applied intervention protocols, the supplements and/or diets. Different kinds of antioxidants (e.g., omega-3 fatty acids, vitamins, different polyphenols subgroups) have been studied, and the doses differ (e.g., ranging from several mg

to 1 g for polyphenols), also the bioavailability of the nutrient and antioxidant supply differs in the habitual and/or study diet (Austermann et al., 2021).

The nutrient and antioxidant supply in the habitual diet and at study entry seem to affect study outcomes. Subjects who have low concentrations of circulating antioxidants seem to respond more to enrichment of antioxidative foods in their diet than subjects who have high concentrations of circulating antioxidants (Porrini & Riso, 2008). Moreover, it seems that plasma concentrations of antioxidants cannot be further increased once a certain threshold is reached (Porrini & Riso, 2008). This suggests that antioxidant supplementation to a diet already high in antioxidants does not further improve bone health. Unfortunately, baseline (before BDC) antioxidant status was not analysed in this study and should be considered in future studies to investigate this hypothesis. Regardless, all participants of the Aox-cocktail study were sufficiently supplied with nutrients and antioxidants by the study diet (Austermann et al., 2021), which followed DRI values (Food and Nutrition Board of the Institute of Medicine, National Academies, 2011; German Nutrition Society, Austrian Nutrition Society, Swiss Society for Nutrition Research, Swiss Nutrition Association, 2013; Williamson & Holst, 2008).

When interpreting study outcomes, it is critical to determine their clinical relevance. BMD changes can be considered clinically significant at >2.77*CV% (Shepherd & Lu, 2007). For the Aox-cocktail study, this means a change of >4.15% for whole-body aBMD. At R + 12, the mean reduction in aBMD compared to BDC values was −3.07% in the Aox group and −3.45% in the Con group, which suggest that the changes observed in whole-body aBMD in this study might not be clinically relevant. The same applies to femur aBMD. However, individual data show that five of 20 participants had whole-body aBMD changes >LSC at R + 12; thus, these five individuals experienced a clinical effect that has not recovered at this time point. A > 5.54% change in local vBMD at the tibia and the radius would be considered clinically significant. Yet, neither average, cortical nor trabecular vBMD of the radius and tibia showed a change from baseline in this magnitude at HDBR60 or R + 30 for any volunteer. The reduction in BMD and deterioration of bone structure is likely to become clinically relevant as the duration of bed rest increases.

Wong et al. found that whole-body aBMD in post-menopausal women supplemented with 120 mg isoflavonoids/d for 2 years was significantly higher (0.64%) than in non-supplemented

controls (Wong et al., 2009). The authors stated that this difference only translates to a minimal clinical effect and that the supplementation neither slowed bone loss at key fracture sites nor affected concentrations of bone turnover markers (Wong et al., 2009). After 6 months of dried plum consumption, postmenopausal osteopenic women had a statistically significant increase in whole-body aBMD compared to values measured in controls (Hooshmand et al., 2016), but this may not be clinically relevant either because the increase was 1.31% versus the required 1.52% (clinical relevance: >2.77*0.55 = 1.52). The same applies to the results of a study by Ornstrup et al. (Ornstrup et al., 2014): The 2.6% increase in trabecular vBMD at the lumbar spine in obese men after 1000 mg/d resveratrol administration for 16 weeks (Ornstrup et al., 2014) is likely not clinically relevant (clinical relevance: >2.77*1.3% = 3.60%). It is important to state that longer intervention durations might increase the BMD further and that clinically relevant effects might be detectable at a later point in time (e.g., after 1–2 years), especially if the individual was consuming a diet with insufficient amounts of antioxidants before the intervention. However, further investigation would be needed to determine whether prolonged antioxidant supplementation in addition to a balanced diet rich in antioxidants can reduce a clinical effect on bone.

Apart from the previously reported limitations (Austermann et al., 2021), only men were included in this study, to exclude the effect of hormonal changes, especially oestrogen, during the menstrual cycle as an influencing factor. The goal was to study the effects in women, depending on the outcomes of this clinical trial in men.

Finally, the antioxidant supplementation in the present study did not affect BMD, BMC and bone structure parameters. This confirms our previous conclusion that supplementation of antioxidants in addition to an already well-balanced diet may not further improve BMD. However, bone loss-related diseases such as osteoporosis are a common health issue in ageing populations –for both women and men– and dietary strategies for primary and secondary prevention are needed. Circulating antioxidant levels are often low in older people, and their intake of antioxidants such as vitamins C, A and E is often reduced (Fletcher et al., 2003; Volkert et al., 2004). Therefore, antioxidant supplementation might be beneficial to reduce the risk of bone loss-related diseases in the elderly. Regarding bioavailability and bioactivity, future nutritional intervention studies should focus on holistic approaches including different amounts of vegetables and fruits high in certain antioxidants in the study diet rather than supplementation of single antioxidative compounds or antioxidant cocktails.

Current recommendations for astronauts emphasise that nutrients should be provided by foods rather than supplements whenever possible. The findings presented here similarly do not support a recommendation or antioxidant supplementation. We are about to embark on missions to send the first woman and the first person of colour to the moon in the coming years and are in early planning to send humans to Mars in the coming decades. The higher radiation profiles expected on these missions beyond low-Earth orbit will require further evaluation for the potential of antioxidants as countermeasures when food cannot meet the requirements for these environments. Food and nutrition will be the only countermeasure guaranteed on these exploration-class missions, and so we need to optimise their full potential to protect and maintain human health in these off-planet explorations.

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Authors' contributions

The author's responsibilities were as follows: MH & NB,SRZ and SMS designed the study; KA, NB and MH conducted the study; KA and MH analysed data; KA and RF performed statistical analysis; KA wrote the manuscript; and KA and MH have primary responsibility for the final content. All authors have read and revised drafts of the manuscript and approved the final manuscript.

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General Discussion

The Aox-cocktail study aimed to mitigate bone loss in HDBR by supplementing with an antioxidant cocktail, containing polyphenols, omega-3-fatty acids, vitamin E and selenium which was administered with the study meals. The data demonstrate that a supplementation of this antioxidant supplement to a well balanced diet did not affect whole body, lumbar spine and femur aBMD and BMC, trabecular and cortical vBMD, thickness at the radius and tibia, calcium homeostasis, and biochemical markers of bone formation and bone resorption in healthy male volunteers aged between 20 and 45 years during 60 days of bed rest [57,58].

This is partly in line with previous human intervention study results, but there are also studies that show opposite results [59]. The high variability in the different study outcomes might be attributed to a combination of the following aspects: i) methodological differences (study standardization, outcome parameters, study duration and antioxidant dosage); ii) differences in volunteers initial antioxidant status and antioxidant content in study or habitual diet apart from the antioxidant intervention; iii) intake control of bone affecting nutrients (e.g. calcium, sodium, phosphorus, vitamin D and vitamin K) iv) volunteer characteristics (sex, age, genetics, ethnicity and bone health status); and v) antioxidant bioavailability.

Based on the findings in the systematic literature search [59] highly controlled study conditions were applied in the Aox-cocktail study to ensure that any changes observed are indeed due to the antioxidant administration [57,58]. During the 60 days intervention period (HDBR) all volunteers were immobilized and spend 24 h per day in a 6° head-down tilt bed rest. The study diet was strictly controlled and individually tailored with no differences in nutrient supply between the intervention and control group (**Table 2-2**). The participants stayed in a metabolic ward (MEDES) for the whole study duration (88 days). This experimental area is a strictly controlled environment with temperature conditions within 20-25°C, humidity between 45-55%, light intensity between 0 and 500 Lux and acoustic isolation from outside (-60 dB). To monitor the bed rest standards [60] volunteers were video-monitored during their hospitalization and the 6° angle of the beds was controlled two times per day. To maintain a normal day-night cycle volunteers were woken up between 6:30 and 7 a.m. and lights were turned off at 11 p.m.

The high similarity of the control group data to findings of previous bed rest studies highlights the good standardization and methodological approach of the Aox-cocktail study. The observed bed rest related changes, an increase of bone resorption markers (**Figure 2-8**) and calcium excretion (**Figure 2-7**) and unchanged bone formation marker concentrations (**Figure 2-6**) during HDBR are comparable to previous bed rest studies of similar duration [61–63]. The increase of bone resorption during HDBR resulted in a decrease of average and cortical vBMD at the distal radius. During recovery period, when the bone resorption marker decreased and bone formation increased radial average and cortical vBMD increased again. These changes in vBMD are also reflected in the cortical thickness. Trabecular vBMD at the radius did not change during the whole study duration (**Table 3-4**). These changes only on the cortical level have also been observed in men during the 2nd Berlin BedRest Study [64]. Data from women, on the other hand, show changes on the trabecular level [65,66]. Thus, our data support the suggestion of a sex specific effect of bed rest. In men the reduction in BMD at the radius seems to be caused by a deterioration at the cortical level, whereas women experience reductions of the trabecular bone [61].

In the Aox-cocktail study a combination of increased cortical vBMD and unchanged trabecular vBMD at the distal tibia during HDBR led to an increase in tibial average vBMD (**Table 3-4**). This initial elevation of cortical vBMD at the tibia has been previously observed in the Berlin BedRest study and in the WISE study in both men and women [66,67]. In both studies cortical vBMD increased during HDBR before dropping during recovery period [66,67], which is in accordance with our data. A possible explanation for this is a time-delayed effect of the bed rest related physiological changes on bone structure. This delayed reduction is also reflected in femur and whole body aBMD (**Table 3-3**). Concurrently, bone resorption marker excretions and concentration (NTX, CTX and βCTX) are already increasing during HDBR (**Figure 2-8**). This indicates that the ellevated bone resorption is only later reflected in BMD and shows that bone biomarkers are affected more quickly than BMD. These differences in response time must be taken into account when comparing the effects of antioxidants in studies that used different bone-related outcome parameters. Human intervention studies conducted so far investigated the effects of antioxidants either only on bone turnover markers [59,68,69]; BMD only [70,71]; or BMD and bone turnover markers [34,72–74] and/or on bone structure parameters [75,76]. A comparison of the different outcome measurements is difficult as they can reflect changes in bone metabolism and their health-related consequences differently and independently from one another. Bone structure

parameters (trabecular architecture) for instance can reveal reductions in bone strength, which are not reflected in BMD [55]. The fact that bone structure changes are not automatically represented in BMD can be observed in the Aox-cocktail study as well. The increase in trabecular thickness at the radius did not result in any changes on radial vBMD (**Table 3-4**). In order to achieve the most comprehensive insight, and to account for their different advantages and limitations a combined examination of BMD, bone biomarkers and bone structure data, like in the Aox-cocktail study, should be used in future studies.

For the assessment of bone markers the collection time point of blood and urine samples is crucial, as the markers undergo a circadian rhythm, with the highest concentrations early in the morning (2 a.m. - 8 a.m.) and lowest in the afternoon and evening (1 p.m. - 11 p.m.) [77-79]. For bAP the diurnal rhythm is opposite with a peak in the afternoon and nadir in the morning [77]. Differences between the highest and lowest concentrations can vary between 20-60% [77]. This shows how important the collection time point is and that standardization is essential for consecutive measurements as used in intervention studies. Bone resorption markers can be analyzed either in serum, spot urine or 24h urine samples [77,80,81]. The same method has to be applied during the entire study duration, as analysis of bone markers from different sample materials can lead to different results. Both the Aox-cocktail study and previous human intervention studies [68,74,76,82–84] showed a good standardization regarding the collection time points. Fasting blood samples, 24h urine or early morning spot urine were taken at the same time on consecutive measurement. Therefore, it can be ruled out that the circadian rhythm of bone biomarkers contributed to variations in study outcomes. Particularly urine bone biomarkers are prone to intra-individual variations (day to day variation), with a 12-35% variation for urinary CTX and NTX excretion rates [77]. However, this effect can be minimized by averaging the results of two consecutive days, as done in the Aox-cocktail study (**Figure 2-3**). Supplementation of 150 mg resveratrol for 16 weeks in obese men did not affect early morning spot urine marker CTX and calcium excretion [76] which is consistent with our findings in 24h urine (**Figure 2-8**). A 54 mg genistein supplementation in postmenopausal osteopenic women for two years led to a reduction of urinary excretion of resorption markers pyridinolin and deoxypyridinolin, measured in early morning spot urine samples [83]. In this study urine samples were taken on single time points and differences in bone marker concentrations might be due to day-to-day variations. Apart from the circadian rhythm, bone marker concentrations are affected by the sex of the participants and concentrations differ throughout the life of men and women [78,80]. Young

adult men (3rd decade) have higher bone turnover marker concentrations than women of the same age. Later in life levels decrease and have their lowest concentrations during the fifth and sixth life decade in men, whereas women show the lowest concentrations 10 years earlier [78,85]. Until the age of about 60 years the bone marker concentrations remain stable in men and after the sixth decade bone resorption markers increase slightly, but bone formation does not change or only marginally increase [78,80,85]. Women, however show the strongest increase in bone marker concentrations during the early postmenopausal phase [86]. Higher bone resorption marker concentrations are caused by an increase of bone remodeling units throughout the skeleton. After menopause the bone formation is impaired and the resorbed bone is not completely replaced in the single bone remodeling units. Higher formation marker rates are caused by an increase in overall bone remodeling units and therefore do not reflect the impaired bone formation at single remodeling sites [78]. This again shows how important it is to combine different methods for bone health assessment to obtain a comprehensive picture and to account for biological and analytical variations. The sex and age specific differences complicate the comparison between women and men and have to be considered in bone marker data interpretation between sexes as well as in the design of future studies. In the Aox cocktail study only young, male participants were included, whereas most intervention studies conducted until now investigated the effects in osteopenic or osteoporotic women aged between 50 and 80 years [59]. This complicates the comaprison of study results and might attribute to different study outcomes.

Another important factor that may account for observed differences is study methodology including variations in study duration and used antioxidant doses. Findings from the literature search show that the study duration varied between 8 weeks and two years (**Table 1-1**). With a duration of 60 days the Aox-cocktail study is at the beginning of this range. However, it is difficult to assess the effective study duration as both, shorter as well as longer intervention periods found effects [76,87], or did not observe any effects of the antioxidant supplementation [70,75,88]. The same applies for the antioxidant dosage used in the intervention studies. Doses vary highly, ranging from several mg [74,89] to 1 g per day [76] for polyphenols and 320-4200 mg EPA/DHA daily [87] and the data do not suggest a dose-dependent effect. For the effects of α-tocopherol on bone turnover Chin et al. suggest a U-shape dose dependency, based on animal studies, with protective effects at lower doses and detrimental effects at higher doses [90]. Negative effects on bone turnover were observed with amounts of 500 - 600 IU/kg diet in rodents [90]. A cross-sectional study found a low

serum α-tocopherol to γ-tocopherol ratio and high serum γ-tocopherol levels to be associated with an increase in serum bAP concentrations [91]. They also indicate that α-tocopherol suppresses γ-tocopherol, therefore might reduce the bone formation favoring effects of γ-tocopherol [91]. Daily supplementation of 400 IU (296 mg) α-tocopherol for eight weeks resulted in a reduction of γ–tocopherol concentrations in men and women [92]. Nutritional vitamin E supplementation in the Aox-cocktail study was with 168 mg below this amount and even with the vitamin E content in the study diet (19.5±1.0 mg/d in HDBR) vitamin E intake did not reach to this level, which suggests no detrimental effects of the vitamin E supply on bone metabolism in the Aox-cocktail study.

The antioxidant supply in the study or habitual diet seems to have an important impact on the study outcomes. The results from the Aox-cocktail study suggest that if the antioxidant supply with the habitual diet is already sufficient a further enrichment of the diet seems not to have any further beneficial effects. In the Aox-cocktail study nutrient and antioxidant supply with the study diet was sufficient according to dietary reference intakes [57]. Findings from other studies indicate a threshold for antioxidant plasma levels and once reached no further increase occurs [93]. It is possible that this level is already reached with the study diet and therefore the further supplementation did not result in any additional effect. A comparison of our outcomes with other human intervention studies shows that most studies investigating the effects of antioxidants on bone metabolism do not provide any information regarding the nutrient and antioxidant supply with the habitual or study diet [72,74–76,88,94]. Marini et al. [83] investigated the effects of genistein on bone metabolism in osteopenic postmenopausal women [83]. The supplementation of 54 mg/d genistein for two years improved BMD and bone turnover markers [83]. During this study participants adhered to a low-isoflavonoid diet and the intake of soy and legumes was prohibited [83]. Hence, the antioxidant supply with the habitual diet might have been insufficient. Therefore, the supplementation of genistein might have only led to an effect, because the initial antioxidant supply may not have been adequat. This supports the idea that the antioxidant effect seems to depend on the habitual and the initial antioxidant supply. However, in order to confirm this hypothesis, studies specifically designed to address this question must be conducted.

Apart from the antioxidant content of the habitual diet, the presence of other nutrients with an impact on bone metabolism in the habitual or study diet is an aspect to consider. Vitamin D plays an important role in the calcium homeostasis and has an important impact on bone

metabolism with its endocrine and immune-modulating effects [95,96]. In the Aox-cocktail study only volunteers with a sufficient vitamin D status were included and 25-OH-D serum levels were monitored throughout the course of the study. Its serum concentrations did not significantly change during the study and did not differ between the study groups (**Table 2-3**) [57]. Thus, vitamin D did not influence the study outcomes. The same applies for the intake of sodium, calcium, phosphorus, vitamin K and the antioxidative vitamins vitamin E, vitamin A and vitamin C [57]. Nutrient intakes did not differ between Aox and Con (**Table 2-2**). When comparing study outcomes, it is important to look at the habitual diet as well as the intervention products. As mentioned above most of the studies conducted so far do not provide any information about the composition of the habitual diet volunteers adhered to before and during the study [68,69,74,76,94,97–102]. Regarding bone affecting nutrients almost all studies, conducted so far, fail to provide data on the intake of nutrients beyond calcium and vitamin D such as sodium, phosphorus, or vitamin K and antioxidative nutrients, such as vitamin C, vitamin A and vitamin E. Thus, their influence on the study outcomes cannot be ruled out. In most studies calcium and vitamin D were supplemented in intervention and control groups to ensure an adequate supply [34,68,74,83,94,99–101]. Other studies did either evaluate the amounts of calcium and vitamin D consumed with the habitual or study diet and did not observe any differences between the study groups [103] or did not provide information regarding vitamin D or calcium intake [69,76,98,102]. In these last-mentioned studies an effect of calcium and vitamin D on the study outcomes cannot be ruled out. Generally, the lack of detailed information about the habitual diet and therefore the intake of bone affecting and antioxidative nutrients complicate the comparison of study outcomes and should be considered in the design of future investigations.

A further important determinant seems to be volunteer characteristics like sex, age, genetics, ethnicity and bone health status. Antioxidant bioavailability is affected by these individual related factors [104]. Data indicates a higher polyphenol modification efficacy (formation of effective metabolites) in women compared to men [104–106] and in Asian vs. Western people [104,107]. Apart from that, the health condition also seems to play an important role [104] and studies conducted so far [57–59] might indicate a therapeutic rather than a preventive effect of antioxidant administration.

The bioavailability of polyphenol subgroups is different as well and can vary from 0.3% for anthocyanins and about 43% for isoflavonoids [108]. Thus, they likely have a different

potency and different doses are needed, which makes a comparison more difficult. Besides the chemical structure, food matrix and meal composition determine antioxidant bioavailability [104,109]. Whereas the presence of fat, protein or lecithin has a positive impact on polyphenol absorption, fibers and chelating agents reduce absorption [93]. Apart from that, the nature of the food matrix solid vs. liquid food also has an effect, with an improved bioavailability from liquid foods [93]. Food processing is another important determinant of polyphenol bioavailability. Cooking of carotinoid-rich vegetables for instance increases their bioavailability by changing the food matrix [93]. When comparing the outcomes of the Aox-cocktail study [57,58] with the studies included in the literature review (**Table 1-1**) it seems that antioxidants in form of supplements might not be as efficient as an increased intake of antioxidant rich foods. This observation may be supported by Léotoing et al. [110]. They demonstrated that the beneficial action of dried plums on bone health in a rat model of postmenopausal osteoporosis was not dependent on its phenolic content [110]. The authors suggest that the unique combination of nutrients and micronutrients of dried plums is responsible for the positive effects on bone health [110]. This supports the idea that the difference in the matrix of the antioxidant administration plays an important role with regard of the effect and that whole foods might be more efficient due to their unique combination of nutrients and the food matrix.

Finally, an important aspect in the interpretation of study results is the clinical relevance of the effects observed. Statistically significant does not automatically mean that the intervention used also has a clinical impact. Bone markers are affected by many biological factors. To ensure that the observed changes are not due to biological variations, but actually reflect changes in bone turnover rates, the concept of least significant change (LSC) was introduced [53]. According to this concept the change has to be greater than the imprecision of the measurement [53]. For bone resorption markers the biological variation is higher than for bone formation markers and a LSC of 60 – 80% for resorption and >25% for bone formation markers should be applied [53]. The LSC for BMD measurements, conducted on the same system, is considered 2.77*CV% [111]. When the observed changes are above these thresholds the results can be considered to have a clinical effect. In the Aox-cocktail study a comparison of pre-bed rest data with the end of HDBR only show a change >60% for the bone resorption marker CTX. Changes of NTX and βCTX were below this threshold. For the bone formation makers bAP and P1NP the percentage change between baseline and HDBR day 60 was below 25%, the change between baseline and FU however showed a percentage

change above 25% for both markers, whereas the change for bAP was lower than for P1NP. This is also reflected in the reference values. The bAP concentration at FU, with 25.4 ± 4.9 U/L for Aox and 23.0 ± 6.5 U/L for the Con is still within the assay manufacturer's reference range of 15.0 – 41.3 U/L. The serum concentration of P1NP however was higher than the reference range of 22 – 87 µg/L (102.2 ± 37.0 µg/L for Aox and 97.6 ± 21.8 µg/L for Con). When considering BMD values above 2.77*CV% as clinically relevant, changes in femur and whole body aBMD as well as local vBMD observed in the Aox-cocktail study are not clinically relevant [58]. However, it is important to mention that the study duration was only 60 days and that the reduction in BMD and deterioration of bone structure is likely to become clinically relevant with an increase in bed rest duration. When applying the concept of LSC to the data from the literature search it seems that even though statistically significant the effects on bone markers and BMD might not be clinically significant as their magnitude is below the mentioned thresholds. Shen et al. found a statistically significant increase in bAP after one month of 500 mg green tea polyphenol supplementation [68]. This increase was only attributed to 5% compared to baseline. Although even a statistical significance was shown it is likely not clinically relevant and the result of a biological variation. A daily consumption of both 50 g and 100 g dried plums led to a significant reduction in TRAcP 5b serum concentrations and an increase of whole body aBMD [74]. The percentage change of -18% for the 50 g dried plum group and -20% for the 100 g dried plum group in TRAcP 5b after six months though might not be clinically significant as the change is below the above mentioned threshold of 60 % [53]. The same applies for aBMD data from this study (observed change of 1.31% vs. LSC=1.52%) [74], as well as the study from Wong et al. [72] and Ornstrup et al. [76]. However, the studies from Hooshmand et al. [74], Shen et al. [68] and Ornstrup et al. [76] only lasted between four and six months. It is possible that clinically relevant effects might occur after longer intervention periods. Moreover, it is possible that the effect, although not clinically relevant at the end of the studies, accumulates with continued antioxidant intake over the course of a lifetime, resulting in a bone preserving effect that cannot be demonstrated in the relatively short period of study.

Conclusion and Prospect

Based on the findings of the systematic literature review a highly standardized human intervention study was designed to examine the synergistic effects of antioxidants on different markers of bone metabolism. The antioxidant mixture applied as a countermeasure in the Aox-cocktail study did not reduce the negative effects of HDBR on bone turnover. We could not show any effect of the tested antioxidant cocktail on BMC, BMD, trabecular and cortical thickness in the radius and tibia, calcium homeostasis, urinary calcium excretion, bone formation and bone resorption markers, although most bone impacting factors were standardized and thereby excluded. The similarity of the bed rest induced effects on bone markers with previous bed rest studies underlines the good methodological approach of this trial and the highly-controlled study conditions are an outstanding characteristic and allowed a comprehensive analysis of the nutritional intake. Based on our results, no recommendations regarding dosage and form of antioxidants used as a contribution to the maintenance of BMD can be drawn.

As stated above the food matrix seems to play an important role for the bioavailability of antioxidants and seems to be higher if antioxidants are consumed in the form they naturally occur (fruits, vegetables, tea and coffee) [104,109]. Therefore, future studies should focus on the investigation of antioxidant-rich foods instead of using polyphenol extracts or isolated polyphenols in form of supplements as intervention products. That way participants benefit not only from the individual substances but also from the combination and interaction of bone-active nutrients in the foods provided. Choosing the dosage and form of application are crucial. That too high doses of isolated antioxidants can even have harmful effects was shown in a meta-analysis of 68 randomized intervention trials [112]. This also supports the idea of using antioxidant rich foods instead of supplements as harmful effects of supra-nutritional doses can be avoided.

So far, no evidence exists that the choice of isolated antioxidants over an antioxidant-rich diet decreases the risk of osteoporosis or other bone loss related diseases. The recommendation should still be an improvement of the habitual diet towards a sufficient supply with all required nutrients rather than using nutritional supplements to meet the nutritional needs. With regard to the prevention or nutritional therapy of certain diseases, this can also mean that the intake of certain food groups that show positive effects is increased.

To investigate whether antioxidants have preventive and/or therapeutic effects future studies might consider the examination of participants with bone related diseases in comparison to healthy volunteers. So far the evidence indicates that omega-3 fatty acids and polyphenols rather have a therapeutic than a preventive effect [57–59]. However, this observation requires further investigation with studies carefully designed to evaluate this hypothesis. In the study designing process it is crucial to take the other confounding factors like, age, sex, habitual diet etc. into account to minimize study bias.

The literature research and our study also revealed that the outcomes might be determined by the characteristics of the study population (e.g., age, sex ethnicity). Thus, it would be an interesting approach to compare different study populations to investigate the magnitude of factors like age, sex and ethnicity, with the goal to develop recommendations for different populations (e.g., women vs. men, different age groups). However, in all these studies a good standardization is crucial.

Finally, investigators should consider the possible effect of the habitual diet of volunteers on study outcomes and include this in the hypothesis and design of their studies. Antioxidant supplementation, in addition to a well-balanced diet, might not have any further beneficial effects. However, this hypothesis needs further investigation.

Summary

Age and disease related inactivity in older people and inactivity during space flight and in ground-based analogs, such as 6° head-down tilt bed rest (HDBR) are associated with bone loss. This bone loss is mainly induced by decreased mechanical loading. Additionally, oxidative stress resulting from excessive formation of reactive oxygen species (ROS) or dysfunction of the antioxidant defense systems leads to increased bone resorption processes. Antioxidants like polyphenols, omega-3-fatty acids, vitamins and micronutrients may mitigate the damaging effects of ROS on bone turnover and mediate the scavenging of free radicals.

To get an overview of the current state of research a literature search was conducted, with focus on the effects of nutritive polyphenols on bone metabolism. Based on these results a randomized, controlled, intervention study, in a parallel design was conducted at the Institute for Space Medicine and Physiology, Toulouse, France with 20 healthy male volunteers (age 34 ± 8 y, weight 74 ± 6 kg). We hypothesized that antioxidant supplementation during 60 days of HDBR would positively affect bone markers, bone mineral content (BMC) bone mineral density (BMD) and bone structure parameters compared to non-supplemented controls. The study was divided into two campaigns and each campaign consisted of a 14-d adaptation (BDC), a 60-d HDBR and a 14-d recovery (R) phase. Ten volunteers participated in each campaign. In both campaigns, five volunteers were randomly allocated to the intervention group and five volunteers to the control group. In the intervention group volunteers received an antioxidant cocktail, consisting of 741 mg polyphenols, 2.1 g omega-3-fatty acids, 168 mg vitamin E and 80 µg selenium. In the control group volunteers received no supplement. All volunteers received an individually tailored and strictly controlled diet. BMC, lumbar spine, femur and whole body BMD, BMD of the cortical and trabecular compartments of the distal radius and tibia and cortical and trabecular thickness, as well as serum calcium, parathyroid hormone, osteocalcin, and bone formation markers aminoterminal propeptide of type I collagen (P1NP) and bone alkaline phosphatase (bAP) were measured at different time points during BDC, HDBR and R, along with urinary calcium and bone resorption markers C-telopeptide of type I collagen (CTX) and N-telopeptide of type I collagen (NTX).

The antioxidant supplement did not affect BMC, lumbar spine, femr and whole-body BMD, cortical and trabecular BMD or thickness in the radius or tibia, calcium homeostasis (serum calcium and parathyroid hormone); bone resorption markers (urinary CTX, urinary NTX, and

serum β-CTX); bone formation markers (bAP, P1NP and osteocalcin); or urinary calcium excretion. In both groups, typical bed rest related changes were observed (increase of bone resorption markers, unchanged bone formation makers, decrease of BMC, BMD and changes in bone structure).

Supplementation of an antioxidant cocktail to a diet matching the DRIs did not affect bone turnover during 60-d HDBR in young, healthy, male subjects.

Zusammenfassung

Alters- und krankheitsbedingte Inaktivität bei älteren Menschen sowie Immobilität während des Aufenthalts in Schwerelosigkeit und in bodengebundenen Analogstudien, wie z. B. der 6°-Kopftielflage (HDBR), sind mit Knochenverlust verbunden. Dieser Knochenverlust wird vor allem durch verminderte mechanische Belastung hervorgerufen. Zusätzlich führt oxidativer Stress, der aus einer übermäßigen Freisetzung reaktiver Sauerstoffspezies (ROS) oder einer Fehlfunktion des antioxidativen Abwehrsystems resultiert, zu erhöhten Knochenresorptionsprozessen. Antioxidantien wie Polyphenole, Omega-3-Fettsäuren, Vitamine und Mikronährstoffe scheinen die schädigenden Auswirkungen von ROS auf den Knochenumsatz mildern zu können.

Um einen Überblick über den aktuellen Stand der Forschung zu erhalten, wurde eine Literaturrecherche durchgeführt, wobei der Fokus auf den Auswirkungen von nutritiven Polyphenolen auf den Knochenstoffwechsel lag. Basierend auf diesen Ergebnissen wurde eine randomisierte, kontrollierte Interventionsstudie im Parallel-Design im Institut für Raumfahrtmedizin und Physiologie, Toulouse, Frankreich, mit 20 gesunden männlichen Probanden (Alter 34 ± 8 Jahre, Gewicht 74 ± 6 kg) durchgeführt. Die Hypothese war, dass eine Antioxidantien-Supplementierung während einer 60-tägigen HDBR Knochenmarker, Knochenmineralgehalt (BMC), Knochendichte (BMD) und Knochenstrukturparameter positiv beeinflusst. Die Studie war in zwei Kampagnen unterteilt, und jede Kampagne bestand aus einer 14-tägigen Anpassungs- (BDC), einer 60-tägigen 6° Kopftieflage-Phase (HDBR) und einer 14-tägigen Erholungsphase (R). An jeder Kampagne nahmen zehn Probanden teil. In beiden Kampagnen wurden fünf Probanden nach dem Zufallsprinzip in die Interventionsgruppe und fünf Probanden in die Kontrollgruppe eingeteilt. In der Interventionsgruppe erhielten die Probanden einen Antioxidantien-Cocktail, bestehend aus 741 mg Polyphenolen, 2,1 g Omega-3-Fettsäuren, 168 mg Vitamin E und 80 µg Selen. In der Kontrollgruppe erhielten die Probanden kein Supplement. Alle Probanden erhielten eine individuell abgestimmte und streng kontrollierte Diät. Ledenwirbelsäule, Femur und Ganzkörper BMD und BMC, BMD der kortikalen und trabekulären Kompartimente des distalen Radius und der Tibia und die kortikale und trabekuläre Dicke, sowie Serum-Calcium, Parathormon, Osteocalcin, und die Knochenformationsmarker aminoterminales Propeptid von Typ-I-Kollagen (P1NP) und alkalische Knochenphosphatase (bAP) wurden zusammen mit der Calciumausscheidung und den Knochenresorptionsmarkern C-Telopeptid des Typ-I-

Kollagens (CTX) und N-Telopeptid des Typ-I-Kollagens (NTX) im 24 h Urin zu verschiedenen Zeitpunkten während BDC, HDBR und R gemessen.

Die Antioxidantiengabe hatte keinen Einfluss auf die Ledenwirbelsäule, Femur und Ganzkörper Knochendichte und den Knochenmineralgehalt, die kortikale und trabekuläre Knochendichte und Dicke im Radius und der Tibia, Calciumhomöostase (Serum Calcium- und Parathormonkonzentrationen), Exkretion der Knochenresorptionsmarker (CTX im Urin, NTX im Urin und Serum β-CTX), Serumkonzentrationen der Knochenaufbaumarker (bAP, P1NP und Osteocalcin) oder die Calciumausscheidung im Urin. In beiden Gruppen wurden typische bettruhebedingte Veränderungen beobachtet (Anstieg der Knochenresorptions-marker, unveränderte Konzentrationen der Knochenformationsmarker, Reduktion der BMC, BMD und Veränderungen der Knochenstruktur).

Die Supplementierung eines Antioxidantien-Cocktails zu einer Diät, die den Referenzwerten entsprach, hatte keinen Einfluss auf den Knochenumsatz gesunder, junger Männer während einer 60-tägigen 6° Kopftieflage.

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