nutrients

Special Issue Reprint

Dietary Factors in Bone Health

Edited by
Qian Zhang

mdpi.com/journal/nutrients

MDPI

Dietary Factors in Bone Health

Dietary Factors in Bone Health

Editor

Qian Zhang

MDPI

Basel • Beijing • Wuhan • Barcelona • Belgrade • Novi Sad • Cluj • Manchester

Editor
Qian Zhang
Department of Nutrition and
Health
China Agricultural University
Beijing
China

Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Nutrients* (ISSN 2072-6643) (available at: https://www.mdpi.com/journal/nutrients/special_issues/Bone_elder).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-2547-9 (Hbk)
ISBN 978-3-7258-2548-6 (PDF)
doi.org/10.3390/books978-3-7258-2548-6

Contents

About the Editor

Qian Zhang

Qian Zhang is a professor in the Department of Nutrition and Health at China Agricultural University. Dr. Zhang received her Ph.D. at the Institute for Nutritional Science, Chinese Academy of Science in Shanghai, China, and completed postdoctoral training at Johns Hopkins University. Her research is focused on the regulation of osteoporosis by nutritional factors and the development of bone marrow stromal cells. She has authored over 30 original publications, and has served as a reviewer for Bone Research, Nutrients, The Journal of Translational Medicine, and other journals. She is an editorial board member of Bone research and National Science Open.

nutrients

MDPI

Editorial

New Insights into Nutrients for Bone Health and Disease

Qian Zhang [1,2]

[1] Department of Nutrition and Health, China Agricultural University, Beijing 100193, China; qianzhang@cau.edu.cn
[2] Food Laboratory of Zhongyuan, Luohe 462300, China

Bone health includes the health of bone minerals, mass, geometry, and microstructure. Keeping bone healthy protects from osteoporosis, fragile bones, and bone fractures with aging. In addition to age, other factors that affect bone health are sex, ethnicity, family history, hormone, physical activity, muscle strength, and nutrition [1]. Although bone loss happens more in middle-aged and older women, it can affect young people too. Nutritional disorders and defective dietary components can influence bone health in multiple ways. For example, micronutrients and macronutrients can benefit and hurt bone health depending on exposure, and dietary alcohol is a risk factor for osteoporosis. Although there has been progress in nutritional targets in bone health, many conclusions remain controversial or undiscovered. This Special Issue is focused on uncovering the underlying molecular mechanisms of all kinds of nutritional factors to bone health in patients or animal models. The published paper studied various interesting aspects of nutrition, such as calcium (Ca), vitamin D, vitamin K, and polyphenols, and explored their effect in samples with diversity.

Calcium is the most studied and most crucial nutrition factor in bone homeostasis. According to Mayoclinic [2], the recommended calcium intake for adult men under 70 and adult women under 50 is 1000 mg per day and 1200 mg for older persons. Bone is the biggest organ in the human body containing about 60–70% inorganic mineral, which is mainly composed of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, and 30–40% organic constituents, which is primarily composed of type I collagen [3]. Therefore, calcium is the most abundant mineral element in bone. Hormones such as parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), calcitonin, sclerostin, and osteocalcin help to maintain the dynamic balance of calcium between bone and serum. Some studies do not support the positive effect of adequate on bone health in aged people, perhaps because of the decreased osteoblast number, weak absorption, or other reasons [4]. Much work needs to be conducted to clarify the calcium intake range with the people's age, gender, and lifestyle.

It is well known that vitamin D deficiency is a risk factor for osteoporosis. Vitamin D canbe synthesized by the skin after sunshine exposure, and also absorbed via the intestine. Vitamin D is metabolized in the liver to 25[OH]D and then transported to the kidney to develop into its active form, $1,25(OH)_2D_3$ [calcitriol], which helps with calcium absorption by enhancing the intestinal transcellular calcium transport process [5]. $1,25(OH)_2D_3$ induces the biosynthesis of the epithelial calcium channel, transient receptor potential vanilloid type 6, and the calcium-binding protein calbindin-D_{9k} [6]. Although it is well recognized that vitamin D helps build bones, the amounts still need investigation. In 2019, a clinical trial published in JAMA found that compared with moderate vitamin D intake, 10 or more times higher vitamin D intake did not result in more bone mineral density or bone strength in healthy adults. The toxicity or harm of the higher dose of vitamin D needs further research [7]. Vitamin K is a cofactor for the gamma-carboxylation of osteocalcin, which attract calcium to promote mineralization and bone formation [8].

Some studies have shown that a particular fruit and vegetable intake volume lowers the risk of bone fracture [9], which might be partially because of their polyphenols. Due to the antioxidative effect, polyphenols protect the oxidative damage caused by the

Citation: Zhang, Q. New Insights into Nutrients for Bone Health and Disease. *Nutrients* **2023**, *15*, 2648. https://doi.org/10.3390/nu15122648

Received: 8 May 2023
Accepted: 23 May 2023
Published: 6 June 2023

accumulated reactive oxygen species (ROS) in bone marrow, thereby decreasing the risk of osteoporosis. In addition, polyphenols also downregulate inflammatory cytokine, including the osteoclast differentiation factors like RANKL and TNFa, resulting in reduced osteoclast activities and less bone absorption [10]. Polyphenols were divided into four groups: flavonoids, phenolic acids, polyphenolic amides, and others. Each group contains numerous polyphenols and many new polyphenols associated with bone health need to be discovered.

In this Special Issue, research articles highlight novel findings to contribute insight into the dietary nutrients affecting bone health at the molecular and biomedical levels, advancing knowledge in scientific studies and clinical practice.

Funding: This work was supported by the National Natural Science Foundation of China Grant 82272566, Beijing Nova Program 20220484144, and the 111 Project from the Education Ministry of China B18053.

Conflicts of Interest: The author declares no conflict of interest.

References

1. *Osteoporosis: Assessing the Risk of Fragility Fracture*; National Institute for Health and Care Excellence (NICE): London, UK, 2017.
2. Calcium and Calcium Supplements: Achieving the Right Balance. Available online: https://www.mayoclinic.org/healthy-lifestyle/nutrition-and-healthy-eating/in-depth/calcium-supplements/art-20047097 (accessed on 29 May 2023).
3. Vannucci, L.; Fossi, C.; Quattrini, S.; Guasti, L.; Pampaloni, B.; Gronchi, G.; Giusti, F.; Romagnoli, C.; Cianferotti, L.; Marcucci, G.; et al. Calcium Intake in Bone Health: A Focus on Calcium-Rich Mineral Waters. *Nutrients* **2018**, *10*, 1930. [CrossRef] [PubMed]
4. Tai, V.; Leung, W.; Grey, A.; Reid, I.R.; Bolland, M.J. Calcium Intake and Bone Mineral Density: Systematic Review and Meta-Analysis. *BMJ* **2015**, *351*, h4183. [CrossRef] [PubMed]
5. Qiao, W.; Yu, S.; Sun, H.; Chen, L.; Wang, R.; Wu, X.; Goltzman, D.; Miao, D. 1,25-Dihydroxyvitamin D Insufficiency Accelerates Age-Related Bone Loss by Increasing Oxidative Stress and Cell Senescence. *Am. J. Transl. Res.* **2020**, *12*, 507–518. [PubMed]
6. Veldurthy, V.; Wei, R.; Oz, L.; Dhawan, P.; Jeon, Y.H.; Christakos, S. Vitamin D, Calcium Homeostasis and Aging. *Bone Res.* **2016**, *4*, 16041. [CrossRef] [PubMed]
7. Burt, L.A.; Billington, E.O.; Rose, M.S.; Raymond, D.A.; Hanley, D.A.; Boyd, S.K. Effect of High-Dose Vitamin D Supplementation on Volumetric Bone Density and Bone Strength: A Randomized Clinical Trial. *JAMA* **2019**, *322*, 736–745. [CrossRef] [PubMed]
8. van Summeren, M.; Braam, L.; Noirt, F.; Kuis, W.; Vermeer, C. Pronounced Elevation of Undercarboxylated Osteocalcin in Healthy Children. *Pediatr. Res.* **2007**, *61*, 366–370. [CrossRef] [PubMed]
9. Benetou, V.; Orfanos, P.; Feskanich, D.; Michaelsson, K.; Pettersson-Kymmer, U.; Eriksson, S.; Grodstein, F.; Wolk, A.; Bellavia, A.; Ahmed, L.A.; et al. Fruit and Vegetable Intake and Hip Fracture Incidence in Older Men and Women: The Chances Project. *J. Bone Miner. Res.* **2016**, *31*, 1743–1752. [CrossRef] [PubMed]
10. Nicolin, V.; De Tommasi, N.; Nori, S.L.; Costantinides, F.; Berton, F.; Di Lenarda, R. Modulatory Effects of Plant Polyphenols on Bone Remodeling: A Prospective View from the Bench to Bedside. *Front. Endocrinol.* **2019**, *10*, 494. [CrossRef] [PubMed]

nutrients

MDPI

Article

Quantitative Diet, Body Composition and Sprint Performance in Female Professional Beach Handball Players

Alejandro Martínez-Rodríguez [1,2,*], Javier Sánchez-Sánchez [3], María Martínez-Olcina [1], Manuel Vicente-Martínez [4], Marcelo Peñaranda-Moraga [1], Nuria Asencio-Mas [1], Lucía Gonzálvez-Alvarado [1], Piotr Matlosz [5], Rodrigo Yáñez-Sepúlveda [6], Guillermo Cortés-Roco [7] and Juan Antonio Sánchez-Sáez [8,*]

[1] Department of Analytical Chemistry, Nutrition and Food Science, University of Alicante, 03690 Alicante, Spain
[2] Alicante Institute for Health and Biomedical Research (ISABIAL), 03010 Alicante, Spain
[3] School of Sport and Science, European University of Madrid, 28670 Madrid, Spain
[4] Faculty of Health Science, Miguel de Cervantes European University, 47012 Valladolid, Spain
[5] Institute of Physical Culture Sciences, Medical College of Rzeszów University, University of Rzeszow, 35-310 Rzeszów, Poland
[6] Faculty of Education and Social Sciences, Universidad Andres Bello, Viña del Mar 2520000, Chile
[7] Escuela de Educación, Pedagogía en Educación Física, Entrenador Deportivo, Universidad Viña del Mar, Viña del Mar 2520000, Chile
[8] Grupo de Investigación GDOT-Gestión Deportiva, Ocio y Tecnología, Faculty of Sport, Catholic University of Murcia, 30107 Murcia, Spain
* Correspondence: amartinezrodriguez@ua.es (A.M.-R.); jasanchez419@ucam.edu (J.A.S.-S.)

Abstract: Women's elite sports have experienced an exponential increase in the last decade, as has beach handball (BH). The high demands of this sport mean that athletes need to be in superior physical condition, so nutrition and body composition are determining factors in their sporting performance. For this reason, the aim of this study was to analyze, compare and correlate the most relevant variables of food intake (quantitative), body composition (focus on the bone mass characteristics) and sprint performance in female professional BH players. Thirty-three women from the National Spanish Team participated in this study. Dietary assessment, anthropometric measurements and sprint tests were performed. In general, the players had a low carbohydrate intake and adequate protein intake, with no significant differences depending on the category and playing position. For senior players, positive correlations were found between protein intake and bone mass ($r = 0.584$, $p = 0.022$), polyunsaturated fatty acid intake and muscle mass ($r = 0.387$, $p = 0.026$) and finally between fat mass and animal protein intake ($r = 0.569$, $p = 0.027$). Body composition was similar in both categories; however, goalkeepers had the highest fat ($22.6 \pm 3.86\%$, 16.2 ± 4.84 kg) component (vs. wings: $17.4 \pm 3.53\%$, $p = 0.031$/vs. specialists: 11.1 ± 1.91 kg, $p = 0.034$), and senior players had higher muscle mass (kilograms). It is worth noting the finding that players with a greater trochanter height had significantly lower sprint times ($p = 0.014$ and $p = 0.048$ for 5 and 10 m, respectively). Certain bone characteristics, such as iliospinale height, biacromial and bimalleolar diameters, mesosternal perimeter and biceps skinfold, differ depending on the position. In addition, the greater speed of the senior players may be due to the greater specialization, number of training sessions performed and specific bone characteristics, such as trochanter height. In this regard, the data provided in this study will assist with establishing criteria for the selection of talent for this sporting discipline.

Keywords: body composition; team sports; bone; somatotype

Citation: Martínez-Rodríguez, A.; Sánchez-Sánchez, J.; Martínez-Olcina, M.; Vicente-Martínez, M.; Peñaranda-Moraga, M.; Asencio-Mas, N.; Gonzálvez-Alvarado, L.; Matlosz, P.; Yáñez-Sepúlveda, R.; Cortés-Roco, G.; et al. Quantitative Diet, Body Composition and Sprint Performance in Female Professional Beach Handball Players. *Nutrients* **2023**, *15*, 138. https://doi.org/10.3390/nu15010138

Academic Editor: Qian Zhang

Received: 29 November 2022
Revised: 19 December 2022
Accepted: 23 December 2022
Published: 28 December 2022

1. Introduction

Beach handball is characterized by motor characteristics such as accelerations, sprints or jumps, as well as rapid changes of direction and a high number of physical collisions [1,2].

The nutritional requirements of beach handball players, as seen in other team sports [3], will be variable throughout the season, affecting the intake of macronutrients, micronutrients and total energy. Currently, carbohydrate recommendations are between 5 and 12 g/kg body weight for moderate- to high-intensity exercise (duration of 1–4 h) [4], decreasing to 3–5 g/kg body weight for low-intensity exercise of up to one hour in duration [4]. Regarding proteins, with the objective of helping with muscle protein synthesis and recovery, recommendations of 1.2–2 g/kg body weight are established. As for micronutrients, female athletes have higher requirements, highlighting the importance of B vitamins [5], iron, calcium and vitamin D in athletes with a low caloric intake. The estimation of energy requirements in female beach handball players is a considerable challenge, as requirements may increase or decrease depending on age, general level of daily activity, training conditions and body composition [6].

In this sense, in the field of sport, the assessment of body composition is fundamental because it is one of the factors that can determine athletic potential and the probability of success in a particular sport, in combination with technical/tactical, physical, functional and psychosocial factors [7,8]. Body composition involves the analysis of the human body based on the fragmentation of total body mass. For beach handball, body fat should be monitored, as adequate fat levels allow players to move more efficiently during training and matches. Lean mass—in particular, muscle mass—should also be controlled, as inadequate training loads (excessive or insufficient) can lead to changes in physique which could affect performance factors such as speed, strength, power and injury risk [9,10].

In terms of bone mass, an article was recently published in which reference was made to the bone quality of these players [11]. It was observed that, after assessment of bone quality using a heel ultrasound densitometer, the broadband ultrasound attenuation (BUA) and speed of sound (SOS) values of female beach handball players were higher than those of both long- and short-distance runners [12–14], gymnasts [13] and powerlifters [15]. It seems, therefore, that the practice of this sport in the sand, as well as the repeated impacts after jumps, turns and sprints, favors the development and bone quality of the growing skeleton.

Bone is a highly dynamic tissue that adapts to changes in systemic signals, including hormones, as well as to mechanical stresses induced by physical activity [16]. Fatty acid intake is associated with increased bone mineral density, even reducing the risk of fracture [17]. In the case of female athletes, in order to maintain bone health, there are situations that may be relevant; including low energy availability, low carbohydrate availability, protein intake, vitamin D and dermal losses of calcium and sodium [18].

This highlights the critical role that properly planned and personalized nutrition plays in the bone health of female athletes and the necessity of a nutritional assessment of each athlete. This permits the determination of whether the required amounts of key nutrients are being consumed to support both bone health and optimal athletic performance. In fact, some morphological factors related to bone mass, such as leg length, have been found to regulate stride length, thus contributing to sprint performance [19]. Although previous studies described the anthropometric profile of beach handball [11,20,21] and handball players [22–27], none provided a complete and detailed description of all the bone diameters, lengths and body heights that are included in the complete ISAK (International Society for Advancement in Kinanthropometry) profile [28]. This is of particular interest to respond to different performance results, detect sporting talent and establish the athletic characteristics of athletes.

The main objective of this study was to describe and compare the dietary intake, anthropometrics, body composition, somatotypes and proportionality profiles of female professional beach handball players, according to age category (junior vs. senior) and playing position. Additional objectives were to correlate the different anthropometric variables with the results of the 5, 10 and 15 m sprints and provide data that can be useful in detecting sports talent.

The hypothesis proposed is that senior players consume higher-quality diets since they are more aware of the importance of nutrition due to the length of time they have been

practicing sports. It is also expected that protein consumption will be related to muscle mass, and high consumption of saturated fats to fat mass. In terms of body composition, senior female players will present a larger muscular compartment. Considering playing position, it is expected that goalkeepers will have the highest fat compartment. Speed results are expected to correlate with anthropometric characteristics rather than age category.

2. Materials and Methods

2.1. Study Design

A descriptive cross-sectional study was carried out to determine the body composition (fat mass, muscle mass, bone mass and residual mass, somatotype, body height and length, proportionality and speed) of female professional beach handball players. Due to their trajectory on the field, the players of the present research represent the world elite. The Declaration of Helsinki guidelines (revised in Fortaleza, Brazil, in October 2013) and the recommendations of Good Clinical Practice (Document 111/3976/88 of July 1990) were considered in carrying out the research. The ethics committee of the University of Alicante (Spain) (UA-2019-04-09) approved the study protocol.

2.2. Participants

A total of 33 players on the Spanish Beach Handball National Team participated in the research: 18 juniors and 15 seniors. These two national teams were champions and runners-up of the last IHF Women's U18 Beach Handball World Championship (second edition) and the IHF Women's Beach Handball World Championship (ninth edition) held in Greece in 2022; thus, they could be considered world top players. Considering the playing position, the sample was divided into 6 goalkeepers, 10 wings, 8 specialists, 6 pivots and 3 defenders. The exclusion criteria for this study included chronic disease, any injury that prevented the players from performing the established tests or not having completed the informed consent form. The players did not receive any economic compensation for their participation in the study. In the case of underage players, the parents or legal representatives were the ones who signed the consent form. The anonymity of the players was preserved throughout the research.

2.3. Data Collection

2.3.1. Dietary Records

For quantitative determination of players' diets, they were asked to make a dietary record of four consecutive days (three during the week and one on the weekend) in the week prior to the intervention. The method of food weighing was chosen, since it is the method that offers the most information on quantity and frequency, and the record was accompanied by pictures of their meals. The players weighed what they ate and recorded all the foods they consumed during the established period, as well as the ingredients of the recipes or dishes consumed. They also had to indicate whether the food was raw or cooked [29–31]. The use of weighed food diaries has been suggested as the gold standard for assessing dietary intake in athletes [5].

2.3.2. Anthropometric Measures

For the anthropometric evaluation, the guidelines established by the International Society for the Advancement of Kinanthropometry (ISAK) were followed [32]. Measurements were performed by an ISAK-accredited level 2 anthropometrist, considering within-subject technical measurement error (5% for skinfolds and 1% for circumferences, lengths and heights). All measurements were performed in the same location and under the same conditions (room temperature, 22 $\pm$ 1 °C). The 42 measurements included in the complete profile were obtained. Body mass or weight (kg) was measured by using a calibrated scale, Tanita, BC545N (Tokyo, Japan), with an accuracy of 100 g. For height (cm), a mobile anthropometer, Seca 213 (SECA Deutschland, Hamburg, Germany), with a precision of 1 mm, was used, keeping the heads of the female players in the Frankfort plane position.

The 40 × 50 × 30 cm anthropometric bench was also used to measure sitting height. The distance between the points of the middle finger of the right and left hand was expressed in centimeters, and the wingspan was measured with an anthropometer that was placed on the wall and parallel to the floor.

The circumferences (head, arm (relaxed), arm (flexed and tensed), forearm (maximum), wrist (distal styloids), chest (mesosternal), waist, hip, thigh (1 cm gluteal), mid-thigh and calf) were measured with an inextensible metal tape measure. A Harpenden skinfold calliper (England; accuracy, 1 mm) was used for collection of the eight skinfolds (subscapular, tricipital, bicipital, iliac crest, supraspinal, abdominal, anterior thigh and medial leg). For small diameters (humerus, femur, wrist (bistyloid) and bimalleolar), a 22 cm pachymeter was used, and for large diameters (biacromial, bi-ilocristal, transverse chest and anterior–posterior chest depth), lengths and heights (acromiale–radiale, radiale–stylion, midstylion–dactylion, iliospinale height, trochanterion height, trochanterion–tibiale laterale, tibiale-lateral height, tibiale laterale–sphyrion tibiale and foot length), a 60 cm anthropometer and a Holtain segmometer (Holtain, Crymych, UK) were required. All the anthropometric instruments and equipment used were homologated and previously calibrated.

Different formulas were used to calculate body composition: the Rocha [33] equation for bone mass and the Lee et al. (2000) [34] equation for muscle mass. Fat mass was calculated by using the Carter [35], Faulkner [36] and Withers + Siri formulas [37]. All variables were calculated in both percentages and kilograms. Residual mass was calculated from the difference between the total body weight and the sum of the bone, muscle and fat masses.

2.3.3. Somatotype and Proportionality

The mean somatotype of each group of players (junior and senior) and of each playing position was calculated by following the method of Heath and Carter (1967) and classified according to the somatotype categories of Carter and Heath (1990). The phantom stratum was used for proportionality analysis [33].

Each variable was adjusted for phantom size, using the z-score. The z-values have a mean of 0, so a z-value of 0.0 indicates that the given variable is proportionally equal to the phantom; a z-value greater than 0.0 means that the subject is proportionally larger than the phantom for that variable; and, conversely, a z-value less than 0 shows that the subject is proportionally smaller than the phantom for the variable [33].

2.3.4. Sprints

The players performed sprints of 5, 10 and 15 m, starting from a ready position behind the starting line [38]. The sprint time was recorded by using two photocells (Witty Gate, Microgate, Mahopac, NY, USA) located at the starting line and at 5, 10 and 15 m, depending on the test to be recorded. All tests were performed on a sand surface, and the players were barefoot. The assessment started 15 min after the specific warm-up, based on Sánchez-Malia et al. [39]. The subjects first performed articular mobility exercises, and then they ran two 10 m races on a beach handball court, with the court measurements and sand characteristics according to the regulations of the International Federation. A standardized 10 min warm-up protocol was performed, consisting of different types of movement and five 10 m accelerations at a progressive intensity, with the last one at maximum speed. The rest period between warm-up repetitions was 1 min.

2.4. Statistical Analyses

Jamovi statistical software (version 1.6.15, The JAMOVI Project, Sydney, Australia) was used for data analysis. Descriptive calculations were performed for all variables included (mean and standard deviation), both overall and by age category and playing position. The Shapiro–Wilk test was used to test the normality of the distribution of descriptive variables. To evaluate the homogeneity of the data, the Levene test was used. To test for differences in basic measurements, body composition, somatotype, proportionality,

anthropometric characteristics and sprints, both by category and by playing position, an analysis of variance (ANOVA), with a Bonferroni correction and a Tukey test, was applied. Statistical significance was set at $p < 0.05$. In addition, the effect size was calculated by using partial eta-squared (η^2p), considering <0.25, 0.26–0.63 and >0.63 as small, medium and large effect sizes, respectively [40]. Partial omega squared (ω^2) was also calculated in the case of the analyses by playing position, since the sample size of each group was smaller [41] (0.01–0.05, small effect; 0.06–0.13, moderate effect; and >0.14, large effect). An Excel template was used to obtain the z-phantom scores, which were represented as a graphic. The *t*-test was used to compare the differences in dietary intake between junior and senior players. Cohen's d was used as a measure of the effect size (ES), considering small (d = 0.2), moderate (d = 0.6), large (d = 1.2), very large (d = 2.0) and extremely large (d = 4.0). Pearson's correlation coefficient (*r*) was used to indicate how closely the variables were associated with each other. The relationship (or the correlation) between the variables was denoted by the letter "r" and quantified with a number, which varied between −1 and +1. A 0 means that there is no correlation, whereas a 1 means a complete or perfect correlation. The sign of the r shows the direction of the correlation. A negative r means that the variables are inversely related. The strength of the correlation increased both from 0 to +1 and from 0 to −1 [38].

3. Results

A total of 33 players (18 juniors and 15 seniors) of the Spanish Beach Handball National Team participated in the present study. Following quantitative evaluation of the players' diets, as shown in Table 1, no significant differences were found between junior and senior players. Therefore, diet was not a confounding factor when analyzing the rest of the variables studied. However, if the results are compared with the dietary reference intakes (DRIs) for the Spanish population [42], the female players in the present study had lower intakes of most micronutrients than they should. When considering macronutrients [43], it seems that, in general, female players have a low carbohydrate (CH) intake compared to the recommendations, 5–7 g/kg/day. The same is true for proteins; it is estimated that sportswomen should consume between 1.6 and 1.8 g/kg/day, and the average coincided with these values. After the same analysis was performed by playing position, only one tendency ($p = 0.052$) was observed for the % CH variable; however, following the post hoc analyses, no significant differences were observed.

Regarding basic anthropometric measurements, the mean age was 16.7 ± 0.50 for the junior players and 24.8 ± 4.71 for the senior players. The descriptive data (mean ± standard deviation) and the ANOVA to test for differences between the basic measurements (wingspan, height, sitting height, weight and BMI), depending on the category, are shown in Table 2. There were no significant differences in any of the variables.

If these variables are analyzed according to playing position (goalkeepers, wings, specialists, pivots, and defenders), significant differences are observed in both wingspan and height (Table 3). There was a tendency ($p = 0.053$) for pivots to have a greater variable wingspan than wings. The same occurred for the height variable; however, in this case, the difference was significant ($p = 0.014$).

Tables 4 and 5 show the following body composition variables: sum (∑) of six skinfolds (mm), ∑ of eight skinfolds (mm), fat mass with the Carter, Faulkner and Withers + Siri equations, both in percentage and kilograms, muscle mass (Lee et al. 2000 Equation), bone mass (Rocha's Equation) and residual mass. Table 4, which shows the total statistics and those by age category, presents a significant difference ($p = 0.013$) in the variable kilograms of muscle mass, with the senior group reporting higher values (24.7 ± 1.83 kg) than the junior group (22.9 ± 1.96 kg).

Consideration of the playing positions changes the results. As shown in Table 5, there were significant differences in the variable fat mass (both in % and in kilograms) calculated with the Faulkner formula ($p = 0.018$ and $p = 0.025$, % and kilograms, respectively) and in the variable kilograms of bone mass ($p = 0.049$). Following the post hoc analyses, fat mass

was observed to differ between goalkeepers and wings ($p = 0.031$) and between goalkeepers and specialists ($p = 0.034$), with fat mass being higher in goalkeepers in both cases.

Table 1. Quantitative analysis of the players' diets.

	Junior (*n* = 18)		**Senior (*n* = 15)**		***t*-Test**		
	Mean	**SD**	**Mean**	**SD**	***p***	**MD**	**ES**
Energy (kcal)	1684.85	355.219	1701.39	307.361	0.888	−165.433	−0.0495
Energy (kcal/kg/day)	31.81	6.694	32.11	5.794	0.890	−0.308	−0.0488
Carbohydrates (g/kg/day)	3.33	0.894	3.29	0.797	0.877	0.047	0.0548
Protein (g/kg/day)	1.69	0.358	1.81	0.389	0.346	−0.124	−0.3343
Sodium (mg)	1975.23	703.609	1922.95	700.834	0.833	522.867	0.0744
Cholesterol (mg)	289.03	97.276	362.31	116.765	0.058	−732.733	−0.6879
Carbohydrate (g)	176.24	47.665	173.87	41.358	0.881	23.711	0.0528
Protein (g)	89.12	19.469	95.79	20.898	0.350	−66.767	−0.3317
Lipids (g)	69.45	18.803	68.98	13.875	0.937	0.470	0.0280
Fiber (g)	20.33	8.448	20.18	5.809	0.953	0.153	0.0208
Potassium (mg)	2714.18	913.604	2846.20	668.261	0.645	−1.320.167	−0.1626
Calcium (mg)	595.26	268.639	613.64	222.063	0.834	−183.789	−0.0739
Magnesium (mg)	277.12	104.975	279.42	80.273	0.945	−22.978	−0.0243
Phosphorus (mg)	1120.11	278.005	1221.46	292.407	0.316	−1.013.544	−0.3561
Iron (mg)	13.56	5.164	13.01	4.246	0.744	0.549	0.1150
Selenium (mg)	71.34	26.352	83.03	27.562	0.223	−116.944	−0.4347
Zinc (mg)	8.28	2.363	10.68	4.429	0.056	−23.967	−0.6941
Vitamin B12 (µg)	4.69	2.422	5.65	2.326	0.258	−0.959	−0.4031
Folate (µg)	242.19	82.022	263.42	74.447	0.446	−212.256	−0.2697
Vitamin D (µg)	3.73	6.019	4.35	5.174	0.756	−0.619	−0.1095
Carbohydrates (%)	41.74	7.007	40.77	4.977	0.656	0.971	0.1573
Protein (%)	21.34	3.012	22.49	2.482	0.247	−11.489	−0.4125
Lipids (%)	36.99	6.115	36.60	4.813	0.843	0.389	0.0699

kcal = kilocalories; kg = kilograms; mg = milligrams; g = grams; µg = micrograms; % = percentage; SD = standard deviation; MD = mean differences; ES = effect size.

Table 2. Descriptive data and differences in basic measures according to category.

	Total (*n* = 33)		**Junior (*n* = 18)**		**Senior (*n* = 15)**		**ANOVA**			
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	***p***	**η^2p**	**ω^2**
Wingspan (cm)	171	5.94	170	6.23	172	5.52	1.14	0.295	0.035	0.004
Height (cm)	168	5.15	167	4.90	169	5.31	1.73	0.198	0.053	0.022
Sitting height (cm)	88.2	2.53	88.3	2.76	88.1	2.31	0.07	0.787	0.002	−0.029
Weight (kg)	63.6	7.54	62.4	7.29	64.9	7.87	0.85	0.361	0.027	−0.004
BMI (kg/m^2)	22.6	2.47	22.5	2.28	22.8	2.75	0.08	0.767	0.003	−0.028

n = number per group; SD = standard deviation; cm = centimeters; kg = kilograms; m = meters; BMI = body mass index; η^2p = partial eta-squared; ω^2 = omega squared.

Table 3. Descriptive data and differences in basic measures according to playing position.

	Goalkeepers (n = 6)		Wings (n = 10)		Specialists (n = 8)		Pivots (n = 6)		Defenders (n = 3)		ANOVA			
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	p	η^2p	ω^2
Wingspan (cm)	174	6.15	168 *	3.41	168	4.92	175 *	5.58	175	7.18	3.93	0.012	0.359	0.262
Height (cm)	169	3.72	164 *	3.58	166	4.89	172 *	3.96	172	6.60	4.46	0.006	0.389	0.295
Sitting height (cm)	88.7	2.57	87.2	2.92	87.6	2.51	89.1	0.971	90.5	2.14	1.55	0.216	0.181	0.062
Weight (kg)	70.8	9.30	60.2	8.07	61.2	4.12	64.4	4.38	64.8	7.12	2.55	0.062	0.267	0.158
BMI (kg/m^2)	24.9	3.60	22.2	2.50	22.3	1.45	21.8	1.72	21.8	1.31	1.85	0.148	0.209	0.093

n = number per group; SD = standard deviation; cm = centimeters; kg = kilograms; m = meters; BMI = body mass index; η^2p = partial eta-squared; ω^2 = omega squared; * = significant differences $p < 0.05$.

Table 4. Skinfolds and body composition by age category.

	Total (n = 33)		Junior (n = 18)		Senior (n = 15)		ANOVA		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	p	η^2p
∑ 8 Skinfolds (mm)	84.5	25.6	87.5	26.2	80.8	25.3	0.557	0.461	0.018
∑ 6 Skinfolds (mm)	106	30.4	109	31.9	103	29.1	0.346	0.561	0.011
FM Carter (%)	16.7	3.96	17.1	4.05	16.1	3.92	0.557	0.461	0.018
FM Faulkner (%)	18.3	3.64	18.6	3.66	18.0	3.71	0.175	0.679	0.006
FM Faulkner (kg)	11.9	3.75	11.8	3.67	11.9	3.96	0.012	0.911	0.000
FM Withers + Siri (%)	20.4	4.91	21.6	4.70	18.9	4.92	2.540	0.121	0.076
FM Withers + Siri (kg)	13.2	4.70	13.8	4.66	12.6	4.84	0.490	0.489	0.016
MM Lee 2000 (kg)	23.7	2.07	22.9 *	1.96	24.7 *	1.83	6.920	0.013	0.182
MM Lee 2000 (%)	37.6	3.92	36.9	2.72	38.4	4.97	1.280	0.266	0.040
Bone Mass (kg)	9.42	0.796	9.32	0.897	9.55	0.661	0.714	0.404	0.023
Bone Mass (%)	14.9	1.26	15.0	1.19	14.9	1.37	0.096	0.759	0.003
Residual Mass (kg)	17.1	2.37	16.4	1.36	18.0	3.02	4.060	0.053	0.116
Residual Mass (%)	27.0	2.56	26.4	2.09	27.7	2.96	1.960	0.172	0.059

n = number per group; SD = standard deviation; mm = millimeters; kg = kilograms; % = percentage; FM = fat mass; ∑ = summatory; MM = muscle mass; η^2p = partial eta-squared; ω^2 = omega squared; * = significant differences $p < 0.05$.

For bone mass, following post hoc analysis, significant differences were observed between wings and pivots ($p = 0.010$) and between specialists and pivots ($p = 0.021$). The pivots had the highest bone mass (10.0 ± 0.627 kg), followed by the specialists (9.10 ± 0.653 kg) and the wings (9.03 ± 0.793 kg).

Following the correlation of these variables with dietary intakes (Table 1), in senior female players, positive correlations were observed between fat mass and animal-protein intake, both in % ($r = 0.569$; $p = 0.027$ and $r = 0.552$; $p = 0.033$ with the Faulkner and Withers formulas, respectively) and in kilograms ($r = 0.590$; $p = 0.021$ and $r = 0.577$; $p = 0.024$). Muscle mass (kg) was also positively correlated with grams of polyunsaturated fatty acids ingested ($r = 0.636$; $p = 0.011$). Bone mass (kg) was positively related with total grams of protein ingested ($r = 0.593$; $p = 0.020$), grams of protein/kg body weight and day ($r = 0.584$; $p = 0.022$) and monounsaturated fatty acids ($r = 0.531$; $p = 0.042$). No relevant correlations were found for junior players.

Regarding somatotype, Figure 1 shows the mean somatotypes of the players by age category and playing position. There were no significant differences between the values obtained from junior and senior players in any of the three components: endomorphy

(3.57 ± 1.12 vs. 3.16 ± 1.14, junior and senior, respectively), mesomorphy (2.48 ± 0.90 vs. 2.78 ± 0.951) and ectomorphy (2.66 ± 1.04 vs. 2.74 ± 1.17).

Table 5. Skinfolds and body composition according to playing position.

	Goalkeepers (*n* = 6)		Wings (*n* = 10)		Specialists (*n* = 8)		Pivots (*n* = 6)		Defenders (*n* = 3)		ANOVA			
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	***p***	**η^2p**	**ω^2**
∑ 8 Skinfolds (mm)	111	25.1	80.2	29.1	81.0	14.0	78.7	25.4	67.0	7.19	2.50	0.065	0.263	0.154
∑ 6 Skinfolds (mm)	137	28.1	101	34.9	104	18.9	98.3	29.9	86.3	9.26	2.36	0.078	0.252	0.142
FM Carter (%)	20.7	3.88	16.0	4.50	16.1	2.16	15.8	3.93	14.0	1.11	2.50	0.065	0.263	0.154
FM Faulkner (%)	22.6	3.86	17.4	3.53	18.0	2.56	17.3	2.93	15.9	1.51	3.58	0.018	0.338	0.238
FM Faulkner (kg)	16.2	4.84	10.7	3.77	11.1	1.91	11.2	2.34	10.4	2.04	3.27	0.025	0.319	0.216
FM Withers + Siri (%)	24.6	5.87	19.6	5.95	20.1	2.91	19.3	3.81	17.6	1.83	1.59	0.204	0.185	0.067
FM Withers + Siri (kg)	17.8	6.44	12.2	5.42	12.4	2.26	12.5	2.79	11.4	2.11	1.93	0.133	0.216	0.101
MM Lee 2000 (kg)	24.7	2.84	23.3	2.06	22.4	1.09	24.1	1.85	25.6	0.653	2.14	0.102	0.234	0.121
MM Lee 2000 (%)	35.0	2.60	39.3	5.78	36.7	1.24	37.5	2.20	39.8	3.98	1.59	0.206	0.185	0.066
Bone Mass (kg)	9.71	0.806	9.03	0.793	9.10	0.653	10.0	0.627	9.79	0.599	2.72	0.049	0.280	0.173
Bone Mass (%)	13.8	1.29	15.1	1.23	14.9	1.16	15.6	1.06	15.2	1.23	1.91	0.137	0.214	0.099
Residual Mass (kg)	18.6	1.44	15.6	2.53	17.2	1.62	17.7	1.48	17.9	4.51	2.14	0.102	0.234	0.121
Residual Mass (%)	26.6	3.04	25.9	2.86	28.2	1.65	27.6	1.72	27.4	3.81	1.04	0.406	0.129	0.004

n = number per group; SD = standard deviation; mm = millimeters; kg = kilograms; % = percentage; FM = fat mass; ∑ = summatory; MM = muscle mass; η^2p = partial eta-squared; ω^2 = omega squared.

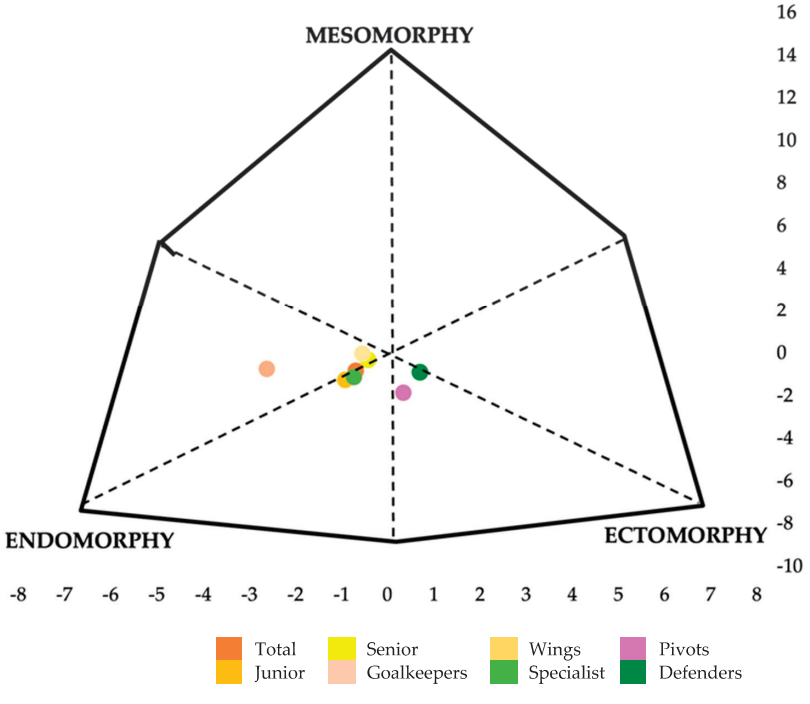


Figure 1. Somatotype distribution in elite female beach handball players.

Regarding playing position, a significant difference was observed in the endomorphic component ($p = 0.047$). However, in the post hoc analysis with a Bonferroni correction, no significant differences were observed between any group. Following the analysis of this variable without correction, significant differences were observed between the position's goalkeepers and wings ($p = 0.014$), goalkeepers and pivots ($p = 0.013$), and goalkeepers and defenders ($p = 0.010$), with endomorphy values decreasing in the following order: goalkeepers (4.55 ± 1.21) > wings (3.17 ± 1.14) > pivots (2.98 ± 0.88) > defenders (2.56 ± 0.75).

The anthropometric dimensions and proportionality profile (of the 21 measurements that correspond to the restricted ISAK profile) of all the players and separated by age category are shown in Figure 2. After the analysis, no significant differences were observed in any of the variables.

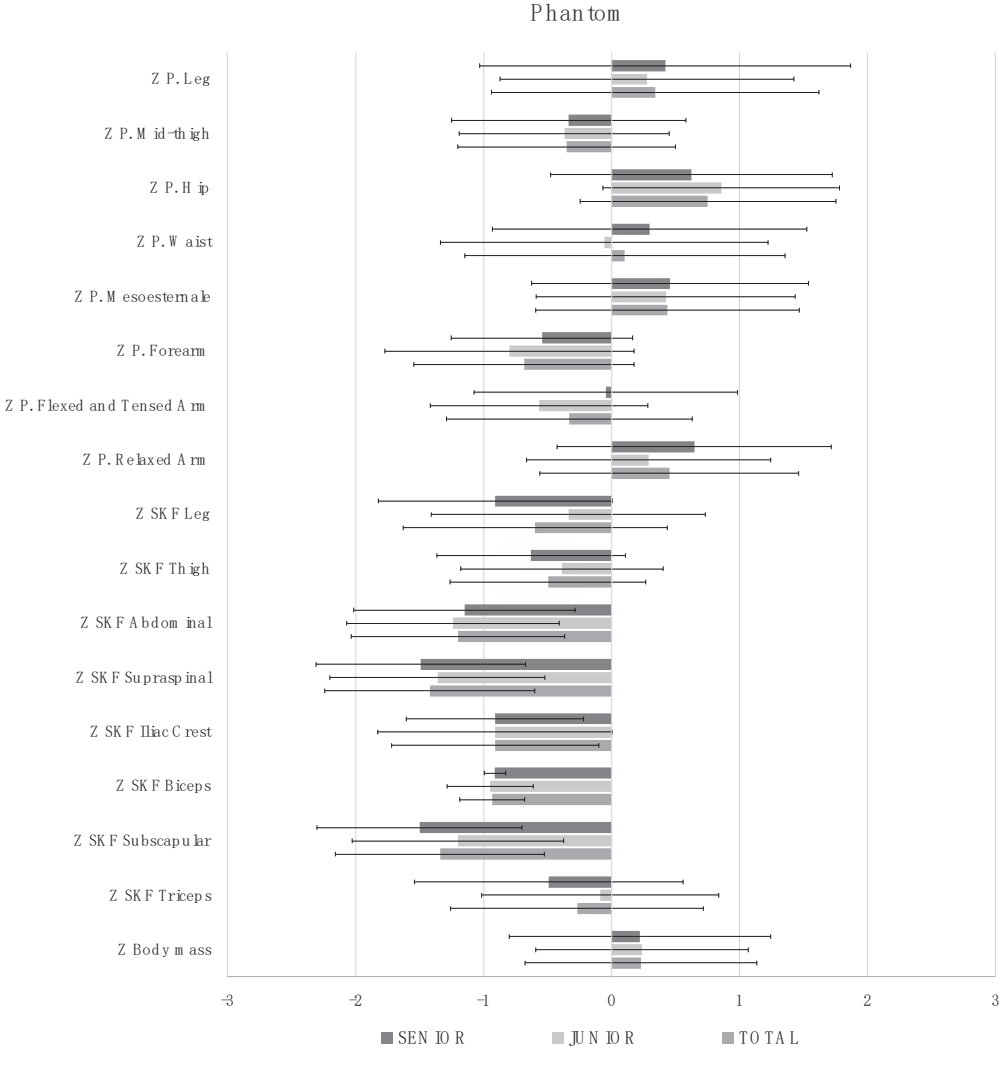


Figure 2. Proportionality with respect to the mannequin of the variables included in the ISAK restricted profile. SKF = skinfolds; P = perimeters.

In addition to skinfolds and perimeters, lengths, heights and large bone diameters were also collected. By age category, significant differences ($p = 0.015$) were only observed in the case of tibiale-laterale height, which was greater in senior players (46.3 ± 1.91) than in juniors (44.4 + 2.38). The descriptive data and differences for each of these variables, depending on the playing position, are shown in Table 6. Significant differences were observed in the variables iliospinale height ($p = 0.032$), biacromial diameter ($p = 0.021$), bimalleolar diameter ($p = 0.025$), mesosternal perimeter ($p = 0.035$) and hip perimeter ($p = 0.014$). Following the post hoc analyses, in the case of the iliospinale height, the female defenders had higher values than the female wings ($p = 0.042$). For biacromial and bimalleolar diameters, mesosternal perimeter and biceps skinfold, no significant differences were found after the post hoc analysis. Hip circumference was significantly higher in specialist players than in defenders ($p = 0.023$), and abdominal fold was significantly higher in goalkeepers than in wings ($p = 0.040$) and defenders ($p = 0.016$).

Table 6. Lengths, heights, diameters, perimeters and skinfolds depending on the playing position.

	Goalkeepers (*n* = 6)		**Wings (*n* = 10)**		**Specialists (*n* = 8)**		**Pivots (*n* = 6)**		**Defenders (*n* = 3)**		**ANOVA**		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	**df2**	***p***
L Acromiale–Radiale	32.5	1.91	30.9	1.12	31.9	0.975	33.1	1.12	33.1	1.82	3.526	9.18	0.053
L Radiale–Stylion	25.2	1.84	24.1	0.693	24.1	1.03	24.9	1.54	24.8	1.37	0.859	8.80	0.524
L Stylion Medio-Dactylion	19.5	0.632	19.1	0.717	19.0	0.824	19.8	0.771	19.6	0.100	2.113	12.95	0.137
H Iliospinale	95.0	3.08	91.2	1.73	92.0	3.45	98.0	4.05	95.1	4.86	4.380	8.67	0.032
H Trochanterion	90.8	5.08	89.7	4.91	88.0	5.27	92.4	4.76	88.7	2.53	0.733	11.28	0.588
H Trochanterion–Tibiale Laterale	43.3	1.59	42.3	1.54	42.0	1.79	44.9	2.74	42.7	1.44	1.372	9.89	0.312
H Tibiale Laterale	46.1	1.49	44.1	1.60	44.3	2.22	47.2	2.37	46.0	4.10	2.512	9.12	0.115
L Foot	25.8	0.912	24.9	0.810	25.1	0.811	26.1	1.27	25.9	0.971	1.695	9.47	0.231
L Tibialis Mediale–Sphyrion Tibiale	39.7	1.64	37.2	1.45	37.1	1.39	39.5	2.61	40.0	2.93	3.275	9.05	0.064
D Biacromial	37.7	1.97	36.1	0.867	36.1	1.59	34.8	4.26	39.5	1.18	5.012	9.08	0.021
D Antero-Posterior Abdomen	20.3	1.96	17.6	1.56	17.4	1.34	17.4	1.57	18.1	2.45	2.175	9.26	0.151
D Bi-Iliocristal	29.1	1.79	26.4	2.07	27.1	1.02	29.4	3.70	27.5	0.819	2.157	10.79	0.143
D Transverse Thoracic	31.8	3.72	28.6	1.05	28.4	0.952	28.4	1.10	29.4	0.961	1.436	9.73	0.294
D Antero-Posterior Thorax	16.9	1.91	15.4	1.62	15.7	1.43	15.4	1.14	15.5	0.737	0.694	11.36	0.611
D Humerus	6.23	0.294	6.11	0.256	6.24	0.200	6.40	0.261	6.30	0.200	1.063	9.97	0.424
D Bistyloid	4.93	0.242	4.91	0.185	4.86	0.226	5.08	0.147	5.03	0.057	1.877	12.75	0.176
D Femur	9.17	0.513	8.76	0.610	8.79	0.181	8.97	0.333	8.73	0.115	1.208	11.75	0.359
D Bimalleolar	6.83	0.339	6.50	0.226	6.66	0.213	6.88	0.240	7.07	0.208	4.518	9.82	0.025
P. Head	56.5	3.57	55.0	2.16	54.9	0.888	54.8	1.07	54.1	1.42	0.479	9.47	0.751
P. Neck	33.0	1.97	31.3	1.91	31.7	1.63	32.2	1.00	32.2	0.462	0.762	12.88	0.568
P. Relaxed Arm	29.3	3.06	27.1	2.29	26.7	1.94	27.3	1.82	27.7	0.153	1.030	12.49	0.430
P. Arm Flex Contra	30.2	2.82	27.9	1.69	27.1	2.10	27.8	2.28	28.8	1.02	1.385	11.00	0.301
P. Forearm	24.5	1.66	23.4	1.31	23.7	0.793	23.7	1.24	24.3	0.987	0.667	9.71	0.630
P. Wrist	14.8	0.625	15.4	3.08	14.4	0.431	14.8	0.210	14.7	0.200	1.510	11.43	0.263
P. Mesoternal	93.5	5.39	86.6	3.61	88.5	4.27	87.0	2.65	90.0	0.751	3.583	13.16	0.035
P. Waist	76.4	6.64	69.1	5.40	70.7	4.36	70.3	1.87	71.5	4.45	1.165	9.47	0.385

Table 6. *Cont.*

	Goalkeepers (n = 6)		Wings (n = 10)		Specialists (n = 8)		Pivots (n = 6)		Defenders (n = 3)		ANOVA		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**F**	**df2**	***p***
P. Hip	103	6.11	95.2	6.72	95.0	2.94	97.1	2.90	99.9	1.22	4.821	12.71	0.014
P. Thigh 1 cm	61.0	5.43	55.7	3.40	59.9	15.3	54.9	2.45	57.6	1.66	1.938	11.66	0.170
P. Mid-Thigh	54.4	4.77	49.9	3.77	49.3	2.18	50.3	3.48	51.5	0.551	2.438	13.03	0.099
P. Leg	36.7	2.97	35.7	3.42	33.9	1.86	35.6	2.36	36.1	0.586	2.176	13.08	0.129
P. Ankle	21.7	1.97	21.1	1.06	20.9	1.15	21.3	0.819	21.1	0.603	0.213	10.93	0.925
SKF Triceps	18.3	5.52	13.3	4.84	13.5	1.51	13.0	3.57	10.8	2.39	1.733	9.30	0.224
SKF Subscapular	14.5	4.73	9.43	3.85	10.0	3.54	8.63	1.78	8.33	2.45	1.857	10.14	0.194
SKF Biceps	8.98	3.36	6.44	3.97	6.14	1.80	6.91	1.56	4.58	0.749	3.634	12.62	0.035
SKF Iliac Crest	19.4	5.10	15.1	5.94	17.1	5.20	13.8	5.01	13.1	2.91	1.500	11.16	0.268
SKF Supraspinal	12.3	3.51	7.93	3.72	8.99	2.88	8.08	3.47	6.83	2.25	1.926	10.50	0.179
SKF Abdominal	23.9	5.79	13.9	5.39	15.0	4.80	14.2	6.15	11.8	1.72	4.930	12.53	0.013
SKF Thigh	27.2	3.35	22.6	7.81	21.2	3.78	21.0	8.03	19.0	3.66	3.227	10.24	0.059
SKF Leg	14.5	4.68	13.0	6.45	12.3	2.87	13.8	5.09	10.3	2.25	0.896	10.94	0.499

SD = standard deviation; L = length; H = height; D= diameter; P = circumference; SKF = Skinfold; df2 = degrees of freedom 2Regarding sprint speed.

Figures 3 and 4 show the results by age category and playing position, respectively. As can be seen in the figure, there were significant differences for the 5 m (p = 0.018), 10 m (0 = 0.020) and 15 m (p = 0.022) sprint, with the senior players being faster than the juniors in all cases. No significant differences were observed according to playing position (Figure 4). The results of a Pearson p-correlation analysis between the sprints and the different anthropometric variables showed significant positive correlations between the different tests of sprints and weight (r = 0.521; p = 0.002 and r = 0.576; p < 0.001 for 10 m and 15 m, respectively), transverse chest diameter (r = 0.390; p = 0.025 and r = 0.442; p = 0.010), neck perimeter (r = 0.390; p = 0.025 and (r = 0.420; p = 0.015), relaxed arm perimeter (r = 0.402; p = 0.020 and r = 0.427; p = 0.013), mesosternal perimeter (r = 0.452 and 0.455; p = 0.008 in both cases), waist (r = 0.445; p = 0.010 and r = 0.487; p = 0.004), hip (r = 0.586 and r = 0.582; p < 0.001 in both cases) and thigh (r = 0.463; p = 0.007 and r = 0.452; p = 0.008).

The same occurred for the antero-posterior chest diameter (r = 0.426; p = 0.013, r = 0.510; p = 0.002 and r = 0.473; p = 0.005 for 5, 10 and 15 m, respectively), $\sum$ of six and eight skinfolds (mm), and % of fat mass (kg), with all the formulas used, in the case of 5 m p < 0.005 and for 10 and 15 m p < 0.001. It should be noted that there were also negative correlations between 5 m and 10 m sprints with trochanterion height (r = −0.423; p = 0.014 and r = −0.347; p = 0.048, respectively); therefore, those female players with a greater trochanterion height were faster. A similar pattern occurred with respect to % muscle mass and % bone mass. Significant negative correlations were observed with the 5 m sprint (r = −0.403 and r = −0.428 for muscle mass and bone mass, respectively; p = 0.013 in both cases), 10 m (r = –0.555; p < 0.001 and r = –0.529; p = 0.002) and 15 m (r = −0.586; p < 0.001 and r = −0.537; p = 0.001); therefore, the players with more muscle mass and more bone mass had faster results in the speed tests.

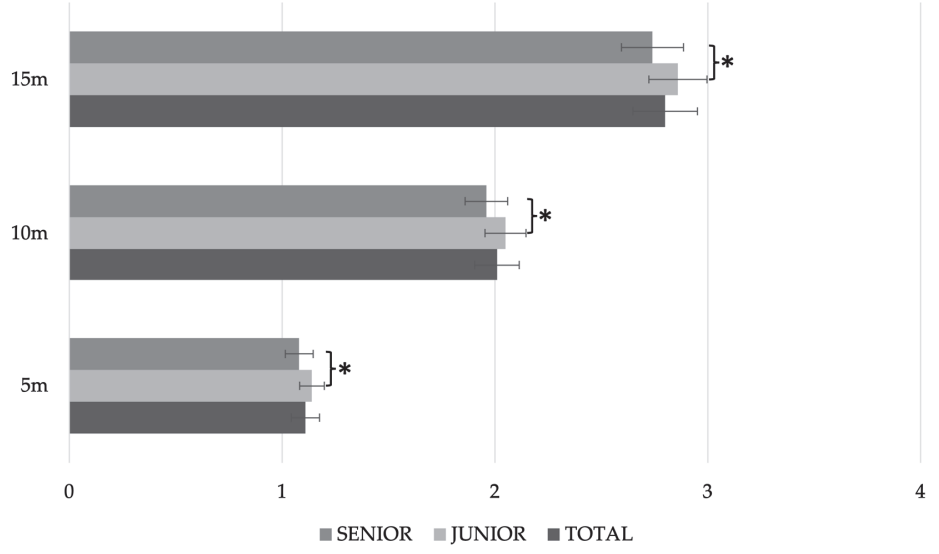


Figure 3. Sprint speed according to age category (junior vs. senior). * = significant differences $p < 0.05$.

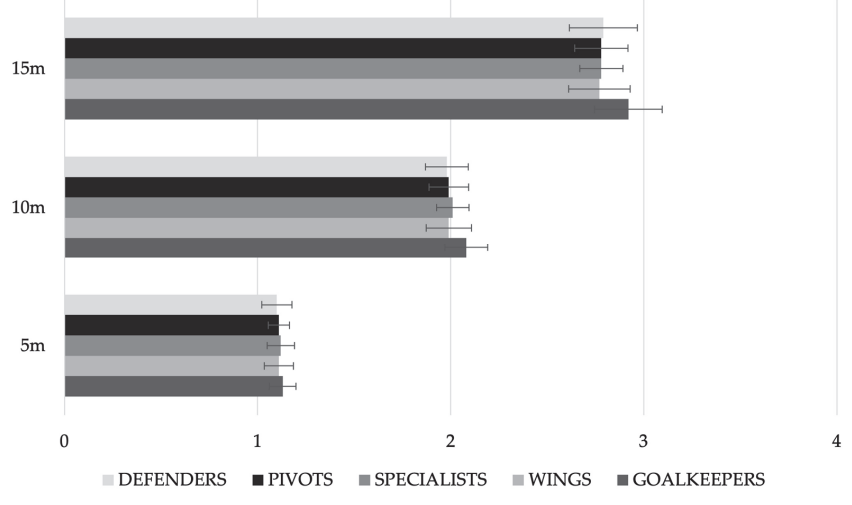


Figure 4. Sprint speed depending on playing position.

4. Discussion

The objectives of this research were to describe and compare the dietary, anthropometric, body composition, somatotype and proportionality profiles of professional female beach handball players according to age category (junior and senior) and playing position. Different anthropometric variables were also correlated with speed results (5, 10 and 15 m sprints).

Previous research has evaluated anthropometric characteristics, somatotype and body composition in beach handball players [9]; however, this study is the first to analyze the dietary intake of female players and to show the complete anthropometric profile of beach handball players as a function of playing position. These data were necessary, as this information could be of great help, together with fitness measurements, to determine physical preparation for competition and to monitor the effects of training and dietary interventions on body composition status and vice versa. However, in reference to the

junior category, the players may be at different stages of development, and this should be taken into account to facilitate an effective transition of performance between the different categories (junior to senior) on the pathway to talent [44]. However, our analysis of the results indicated that the differences between categories (junior through senior) are not so great; therefore, these junior players may be close to the level of development of an elite senior female player.

One of the main findings following the quantitative dietary assessment was that carbohydrate intake was below the recommendations for players performing moderate training of approximately one hour per day (5–7 g/kg body weight) [4]: 3.33 ± 0.89 g/kg body weight in the case of juniors and 3.29 ± 0.797 in seniors.

However, protein intake was indeed in line with current recommendations (1.2–2 g/kg body weight) [4], being 1.69 ± 0.36 and 1.69 ± 0.36 in juniors and seniors, respectively. Mean energy intake was 1684.85 ± 355.21 in juniors and 1701.39 ± 307.36 in seniors, lower than the 1870.46 ± 576.24 previously estimated in female soccer players [45] and 2073.13 and 2512.19 kilocalories observed in other team sports (soccer, tennis, basketball, football, golf, lacrosse, baseball and hockey) [46,47].

Considering that the mean intake of calcium and iron is 1000 mg/day and 18 mg/day, respectively, in both cases, the intake was lower: 595.26 ± 268.64 and 13.56 ± 5.16 mg/day in juniors and 613.64 ± 222.06 and 13.01 ± 4.24 mg/day in seniors, respectively. The vitamin D intake of the female players was 3.73 ± 6.02 and 4.35 ± 5.17 µg in juniors and seniors, complying with the current recommendations for vitamin D intake in female athletes, i.e., 5 µg [43]. The sodium intake of the players in the present investigation was slightly higher than the recommendations [43]. Considering the climatic conditions in which handball training sessions are held, this was to be expected, since 90% of the players reported consuming sports drinks before, during and after training. There is no concrete recommendation for sodium intake in female athletes, as there is some degree of interindividual variability, depending on sweat rates and individual sweat sodium concentrations [48].

In addition, in female athletes, menstruation, together with high-intensity training, can affect the status of other micronutrients, such as zinc and vitamin B12 [3]. The intakes of the players in the present investigation complied with both zinc (8 mg/day) and vitamin B12 (2 µg/day) recommendations, being 8.28 ± 2.36 and 4.69 ± 2.42 µg, respectively, in juniors and 10.68 ± 4.43 mg and 5.65 ± 2.33 µg, respectively, in seniors.

As for basic anthropometric measurements, the elite junior handball players showed a mean height of 167 ± 4.90 cm and a mean weight of 62.4 ± 7.29 kg, while in the senior players, these were 169 ± 5.31 cm and 64.9 ± 7.87 kg. As observed in male players [49], it seems that these differences are caused by the different age groups of players being in different stages of the maturation process. If these results are compared to those of the 32 senior players (25.3 ± 4.8 years) who were competing in the 2017 European Championship [1], both weight and height are slightly lower; 168 ± 3.86 cm and 60.78 ± 3.87 kg. In a study by Pueo et al. [9], both height 169.1 ± 5.1 and weight 62.9 ± 5.3 were more similar in the case of female players (24.1 ± 4.7 years).

Regarding these variables by playing position, an adequate comparison cannot be performed, since in the previous literature, players were grouped according to three playing positions [9]: goalkeepers (mesomorphic endomorph), wing-specialists (mesomorph-endomorph) and pivot-defenders (balanced ectomorph), with the latter two being categorized as the same position, despite their differences in specific training characteristics. However, similarly, the players in the present study had a balanced endomorphic anthropometric profile.

With the results obtained by playing position, it is possible to confirm the conclusion reached in a previous systematic review in female indoor handball players [10] that the weight and height values of wings are lower than those of other positions (Table 2). It seems that the reason for this finding is that wings need faster and lighter bodies, as they must be able to make rapid changes in speed and direction to cover as much of the field as possible.

In the body-composition variables, significant differences ($p = 0.013$) were only observed in the variable muscle mass (kg), which was greater in the senior group (24.7 ± 1.83 kg) with respect to the junior group (22.9 ± 1.96 kg). However, after dividing the sample by playing position, significant differences were observed in fat mass calculated with the Faulkner formula, both in percentage ($p = 0.018$) and in kilograms ($p = 0.025$). Goalkeepers had significantly higher % fat mass (22.6 ± 3.86%) than wings (17.4 ± 3.53%) and greater fat mass (16.2 ± 4.84 kg) than specialists (11.1 ± 1.91 kg). This could be due to the goalkeepers' own role in the team, as they move less than the other players and therefore have a lower energy expenditure. Differences were also observed in bone mass ($p = 0.049$); however, following the post hoc analysis with a Bonferroni correction, no significant differences were observed between positions.

When the results of the present research were compared with those previously mentioned, it was affirmed that the players in the present research showed slightly higher fat mass, 18.9 ± 4.92%, compared to 15.4 ± 3.7% [9], calculated in both cases with the Withers + Siri formula. Following a correlation analysis of body composition variables with dietary intake, for senior female players, the consumption of animal protein was positively correlated with fat mass. The current evidence suggests that higher intakes of animal protein, i.e., in the quantities recommended for athletes, are unlikely to be adverse to bone health, assuming that the amount of calcium ingested is adequate [50]. However, it seems to have a negative effect on the accumulation of the fat-mass component, probably because the quality of the products chosen is inadequate, resulting in an increase in the intake of saturated fats [51]. In addition, correlations were found between bone mass (kg) and protein intake. This confirms previous findings [50], which suggested that protein may have an indirect effect on bone not only through its support of muscle mass and function but also through increasing circulating levels of insulin-like growth factor-1 (IGF-1), which has an anabolic effect on bone.

When compared with those of female indoor handball players [10], the sum of six skinfolds (tricipital + subscapular + supraspinal + abdominal + thigh + leg) was slightly higher in female indoor players (93.81 mm vs. 84.5 mm). However, muscle mass was also greater in the case of the players on the national handball team [45], being 26.2 ± 2.6 kg, compared with 23.7 ± 2.07 kg in the players of the present investigation.

The female beach handball players in the present investigation showed a balanced endomorphic somatotype (endomorphy was dominant, while mesomorphy and ectomorphy were similar, not differing by more than 0.5), in both junior (3.6–2.5–2.7; endomorphy–mesomorphy–ectomorphy, respectively) and senior (3.2–2.8–2.7) players. These results do not coincide with those obtained by Pueo et al. [9], where the players presented an average mesomorphic–endomorphic somatotype (3.5–3.3–2.6). Although the endomorphy and ectomorphy components coincided, the mesomorphy of the players in the present study was slightly lower. However, the results are more similar to the results for indoor handball players (3.1–2.5–2.6) in a study by Marijana Cavala et al. [52]. This makes sense, since many of the players who play beach handball in the summer season are also indoor handball players during the winter.

Nevertheless, this is not always the situation, as Ferragut et al. [45] observed, after analyzing the players of the Spanish national handball team, that the top elite women (26.4 ± 4.5 years) presented a slightly higher mesomorphic component than those reported previously (3.8–4.2–2.3). Regarding the playing position, although in the present research post hoc analyses with a Bonferroni correction did not show significant differences, the endomorphic component was higher in goalkeepers with respect to the rest of the positions: 4.55 ± 1.21 (goalkeepers), 3.17 ± 1.14 (wings), 3.40 ± 0.87 (specialists), 2.98 ± 0.88 (pivots) and 2.56 ± 0.75 (defenders).

Regarding proportionality, the profiles of both groups (junior vs. senior) were similar, with no significant differences in any of the variables. Because this is the first research showing a proportionality analysis in female beach handball players, it is not possible to compare our results with those of previous studies. The same is true for the variables

large bone diameter, length and height. It is important to highlight the negative correlation between trochanterion height (vertical distance from the trochanterion mark to the ground) and the results of the 5 and 10 m sprints; a greater leg length was related to greater running speed. The same was true for muscle mass and bone mass; players with more muscle and bone mass were the fastest.

If these sprint results are compared with those of handball players from previous research [53], the players in the present study are faster (1.11 ± 0.07 and 2.01 ± 0.11 vs. 1.25 ± 0.06 and 2.19 ± 0.05 for 5 and 10 m, respectively). The same occurs in a comparison with beach handball players [54]; 1.17 ± 0.06 and 2.04 ± 0.08 s, for 5 and 10 m, respectively.

As in all research, reference should be made to the strengths and limitations presented. This is the first study to show the results of lengths, heights and proportionality of female beach handball players, contributing a complete data set to the literature. This provides useful data for recruiting and selecting players with an optimal anthropometric profile to improve performance and, consequently, team results. However, it is important to emphasize that, when comparing the results by playing position, caution should be exercised, since the groups did not have the same number of participants. It would be of great interest, in addition to obtaining the complete anthropometric profile, to use the gold-standard instrument DXA for the measurement of body composition. Although a quantitative analysis of the players' intake was carried out, it would be necessary to carry out a qualitative analysis over a longer period, at least 7 days. In addition, the size of the sample and subsamples must be considered, so these data should be interpreted with caution. However, it should be recalled that beach handball is not considered a mass sport, despite its exponential growth in the last decade, especially in the women's category, as is the case in other sports. However, in order to conduct this study, we had the opportunity to analyze the players of the Spanish Beach Handball National Team, i.e., world-class players, since in the last IHF Women's Beach Handball World Championship—ninth edition—and in the IHF Women's Youth Beach Handball World Championship (U18)—second edition—held in Greece in 2022, Spain won the silver medal and the gold medal, respectively. Therefore, the authors understand that the sample has a very exclusive value due to the difficulty accessing this type of population. Along these lines and for future research, players of different nationalities and a larger sample should be considered. Finally, it should be noted that this is a cross-sectional study, so it is not possible to establish causal relationships.

Practical Implications

The present results can be used by coaches and fitness trainers in the creation of a high-performance plan for beach handball, as well as in the design of a talent discovery program. In this way, training can be specifically oriented to each type of female player, a fundamental aspect of elite performance.

5. Conclusions

Anthropometric characteristics, body composition and somatotype are important in team sports, including beach handball. In this study, the results are presented according to age category (junior vs. senior) and the six playing positions (goalkeepers, wingers, specialists, pivots and defenders). The intake of protein and some micronutrients, such as sodium, vitamin D, zinc and vitamin B12, were adequate in beach handball players. However, the intake of carbohydrates, calcium and iron did not meet the recommendations. In the basic measures, there were no significant differences between junior vs. senior players; however, there were significant differences in terms of playing position for the height variable, which was significantly higher in pivots than in wings. Body composition was similar in both groups; however, it should be noted that female goalkeepers had the highest fat component. Regarding somatotype, in both categories, the somatotype was balanced endomorph. An important finding of this research was the correlation between trochanter height and faster results for sprint speed. These data enrich the literature published so far, offering a reference for female beach handball players.

Author Contributions: Conceptualization, A.M.-R., J.S.-S. and J.A.S.-S.; methodology, A.M.-R., J.S.-S. and J.A.S.-S.; software, A.M.-R., J.S.-S., M.M.-O. and M.V.-M.; validation, J.S.-S. and J.A.S.-S.; formal analysis, A.M.-R., J.S.-S., M.M.-O. and M.V.-M.; investigation, A.M.-R., J.S.-S., M.M.-O., M.V.-M., M.P.-M., N.A.-M., J.A.S.-S., L.G.-A., P.M., R.Y.-S. and G.C.-R.; resources, J.S.-S. and J.A.S.-S.; data curation, A.M.-R., J.S.-S., M.M.-O. and M.V.-M.; writing—original draft preparation, A.M.-R., J.S.-S. and M.M.-O.; writing—review and editing, M.V.-M., M.P.-M., N.A.-M., L.G.-A., P.M., R.Y.-S., G.C.-R. and J.A.S.-S.; visualization, A.M.-R. and J.A.S.-S.; supervision, J.S.-S. and J.A.S.-S.; project administration, A.M.-R. and J.A.S.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the University Human Research Ethics Committee of the University of Alicante (Spain) (UA-2019-04-09).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due their containing personal health information.

Acknowledgments: To the players and coaches of the Spanish Beach Handball National Teams, to the Royal Spanish Handball Federation and to the European Institute of Exercise and Health (EIEH) of Alicante University for their selfless collaboration in this research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zapardiel, J.C.; Asín-Izquierdo, I. Conditional Analysis of Elite Beach Handball According to Specific Playing Position through Assessment with GPS. *Int. J. Perform. Anal. Sport* **2020**, *20*, 118–132. [CrossRef]
2. Karcher, C.; Buchheit, M. On-Court Demands of Elite Handball, with Special Reference to Playing Positions. *Sports Med.* **2014**, *44*, 797–814. [CrossRef] [PubMed]
3. Condo, D.; Lohman, R.; Kelly, M.; Carr, A. Nutritional Intake, Sports Nutrition Knowledge and Energy Availability in Female Australian Rules Football Players. *Nutrients* **2019**, *11*, 971. [CrossRef]
4. Thomas, D.T.; Erdman, K.A.; Burke, L.M. Nutrition and Athletic Performance. *Med. Sci. Sports Exerc.* **2016**, *48*, 543–568. [CrossRef] [PubMed]
5. Heaney, S.; O'Connor, H.; Gifford, J.; Naughton, G. Comparison of Strategies for Assessing Nutritional Adequacy in Elite Female Athletes' Dietary Intake. *Int. J. Sport Nutr. Exerc. Metab.* **2010**, *20*, 245–256. [CrossRef] [PubMed]
6. Mielgo-Ayuso, J.; Maroto-Sánchez, B.; Luzardo-Socorro, R.; Palacios, G.; Palacios Gil-Antuñano, N.; González-Gross, M. Evaluation of Nutritional Status and Energy Expenditure in Athletes. *Nutr. Hosp.* **2015**, *31*, 227–236. [PubMed]
7. Bernal-Orozco, M.F.; Posada-Falomir, M.; Quiñónez-Gastélum, C.M.; Plascencia-Aguilera, L.P.; Arana-Nuño, J.R.; Badillo-Camacho, N.; Márquez-Sandoval, F.; Holway, F.E.; Vizmanos-Lamotte, B. Anthropometric and Body Composition Profile of Young Professional Soccer Players. *J. Strength Cond. Res.* **2020**, *34*, 1911–1923. [CrossRef]
8. Chaouachi, A.; Brughelli, M.; Levin, G.; Boudhina, N.B.B.; Cronin, J.; Chamari, K. Anthropometric, Physiological and Performance Characteristics of Elite Team-Handball Players. *J. Sports Sci.* **2009**, *27*, 151–157. [CrossRef]
9. Pueo, B.; Espina-Agullo, J.J.; Selles-Perez, S.; Penichet-Tomas, A. Optimal Body Composition and Anthropometric Profile of World-Class Beach Handball Players by Playing Positions. *Sustainability* **2020**, *12*, 6789. [CrossRef]
10. Martínez-Rodríguez, A.; Martínez-Olcina, M.; Hernández-García, M.; Rubio-Arias, J.; Sánchez-Sánchez, J.; Sánchez-Sáez, J.A. Body Composition Characteristics of Handball Players: Systematic Review. *Arch. Med. Deport.* **2020**, *37*, 52–61.
11. Martínez-Rodríguez, A.; Sánchez-Sánchez, J.; Martínez-Olcina, M.; Vicente-Martínez, M.; Miralles-Amorós, L.; Sánchez-Sáez, J.A. Study of Physical Fitness, Bone Quality, and Mediterranean Diet Adherence in Professional Female Beach Handball Players: Cross-Sectional Study. *Nutrients* **2021**, *13*, 1911. [CrossRef] [PubMed]
12. Brahm, H.; Ström, H.; Piehl-Aulin, K.; Mallmin, H.; Ljunghall, S. Bone Metabolism in Endurance Trained Athletes: A Comparison to Population-Based Controls Based on DXA, SXA, Quantitative Ultrasound, and Biochemical Markers. *Calcif. Tissue Int.* **1997**, *61*, 448–454. [CrossRef] [PubMed]
13. Lehtonen-Veromaa, M.; Möttönen, T.; Nuotio, I.; Heinonen, O.J.; Viikari, J. Influence of Physical Activity on Ultrasound and Dual-Energy X-Ray Absorptiometry Bone Measurements in Peripubertal Girls: A Cross-Sectional Study. *Calcif. Tissue Int.* **2000**, *66*, 248–254. [CrossRef] [PubMed]
14. Lara, B.; Salinero, J.J.; Gutiérrez, J.; Areces, F.; Abián-Vicén, J.; Ruiz-Vicente, D.; Gallo-Salazar, C.; Jiménez, F.; Del Coso, J. Influence of Endurance Running on Calcaneal Bone Stiffness in Male and Female Runners. *Eur. J. Appl. Physiol.* **2016**, *116*, 327–333. [CrossRef] [PubMed]

15. Jawed, S.; Horton, B.; Masud, T. Quantitative Heel Ultrasound Variables in Powerlifters and Controls. *Br. J. Sports Med.* **2001**, *35*, 274–275. [CrossRef] [PubMed]
16. Hagman, M.; Helge, E.W.; Fristrup, B.; Jørgensen, N.R.; Helge, J.W.; Krustrup, P. High Bone Mineral Density in Lifelong Trained Female Team Handball Players and Young Elite Football Players. *Eur. J. Appl. Physiol.* **2021**, *121*, 2825–2836. [CrossRef]
17. Lavado-García, J.; Roncero-Martin, R.; Moran, J.M.; Pedrera-Canal, M.; Aliaga, I.; Leal-Hernandez, O.; Rico-Martin, S.; Canal-Macias, M.L. Long-Chain Omega-3 Polyunsaturated Fatty Acid Dietary Intake Is Positively Associated with Bone Mineral Density in Normal and Osteopenic Spanish Women. *PLoS ONE* **2018**, *13*, 539. [CrossRef]
18. Moran, J.M. Nutrition and Women's Bone Health. *Nutrients* **2022**, *14*, 763. [CrossRef]
19. Tomita, D.; Suga, T.; Terada, M.; Tanaka, T.; Miyake, Y.; Ueno, H.; Otsuka, M.; Nagano, A.; Isaka, T. A Pilot Study on a Potential Relationship between Leg Bone Length and Sprint Performance in Sprinters; Are There Any Event-Related Differences in 100-m and 400-m Sprints? *BMC Res. Notes* **2020**, *13*, 297. [CrossRef]
20. Lemos, L.F.; Oliveira, V.C.; Duncan, M.J.; Ortega, J.P.; Martins, C.M.; Ramirez-Campillo, R.; Sanchez, J.S.; Nevill, A.M.; Nakamura, F.Y. Physical Fitness Profile in Elite Beach Handball Players of Different Age Categories. *J. Sports Med. Phys. Fitness* **2020**, *60*, 1536–1543. [CrossRef]
21. Lemos, L.; Nevill, A.; Duncan, M.J.; De Oliveira, V.C.; Pino-Ortega, J.; Santos, A.; Martins, C.; Nakamura, F. Sport Specific Skills Differentiates Performance Levels Better Than Anthropometric or Physiological Factors in Beach Handball. *Res. Q. Exerc. Sport* **2021**, *93*, 682–687. [CrossRef] [PubMed]
22. Bon, M.; Pori, P.; Sibila, M. Position-Related Differences in Selected Morphological Body Characteristics of Top-Level Female Handball Players. *Coll. Antropol.* **2015**, *39*, 631–639. [PubMed]
23. Bayios, I.A.; Bergeles, N.K.; Apostolidis, N.G.; Noutsos, K.S.; Koskolou, M.D. Anthropometric, Body Composition and Somatotype Differences of Greek Elite Female Basketball, Volleyball and Handball Players. *J. Sports Med. Phys. Fitness* **2006**, *46*, 271. [PubMed]
24. Vila, H.; Manchado, C.; Rodriguez, N.; Abraldes, J.A.; Alcaraz, P.E.; Ferragut, C. Anthropometric Profile, Vertical Jump, and Throwing Velocity in Elite Female Handball Players by Playing Positions. *J. Strength Cond. Res.* **2012**, *26*, 2146–2155. [CrossRef] [PubMed]
25. Mon-López, D.; Zakynthinaki, M.S.; Cordente, C.A.; García-González, J. The Relationship Between Pistol Olympic Shooting Performance, Handgrip and Shoulder Abduction Strength. *J. Hum. Kinet.* **2019**, *69*, 39–46. [CrossRef] [PubMed]
26. Milanese, C.; Piscitelli, F.; Lampis, C.; Zancanaro, C. Anthropometry and Body Composition of Female Handball Players According to Competitive Level or the Playing Position. *J. Sports Sci.* **2011**, *29*, 1301–1309. [CrossRef] [PubMed]
27. Moss, S.L.; McWhannell, N.; Michalsik, L.B.; Twist, C. Anthropometric and Physical Performance Characteristics of Top-Elite, Elite and Non-Elite Youth Female Team Handball Players. *J. Sports Sci.* **2015**, *33*, 1780–1789. [CrossRef]
28. Cruz, J.R.A.; Armesilla, M.D.C.; De Lucas, A.H.; Riaza, L.M.; Pascual, C.M.; Manzañido, J.P.; Quintana, M.S.; Belando, J.E.S. Protocolo de Valoración de La Composición Corporal Para El Reconocimiento Médico-Deportivo. Documento de Consenso Del Grupo Español de Cineantropometría (Grec)de La Federación Española de Medicina Del Deporte (Femede). Versión 2010. *Arch. Med. Deport.* **2010**, *27*, 330–344.
29. Ravasco, P.; Anderson, H.; Mardones, F. Methods of Valuation of the Nutritional Condition. *Nutr. Hosp. Organo Of. Soc. Esp. Nutr. Parenter. Enter.* **2010**, *25*, 57–66.
30. Salvador, G.; Palma, I.; Puchal, A.; Vilà, M.; Miserachs, M.; Illan, M. Entrevista Dietética. Herramientas Útiles Para La Recogida de Datos. *Rev. Med. Univ. Navarra* **2006**, *50*, 46–55. [CrossRef]
31. Morán Fagúndez, L.J.; Rivera Torres, A.; Eugenia González Sánchez, M.; Lourdes De Torres Aured, M.; López-Pardo Martínez, M.; Antonio, J.; Rocamora, I. Historia Dietética. Metodología y Aplicaciones. *Rev. Esp. Nutr. Comunitaria* **2015**, *21*, 53–57. [CrossRef]
32. Marfell-Jones, M.; Olds, T.; Stewart, A.; Lindsay Carter, J. *International Standards for Anthropometric Assessment*; International Society for the Advancement of Kinanthropometry (ISAK): Potchestroom, South Africa, 2006; p. 115.
33. Rocha, M.S. Pesso Ósseo de Brasileiros de Ambos Os Sexos de 17 a 25 Anos. *Arq. Anat. Antropol.* **1975**, *n1*, 445–451.
34. Valensise, H.; Andreoli, A.; Lello, S.; Magnani, F.; Romanini, C.; De Lorenzo, A. Total-Body Skeletal Muscle Mass: Development and Cross-Validation of Anthropometric Prediction Models. *Am. J. Clin. Nutr.* **2000**, *72*, 796–803. [CrossRef]
35. Carter, J.E.L. Body Composition of Montreal Olympic Athletes. In *Physical Structure of Olympic Athletes*; Karger Publishers: Basel, Switzerland, 1982; Volume 16, pp. 107–116.
36. Faulkner, J.A. Physiology of Swimming. *Res. Q. Am. Assoc. Heal. Phys. Educ. Recreat.* **1966**, *37*, 41–54. [CrossRef]
37. Withers, R.T.; Craig, N.P.; Bourdon, P.C.; Norton, K.I. Relative Body Fat and Anthropometric Prediction of Body Density of Male Athletes. *Eur. J. Appl. Physiol. Occup. Physiol.* **1987**, *56*, 191–200. [CrossRef] [PubMed]
38. Akoglu, H. User's Guide to Correlation Coefficients. *Turkish J. Emerg. Med.* **2018**, *18*, 91–93. [CrossRef]
39. Sánchez-Malia, J.M.; Rodiles-Guerrero, L.; Pareja-Blanco, F.; Ortega-Becerra, M. Determinant Factors for Specific Throwing and Physical Performance in Beach Handball. *Sci. Sport.* **2022**, *37*, 141.e1–141.e6. [CrossRef]
40. Richardson, J.T.E. The Analysis of 2 × 2 Contingency Tables-Yet Again. *Stat. Med.* **2011**, *30*, 890-890. [CrossRef]
41. Delavari, S.; Arabshahi, K.S.; Amini, M.; Aalaa, M.; Pourbairamian, G.; Bahoosh, N.; Asadi, N.; Dalal, B.; Kojuri, J.; Hamidi, H.; et al. The Relationship between Experiences Level and Clinical Decision-Making Skill in Midwifery Students: A Cross-Sectional Study. *Med. J. Islam. Repub. Iran* **2022**, *36*, 80. [CrossRef]
42. Dietética, A.; Herramientas, G.Y. Ingestas Dietéticas de Referencia (IDR) Para La Población Española, 2010. *Act. Diet.* **2010**, *14*, 196–197. [CrossRef]

43. Martínez Sanz, J.; Urdampilleta Otegui, A.; Mielgo Ayuso, J. Necesidades Energéticas, Hídricas y Nutricionales En El Deporte. *Eur. J. Hum. Mov.* **2013**, *30*, 37–52.
44. Woods, C.T.; Bruce, L.; Veale, J.P.; Robertson, S. The Relationship between Game-Based Performance Indicators and Developmental Level in Junior Australian Football: Implications for Coaching. *J. Sports Sci.* **2016**, *34*, 2165–2169. [CrossRef] [PubMed]
45. Ferragut, C.; Vila, H.; Abraldes, J.A.; Manchado, C. Influence of Physical Aspects and Throwing Velocity in Opposition Situations in Top-Elite and Elite Female Handball Players. *J. Hum. Kinet.* **2018**, *63*, 23–32. [CrossRef] [PubMed]
46. Baker, L.B.; Heaton, L.E.; Nuccio, R.P.; Stein, K.W. Dietitian-Observed Macronutrient Intakes of Young Skill and Team-Sport Athletes: Adequacy of Pre, during, and Postexercise Nutrition. *Int. J. Sport Nutr. Exerc. Metab.* **2014**, *24*, 166–176. [CrossRef] [PubMed]
47. Burkhart, S.J.; Pelly, F.E. Dietary Intake of Athletes Seeking Nutrition Advice at a Major International Competition. *Nutrients* **2016**, *8*, 638. [CrossRef]
48. Baker, L.B. Sweating Rate and Sweat Sodium Concentration in Athletes: A Review of Methodology and Intra/Interindividual Variability. *Sport Med.* **2017**, *47*, 111–128. [CrossRef]
49. Martínez-Rodríguez, A.; Martínez-Olcina, M.; Hernández-García, M.; Rubio-Arias, J.; Sánchez-Sánchez, J.; Lara-Cobos, D.; Vicente-Martínez, M.; Carvalho, M.J.; Sánchez-Sáez, J.A. Mediterranean Diet Adherence, Body Composition and Performance in Beach Handball Players: A Cross Sectional Study. *Int. J. Environ. Res. Public Health* **2021**, *18*, 1–14. [CrossRef]
50. Sale, C.; Elliott-Sale, K.J. Nutrition and Athlete Bone Health. *Sport Med.* **2019**, *49*, 139–151. [CrossRef]
51. Hertzler, S.R.; Lieblein-Boff, J.C.; Weiler, M.; Allgeier, C. Plant Proteins: Assessing Their Nutritional Quality and Effects on Health and Physical Function. *Nutrients* **2020**, *12*, 1–27. [CrossRef]
52. Cavala, M.; Katić, R. Morphological, Motor and Situation-Motor Characteristics of Elite Female Handball Players According to Playing Performance and Position. *Coll. Antropol.* **2010**, *34*, 1355–1361.
53. Hammami, M.; Gaamouri, N.; Suzuki, K.; Shephard, R.J.; Chelly, M.S. Effects of Upper and Lower Limb Plyometric Training Program on Components of Physical Performance in Young Female Handball Players. *Front. Physiol.* **2020**, *11*, 1028. [CrossRef] [PubMed]
54. Becerra, M.O.; Espina-Agulló, J.J.; Pueo, B.; Jiménez-Olmedo, J.M.; Penichet-Tomás, A.; Sellés-Pérez, S. Anthropometric Profile and Performance Indicators in Female Elite Beach Handball Players. *J. Phys. Educ. Sport* **2018**, *18*, 1155–1160. [CrossRef]

nutrients

MDPI

Article

Optimal Serum 25(OH)D Level and Vitamin D Intake in Young Korean Women

Hye Ran Shin, Hyeon Ju Park and Sun Yung Ly *

Department of Food and Nutrition, Chungnam National University, Daejeon 34134, Republic of Korea
* Correspondence: sunly@cnu.ac.kr; Tel.: +82-42-821-6838; Fax: +82-42-821-8968

Abstract: Vitamin D status is essential for preventing bone disease. Young Korean women have the highest vitamin D deficiency prevalence compared with other demographic groups. This study aimed to establish the optimal vitamin D intake level for maintaining an adequate serum 25-hydroxyvitamin D (25[OH]D) level by season in young Korean women (mean age: 23.1 years). Each participant (wintertime, n = 101; summertime, n = 117) completed a lifestyle survey, dietary record, bone mineral density, and biochemical tests. Seasonal factors impacting 25(OH)D were identified, vitamin D intake for sufficient 25(OH)D levels was calculated, and the relationship between 25(OH)D and intact parathyroid hormone (iPTH) was analyzed. During summertime, 25(OH)D levels were higher than in wintertime (17.9 vs. 15.0 ng/mL). A 1 μg/1000 kcal increase in vitamin D intake increased 25(OH)D levels by 0.170 ng/mL in wintertime and 0.149 ng/mL in summertime. iPTH levels reached a theoretical plateau corresponding to an 18.4 ng/mL 25(OH)D level. The vitamin D intake threshold for maintaining 25(OH)D levels at ≥20 and ≥18.4 ng/mL was ≥10.97 μg/day. For a sufficient level of 25(OH)D in young Korean women, increasing summertime UV irradiation time and increasing vitamin D supplements and vitamin D-containing foods throughout the year is beneficial.

Keywords: vitamin D; bone disease; young Korean women

Citation: Shin, H.R.; Park, H.J.; Ly, S.Y. Optimal Serum 25(OH)D Level and Vitamin D Intake in Young Korean Women. *Nutrients* **2022**, *14*, 4845. https://doi.org/10.3390/nu14224845

Academic Editor: Qian Zhang

Received: 31 October 2022
Accepted: 14 November 2022
Published: 16 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Serum 25-hydroxyvitamin D (25[OH]D) levels are known to reduce the incidence of osteoporosis [1,2]. Osteoporosis is a major cause of poor quality of life in old age and fracture morbidity and mortality rates are very high [3]. Adequate dietary intake of calcium and vitamin D reduces the risk of fractures in older age [4] and high serum 25(OH)D levels decrease the risk of hip fractures [1]; thus, vitamin D supplementation has been reported to reduce the risk of falls [5]. As such, the nutritional status of vitamin D is important for prevention of bone diseases. However, vitamin D status among individuals is known to be poor worldwide [6], and the nutritional status of vitamin D among Koreans is critical [7].

Bone mineral density (BMD) increases during growth, particularly from puberty until the age of 25–35 years, when growth is complete [8]. At this time, peak bone mass is reached and subsequently, aging begins and bone mass is lost [9]. The degree of maximum bone mass and the rate of bone loss are affected by the interaction of genetic, hormonal, environmental, and nutritional factors [10]. In particular, the incidence of fractures and osteoporosis is very high in older women as their bone density decreases rapidly after menopause [11]. As women with higher peak bone mass may have a lower risk of fractures or osteoporosis after menopause [8], bone health in young women is important in relation to quality of life in old age.

Vitamin D also plays a critical role in women's lives during pregnancy. Several studies have shown that vitamin D deficiency is a risk factor for pregnancy- and lactation-related osteoporosis, which can lead to increased musculoskeletal pain, decreased muscle strength, and often severe hip or back pain during pregnancy, which can lead to a decreased quality of life [12,13]. Furthermore, adequate vitamin D nutritional status during pregnancy improves

gestational anemia and is an indicator of fetal nutrition [14]. Consequently, the vitamin D nutritional status of women in their twenties, who are preparing for pregnancy, is crucial.

On the other hand, when the vitamin D level in the blood is insufficient, calcium absorption is reduced. Decreased calcium levels induce parathyroid hormone (PTH) secretion to increase active vitamin D production, thereby increasing calcium absorption. As a result, it promotes bone turnover and causes bone loss. This phenomenon is called secondary hyperparathyroidism and vitamin D deficiency is considered to be the basic mechanism in the pathogenesis of osteoporotic fractures [15]. Many studies have been conducted based on this hypothesis to find the serum 25(OH)D level at which the PTH level is not increased [16–18].

The blood vitamin D level depends on both the amount of vitamin D synthesized under the skin through sunlight and on the intake of vitamin D from food or supplements. As vitamin D synthesis in the skin decreases during the seasons Which offer is less sunlight irradiation, vitamin D intake becomes important for maintenance of blood vitamin D concentration [19–21]. The Dietary Reference Intakes for Koreans 2020 (2020 KDRIs) sets the adequate intake of vitamin D for adults aged 19 to 64 years as 10 μg/day [22]. However, studies that have confirmed the relationship between bone health and vitamin D nutritional status in Korean subjects mainly focused on postmenopausal women and the elderly [23–25], which lacks the evidence criteria for setting adequate vitamin D intake levels for young adults. In 2009, 2009–2011, and 2013–2014 studies using the Korea National Health and Nutrition Examination Survey (KNHANES), it was reported that the vitamin D nutritional status of young Korean women had the highest deficiency prevalence compared with other age groups and men [7,19,26]. Blood tests for vitamin D have not been conducted in KNHANES since 2014; therefore, so studies to follow-up on the vitamin D status of young Korean women is urgently required.

Therefore, the purpose of this study was to identify the serum 25(OH)D levels required to maintain serum PTH levels and prevent bone loss. In addition, a dietary intake survey for the four seasons, a BMD, and blood and urine tests twice a year were analyzed to infer an optimal vitamin D intake for maintenance of an appropriate serum vitamin D level.

2. Materials and Methods

2.1. Study Participants

This study was conducted on women aged 19–29 years living in Daejeon in Korea. Participants were recruited using online community and offline campus bulletin boards. The selection criteria for participants in this study were those whose body mass index (BMI) was in the normal range ($18 \leq$ BMI < 23) and those who consumed regular meals at least twice a day. Patients with chronic, bone, and hormone-related diseases or other health disorders were excluded. Continued drug use, pregnancy, lactation, and mental illness were also exclusion criteria. The study was conducted from September 2020 to August 2021. Informed consent was obtained from all participants who were involved, prior to the start of the study.

The summertime (June–November) attendees were defined as individuals who underwent the biochemical test and completed both dietary intake surveys in autumn and summer; while the wintertime (December–May) attendees were defined as those who underwent the biochemical test and completed both dietary intake surveys in winter and spring. Participants who matched the selection criteria were screened and 140 individuals were recruited for each summertime and wintertime group; however, 24 and 37 participants dropped out from the summertime and wintertime group, respectively. Seventy individuals participated in both summertime and wintertime. Among the 118 summertime and 103 wintertime participants, 1 summertime and 2 wintertime participants with a daily vitamin D intake of $\geq$100 μg were excluded. Finally, a total of 218 young Korean women (117 summertime and 101 wintertime) were included. This study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Chungnam National University (202005-BR-047-01).

2.2. General and Lifestyle Characteristics

The questionnaire on general and lifestyle characteristics was conducted by the self-reporting method once each in wintertime and summertime. The general questions included age and occupation (students, workers, other). Lifestyle characteristics, including ultraviolet (UV) exposure time, smoking status (no: not smoking for over 6 months), alcohol consumption (no: not drinking alcohol for over 6 months), physical activity, number of ways to avoid UV exposure, and vitamin D supplementation, were evaluated. For the UV exposure time, the total time spent in outdoor activities on one day during the week and one day on the weekend was self-recorded. Of these, the outdoor activity time from 10 a.m. to 3 p.m., the time when UV rays are strongest, was calculated in minutes (min), and the average value of UV exposure time for 2 days was used for analysis. Participants were asked to give multiple responses to ways in which they avoid UV exposure, such as sunscreen, cosmetics with UV-blocking effects, hats, facial masks, parasol, and other methods. Physical activity was investigated via the International Physical Activity Questionnaire short form Korean version [27]. Physical activity was classified into three categories by calculated metabolic equivalents of task (MET): low (<600 MET × min per week), moderate (600–3000 MET × min) and high (>3000 MET × min) physical activity. Vitamin D supplements consumed by participants were investigated, including supplement name, vitamin D content per dose, dose size, and daily intake frequency. The intake of vitamin D supplements per day was calculated as follows:

$$\text{vitamin D content per serving} \times \text{daily intake frequency}$$

2.3. Dietary Assessment

For the dietary assessment, participants submitted their dietary records for 3 days, including 2 weekdays and 1 weekend day for each season (Spring: March–May; Summer: June–August; Autumn: September–November; Winter: December–February). Before start of the study, the participants were provided with a video guide on reporting dietary records, and all menu items, ingredients, and amounts consumed by them were recorded in a self-reported form. A ruler of the same length was provided to each participant, which was to be placed on the table while eating and a picture was to be taken. so that they could put it on the table while eating and take a picture. A trained investigator received all the photos, compared them with the participant's self-reported record, confirmed the intake, and analyzed them. Food intake was analyzed using CAN-Pro 5.0 (web version) developed by the Korean Nutrition Society [28]. The vitamin D content of food was analyzed using the vitamin D database (DB) 2.0, published in 2022 [19]. When entering the dietary records of the participants, the food DB of CAN-Pro was used as a basis. However, if a food was not listed on the CAN-pro, the food DB entry was altered to be entered as closely as feasible to the meal composition of the participants, or a new food DB entry was established and utilized for analysis.

2.4. Anthropometry, Bone Turnover Markers (BMTs), and BMD

Anthropometry and biochemical tests were performed on request at the examination center located in Daejeon. Anthropometry was measured using an automatic height measuring device (X-SCAN PLUS II, Jawon Medical Co., Ltd., Seoul, Republic of Korea). For the blood tests, 10 mL of venous blood was collected from the brachial vein on an empty stomach in the morning using a vacutainer serum separating tube (SST, BD Vacutainer, Netherlands). Blood was centrifuged at 3000 rpm for 10 min to separate serum and was stored in a −75 °C cryogenic freezer until analysis. An automated direct competitive chemiluminescent immunoassay (CLIA, ADVIA Centaur Vitamin D Total, Siemens, Washington DC, WA, USA) was used to determine the serum 25(OH)D level, which detects both 25-hydroxyvitamin D2 and D3. This method has been standardized for liquid chromatography with tandem mass spectrometry [29]. In this study, with reference to the value reported by the American Institute of Medicine and the Endocrine Society of the

United States, serum 25(OH)D < 20 ng/mL was defined as deficiency [30,31]. Intact PTH (iPTH) was analyzed by electrochemiluminescence immunoassay (ECLIEA, Elecsyes PTH, Roche, Indianapolis, IN, USA). The reference value of iPTH for women is 15–65 pg/mL. Serum calcium was analyzed by colorimetric method with o-cresolphthalein complexone (oCPC, Clinimate CA, Sekisui Medical Co., Ltd., Tokyo, Japan). The reference value of serum calcium for women is 8.7–10.3 mg/dL. Bone alkaline phosphatase (BAP) was analyzed by chemiluminescence immunoassay (CLIA, Access Ostase Bone Specific Alkaline Phosphatase, Beckman Coulter Diagnostics, Brea, CA, USA). The reference value of BAP for premenopausal women is $\leq$14.0 μg/L. Urine was collected for N-Telopeptide type I collagen (NTX) analysis at 10 mL each, stored in a conical tube at 4 °C, and measured by the CLIA method (VITROS Immunodiagnostic Products NTX Reagent Pack; Ortho-Clinical Diagnostics, Bridgend, Wales, UK). This value is presented in nanomoles of bone collagen equivalents per liter per millimole of creatinine per liter (nM BCE/mM Cr). The reference value of NTX for premenopausal women is 17–94 nM BCE/mM Cr. The posterior anterior lumbar spine (vertebrae L1–L4) was examined using a dual-energy X-ray absorptiometry instrument (ARIA BHR-1-76, GE Healthcare Korea Co., Ltd., Seongnam, Republic of Korea) to measure areal BMD (g/cm^2).

2.5. Statistical Analysis

The results of the study were obtained from the analysis of winter or summertime survey attendees and the total participants who were combined with summertime and wintertime study attendees. For general and lifestyle characteristics, seasonal nutrient intake was calculated by mean ± standard error of mean for continuous variables and proportions were calculated for categorical variables. The scatter plot and mean were calculated for the results of seasonal serum and urine analysis (serum 25[OH]D, iPTH, BAP, Calcium, urine NTX, and BMD). To verify the difference between wintertime and summertime results, the Student's *t*-test for independent samples was used. The seasonal influencing factors of serum 25(OH)D levels were verified using a stepwise method of multiple linear regression analysis. As independent variables, age, BMI, 10 a.m. to 3 p.m. outdoor time (UV exposure time), occupation, smoking, alcohol consumption, number of ways to avoid UV exposure, physical activity, and vitamin D intake (per 1000 kcal) were used. A stepwise regression was performed, and a p value > 0.10 was used for variable removal.

The relationship between 25(OH)D and iPTH was analyzed using an exponential decay model for total participants. Serum 25(OH)D levels required to achieve plateau iPTH concentrations were calculated using the following method [32,33]:

$$\text{iPTH} = a + b \times \exp(c \times 25[\text{OH}]\text{D})$$

The receiver operating characteristic (ROC) analysis and Youden's index calculation were used to calculate the optimal vitamin D consumption to maintain serum 25(OH)D sufficiency for the whole population. Area under the ROC curve (AUC) and p-values were provided. Pearson's correlation coefficient was used to examine the relationship between serum 25(OH)D levels, BMTs (iPTH, NTX, BAP, and calcium), and BMD.

SPSS software, version 27.0 (IBM Corp., Armonk, NY, USA), was used for statistical analysis and MedCalc® Statistical Software, version 20.111 (MedCalc Software Ltd., Ostend, Belgium), was used to compare the AUCs. Exponential decay was analyzed through GraphPad Prism 9, Version 9.4.0 (GraphPad Software, Inc., San Diego, CA, USA). A p-value < 0.05 was considered statistically significant and all tests were two-sided.

3. Results

3.1. The Status of Participants

Table 1 presents the general and lifestyle characteristics of all the participants. The average age of participants was 23.1 ± 0.16 years and there was no significant difference between wintertime and summertime attendees. There was no significant difference in

height, weight, and BMI between the two groups, and the UV exposure time between 10 a.m. and 3 p.m. was 19.5 min in summertime and 14.5 min in wintertime ($p = 0.052$). There was no significant difference in the distribution between the two groups in occupation, smoking status, alcohol consumption status, number of ways to avoid UV exposure, and physical activity level, however, a greater number of people took vitamin D supplements in the summertime group ($p < 0.05$). The proportion of participants with a serum 25(OH)D level of less than 20 ng/mL was 82.2% (83 attendees) in wintertime and 65.8% (77 attendees) in summertime.

Table 1. General and lifestyle characteristics of the participants.

		Wintertime Attendees	Summertime Attendees	Total Participants	*p*-Value
		(*n* = 101)	(*n* = 117)	(*n* = 218)	
Age (years)		23.2 ± 0.25	23.02 ± 0.22	23.1 ± 0.16	0.527
Height (cm)		162.0 ± 0.56	162.3 ± 0.51	162.1 ± 0.34	0.753
Weight (kg)		56.2 ± 0.96	56.2 ± 0.85	56.2 ± 0.63	0.921
BMI (kg/m^2)		21.5 ± 0.28	21.5 ± 0.25	21.5 ± 0.18	0.853
UV exposure time (min)		14.5 ± 1.82	19.5 ± 1.77	17.2 ± 1.28	0.052
Occupation	Student	70 (69.3)	84 (71.8)	154 (70.6)	0.921
	Worker	19 (18.8)	20 (17.1)	39 (17.9)	
	Other	12 (11.9)	13 (11.1)	25 (11.5)	
Smoking status	Yes	7 (6.9)	9 (7.7)	16 (7.3)	0.830
	No	94 (93.1)	108 (92.3)	202 (92.7)	
Alcohol consumption	Yes	76 (75.2)	88 (75.2)	164 (75.2)	0.995
	No	25 (24.8)	29 (24.8)	54 (24.8)	
Number of ways to avoid UV exposure	Never	11 (10.8)	6 (5.1)	17 (7.8)	0.414
	1	24 (23.8)	29 (24.8)	53 (24.3)	
	2	33 (32.7)	37 (31.6)	70 (32.1)	
	≥3	33 (32.7)	45 (38.5)	78 (35.8)	
Physical activity	Low	40 (39.6)	57 (48.7)	97 (44.5)	0.401
	Moderate	41 (40.6)	40 (34.2)	81 (37.2)	
	High	20 (19.8)	20 (17.1)	40 (18.3)	
Taking vitamin D supplements	Yes	19 (18.8)	36 (30.8)	55 (25.2)	0.043
	No	82 (81.2)	81 (69.2)	163 (74.8)	
Prevalence of 25(OH)D deficiency	Deficiency (<20 ng/mL)	83 (82.2)	77 (65.8)	160 (73.4)	0.006
	Sufficiency (≥20 ng/mL)	18 (17.8)	40 (34.2)	58 (26.6)	

Mean ± SEM or N(%); UV; Ultraviolet; UV exposure time: UV rays were strongest between 10 a.m. and 3 p.m.; Students: college students or higher; worker: full-time worker; other: unemployed or part-time workers; Physical activity: Low, <600 metabolic equivalents of task [MET] × min per week; moderate, 600–3000 MET × min per week; High, >3000 MET × min.

3.2. Serum Levels of 25(OH)D, Calcium, BMTs, and BMD

Figure 1 shows the results of the bone biomarkers of study participants and the difference between the two seasons. The mean serum 25(OH)D level was significantly higher ($p < 0.01$) in summertime (17.9 ± 0.61 ng/mL) than in wintertime (15.0 ± 0.64 ng/mL). iPTH levels was significantly higher in wintertime (43.9 ± 1.40 pg/mL) than in summertime (33.78 ± 0.84 pg/mL, $p < 0.001$). NTX levels were significantly higher ($p < 0.001$) in wintertime (41.0 ± 1.51 nM BCE/mM Cr) than in summertime (30.18 ± 1.16 nM BCE/mM Cr). However, BAP (10.52 ± 0.32 μg/L for wintertime, 10.68 ± 0.37 μg/L for summertime), serum calcium (9.02 ± 0.02 mg/dL for wintertime, 9.05 ± 0.02 mg/dL for summertime),

and BMD (1.18 ± 0.11 g/cm^2 for wintertime, 1.17 ± 0.01 g/cm^2 for summertime) did not differ significantly between the two seasons.

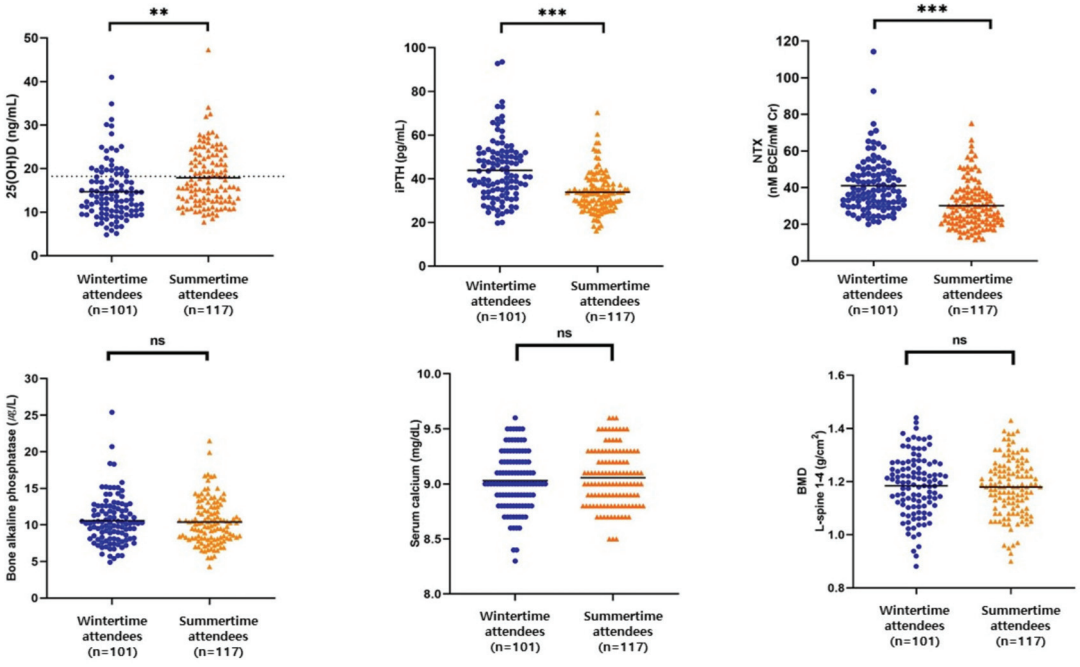


Figure 1. Scatter plots of bone turnover markers and bone mineral density. Solid line: mean value, dotted line on the 25(OH)D graph: 20 ng/mL; iPTH, intact parathyroid hormone; NTX, type I collagen cross-linked N-telopeptide; BMD, bone mineral density; *** $p < 0.001$, ** $p < 0.01$, ns: not significant.

3.3. Seasonal Nutrient Intake

There were no significant differences in total energy intake, calcium intake, vitamin D intake from foods, and vitamin D intake through supplements by season (Table 2). In this study, dietary intake of vitamin D was determined according to a previous research which showed that the contribution of food groups from fish and shellfish, eggs, meat and meat products, milk and dairy products, mushrooms, and grains and grain products to vitamin D intake in Koreans is 99% [19]. In both seasons, dietary intake of vitamin D from fish and shellfish was the highest (contribution to vitamin D intake was 64.9% in wintertime and 54.4% in summertime); however, there was no significant difference in vitamin D intake from all major food groups between the two seasons groups.

3.4. Multiple Linear Regression of Factors Affecting Serum 25(OH)D Level by Season

As a result of multiple linear regression analysis of factors affecting serum 25(OH)D levels in the 218 total participants, the influencing factors of vitamin D intake including supplements per 1000 kcal, ways to avoid UV exposure, and UV exposure time were chosen as variables (Table 3). Serum 25(OH)D levels increased by 0.170 ng/mL for every 1 μg/1000 kcal increase in vitamin D intake ($p < 0.001$). The serum 25(OH)D levels decreased by 1.577 ng/mL for each increase in the participants' number of ways to avoid UV exposure ($p < 0.001$), whereas the serum 25(OH)D levels increased by 0.056 ng/mL for every 1 min increase in UV exposure time ($p < 0.05$). In wintertime, vitamin D intake was the only factor affecting serum 25(OH)D level and in summertime, vitamin D intake, number of ways to avoid UV exposure, and UV exposure time were analyzed as influencing factors. When vitamin D intake was increased by 1 μg/1000 kcal, serum 25(OH)D levels

increased by 0.170 ng/mL and 0.147 ng/mL in wintertime and summertime, respectively ($p < 0.05$). When the number of ways to avoid UV exposure in summertime increased, the serum 25(OH)D level decreased by 2.559 ng/mL ($p < 0.001$) and when the UV exposure time was increased by 1 min, the serum 25(OH)D level increased by 0.059 ng/mL ($p < 0.05$).

Table 2. Energy, calcium, and vitamin D intake in wintertime and summertime.

	Wintertime Attendees	Summertime Attendees	Total Participants	*p*-Value
Dietary intake of nutrients (*n* = 218)				
Energy (kcal/day)	1390.5 ± 37.0	1458.8 ± 36.9	1427 ± 26.2	0.195
Calcium (mg/day)	533.8 ± 23.0	572.46 ± 21.4	554.55 ± 15.7	0.220
Vitamin D (μg/day)	3.19 ± 0.36	3.09 ± 0.29	3.14 ± 0.22	0.827
Dietary intake of vitamin D rich food groups and contribution (μg/day, % [§])				
Fish and shellfish	2.07 ± 0.35 (64.9)	1.68 ± 0.28 (54.4)	1.86 ± 0.22 (59.2)	0.393
Eggs	0.64 ± 0.06 (20.1)	0.73 ± 0.04 (23.6)	0.69 ± 0.04 (22.0)	0.248
Meat and meat products	0.24 ± 0.01 (7.5)	0.33 ± 0.06 (10.7)	0.29 ± 0.04 (9.2)	0.196
Milk and dairy products	0.12 ± 0.03 (3.8)	0.11 ± 0.02 (3.6)	0.12 ± 0.02 (3.8)	0.913
Mushrooms	0.08 ± 0.01 (2.5)	0.10 ± 0.01 (3.2)	0.09 ± 0.01 (2.9)	0.321
Grain and grain products	0.04 ± 0.02 (1.3)	0.06 ± 0.02 (1.9)	0.05 ± 0.02 (1.6)	0.602
Total vitamin D intake (μg/day)				
With supplements [†] (*n* = 55)	24.7 ± 3.22	19.2 ± 2.70	21.1 ± 2.10	0.218
Without supplements [‡] (*n* = 163)	3.32 ± 0.41	3.17 ± 0.36	3.25 ± 0.27	0.789
Overall (*n* = 218)	7.34 ± 1.07	8.10 ± 1.10	7.75 ± 0.77	0.998

Mean ± SEM; [§] Percentage of total dietary vitamin D intake contributions; [†] Taking vitamin D supplements: participants taking vitamin D supplements (wintertime, *n* = 19; summertime, *n* = 36); [‡] Not taking vitamin D supplements: participants not taking vitamin D supplements (wintertime, *n* = 82; summertime, *n* = 81).

Table 3. Regression coefficients for the association between serum 25(OH)D levels and variables by season.

Season	Variable	Regression Coefficient	95% CI	*p* Value
Total participants	Vitamin D intake (with supplement, μg/1000 kcal)	0.170	0.078, 0.263	<0.001
	Way to avoid UV exposure	−1.577	−2.453, −0.702	<0.001
	UV exposure time (min)	0.056	0.012, 0.101	0.013
Wintertime attendees	Vitamin D intake (with supplement, μg/1000 kcal)	0.170	0.030,0.310	0.018
Summertime attendees	Way to avoid UV exposure	−2.559	−3.758, −1.360	<0.001
	Vitamin D intake (with supplement, μg/1000 kcal)	0.147	0.028, 0.266	0.016
	UV exposure time (min)	0.059	0.003, 0.115	0.039

UV exposure time: UV rays were strongest between 10 a.m. and 3 p.m.

3.5. Exponential Decay for Optimal Serum 25(OH)D to Suppress PTH Elevation

As a result of calculating the changes in 25(OH)D and iPTH using an exponential decay function, iPTH increased when the 25(OH)D concentration decreased to 18.44 ng/mL or less (Figure 2). Our resulting equation was as follows:

$$\text{iPTH} = 32.77 + 51.86 \times \exp(-0.1580 \times 25[\text{OH}]\text{D})$$

3.6. ROC Analysis for Optimal Vitamin D Intake at Serum 25(OH)D ≥20 and ≥18.44 ng/mL

The ROC analysis was performed to determine the threshold of vitamin D intake to maintain serum 25(OH)D levels above 20 ng/mL (Figure 3). In this case, the optimal vitamin D intake criterion was 10.97 μg/day (AUC = 0.621, $p < 0.01$). Additionally, the threshold for vitamin D intake based on the serum 25(OH)D level of 18.44 ng/mL analyzed in this study was 10.97 μg/day (AUC = 0.642, $p < 0.001$).

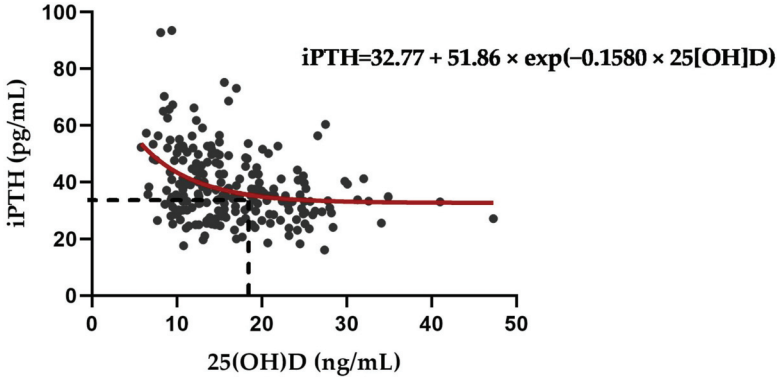


Figure 2. Nonlinear regression between serum 25(OH)D and intact PTH levels in total participants. Serum 25(OH)D levels required to achieve plateau iPTH concentrations were calculated using the following equation: iPTH = a + b × exp (c × 25[OH]D). iPTH increased when the 25(OH)D concentration decreased to 18.44 ng/mL or less.

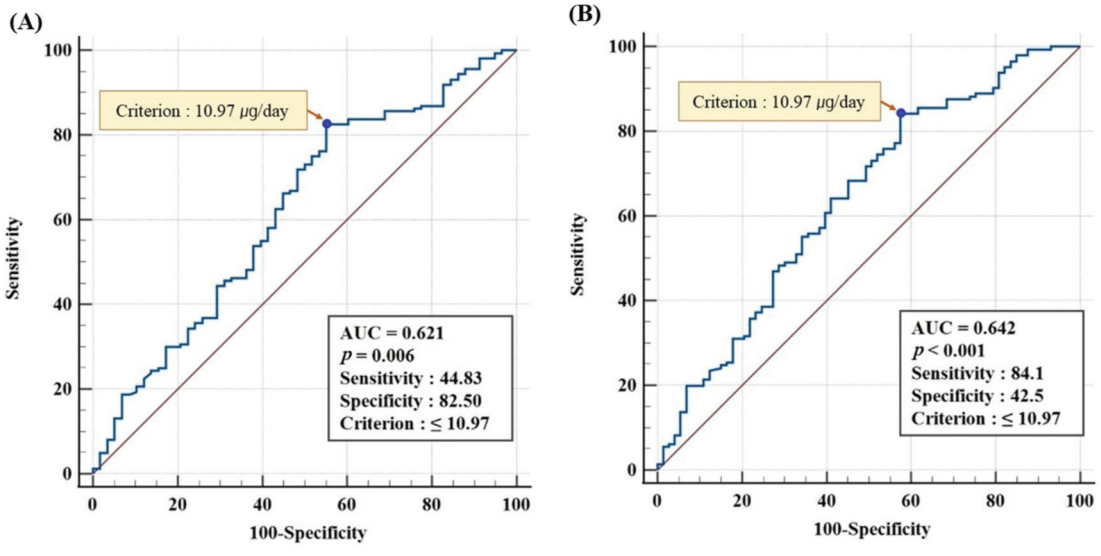


Figure 3. ROC analysis for assessing vitamin D intake at serum 25(OH)D levels ≥ 20 ng/mL (**A**) and ≥18.44 ng/mL (**B**). The vitamin D deficiency cutoff value, as per the American Institute of Medicine and the Endocrine Society of the United States, is 20 ng/mL (**A**) and the appropriate serum 25(OH)D level obtained from the total participants of this study was 18.44 ng/mL (**B**).

3.7. Correlation between Variables

Serum 25(OH)D levels in both, total participants and the wintertime attendee cohort correlated negatively with iPTH ($p < 0.001$), but positively with serum calcium levels in summertime ($p < 0.05$, Table 4). In all cases, NTX and BAP were positively correlated ($p < 0.001$) and serum calcium levels in total participants and wintertime attendees were negatively correlated with iPTH ($p < 0.05$). However, serum 25(OH)D levels and all BMTs were not associated with BMD.

Table 4. Correlation between serum 25(OH)D levels and bone turnover marker levels.

Season	Variables	iPTH	NTX	BAP	Serum Calcium	BMD (L1-4)
Total participants	25(OH)D	−0.302 ***	−0.095	−0.070	0.092	−0.045
	iPTH		0.132	−0.056	−0.134 *	0.053
	NTX			0.480 ***	0.025	−0.087
	BAP				0.037	−0.133
	Serum calcium					−0.010
Wintertime attendees	25(OH)D	−0.335 ***	−0.072	−0.109	−0.037	0.011
	iPTH		−0.058	−0.074	−0.202 *	0.068
	NTX			0.589 ***	−0.063	−0.151
	BAP				−0.083	−0.187
	Serum calcium					0.013
Summertime attendees	25(OH)D	−0.134	0.036	−0.056	0.183 *	−0.086
	iPTH		0.052	−0.033	−0.018	0.015
	NTX			0.490 ***	0.169	−0.053
	BAP				0.116	−0.094
	Serum calcium					−0.029

Pearson's correlation coefficient. iPTH, intact parathyroid hormone; NTX, type I collagen cross-linked N-telopeptide; BMD, bone mineral density; L1–4, lumbar spine 1–4; *** $p < 0.001$, * $p < 0.05$.

4. Discussion

Worldwide, vitamin D deficiency is a serious concern for all ages. Poor nutritional status of vitamin D in young women is a risk factor for post-menopausal fractures and osteoporosis [34]. Based on KNHANES, the serum 25(OH)D levels was found to be the lowest in women aged 19 to 29 years among all age groups; values were at 4.71 ng/mL in 2009 [26], 13.73 ng/mL in 2009–2011 [7], and 13.85 ng/mL in 2013–2014 [19]. In addition, their intake of vitamin D was 2.48 μg in 2009 [26], 2.77 μg in 2009–2011 [7], and 2.48 μg in 2013–2014 [19], which accounted for only 25% of the 2020 KDRIs vitamin D adequate intake value of 10 μg. Even though vitamin D deficiency is a national problem, blood vitamin D has not been measured in KNHANES since 2014. Therefore, this study was carried out to investigate the factors influencing vitamin D nutritional status in young Korean women aged 19–29 years in a metropolitan area, and to determine the vitamin D intake level required to maintain an optimal level of serum 25(OH)D.

The main contributing factor to blood vitamin D levels is the vitamin D synthesis under the skin caused by UV irradiation. In this study, the time spent outdoors during the daytime from 10 a.m. to 3 p.m. was found to be about 17.2 ± 1.28 min in young Korean women. The serum 25(OH)D level increased by 0.056 ng/mL when the UV exposure time was increased by 1 min in this study. A study conducted by Asakura et al. [20] in Japan showed that increasing UV exposure time in summer by 10 min increased serum 25(OH)D_3 level by 0.47 ng/mL. Another study conducted by Goswami et al. [35] on Indian men in the summertime (August to September) found that serum 25(OH)D level increased by 2.03 ng/mL per hour of sun exposure, showing similar results to this study.

In addition, serum 25(OH)D levels decreased by 1.57 ng/mL as the number of ways to avoid UV exposure increased. However, none of the variables associated with UV exposure in wintertime had a statistically significant effect on serum vitamin D levels. Under the skin, 7-dihydrocholesterol is synthesized effectively on exposure to UVB radiation in the 290–315 nm wavelength range. Korea lies between 33° and 38° north latitude and winter UVB radiation is only a quarter of summer levels [36]. Park et al. [37], who conducted a study on the UV exposure time required for vitamin D synthesis in Koreans, stated that the threshold exposure time for vitamin D synthesis in summer was less than 30 min, but more than 100 min in winter. It was also reported that sufficient vitamin D synthesis was impossible with UV exposure alone in winter. In particular, in the northern hemisphere, winter temperatures are low; therefore, people wear long sleeves and pants. As a result,

skin is less exposed to UV radiation during outdoor activities, which also contributes to vitamin D deficiency.

Aside from UV exposure time, daily vitamin D intake, including supplement intake, was discovered to be an influential variable in this study. In total participants, it was found that the vitamin D intake increased by 1 μg/1000 kcal with serum vitamin D level increase of 0.170 ng/mL These effects of vitamin D intake were also shown in wintertime study. In a study conducted on the Japanese population, the effect of vitamin D intake on serum vitamin D level was reported to be higher than that observed in our study; an increase in vitamin D intake of 1 μg/1000 kcal increased serum 25(OH)D_3 by 0.88 ng/mL in summer and 1.7 ng/mL in winter [20]. However, in a study that confirmed the serum 25(OH)D level as a result of vitamin D supplementation, when vitamin D was additionally consumed at 1000 IU/day (25 μg/day), serum 25(OH)D increased by 12 nmol/L (4.81 ng/mL) [38]. Another meta-analysis of the relationship between vitamin D-fortified foods and serum 25(OH)D levels revealed that intake of 100 IU/day (2.5 μg/day) dietary vitamin D could increase serum 25(OH)D by 2 nmol/L (0.80 ng/mL) [39]. Researchers report the effects of vitamin D intake on blood vitamin D levels slightly differently. In the latter two studies, the effects of vitamin D intake on serum vitamin D (0.19 ng/mL and 0.32 ng/mL of serum 25(OH)D per μg of vitamin D intake, respectively) were similar to or slightly higher than the results of this study. Previous research on Koreans has shown that vitamin D intake has a positive effect on serum 25(OH)D levels [7,26]. A study conducted on Koreans that confirmed a positive correlation between vitamin D intake and serum 25(OH)D level during low UV irradiation, pointed out the importance of vitamin D intake in winter [19]. In this study, the effect of vitamin D intake on serum 25(OH)D levels was greater in wintertime, thereby, supporting the results of previous studies.

In the KNHANES, serum vitamin D levels were measured only between 2008–2014. The average serum 25(OH)D concentration of Korean women aged 19–29 years in the 2013–2014 KNHANES was 13.85 ng/mL [19], which was lower than the 16.54 ng/mL for the total participants in this study. Expert groups, such as the American Institute of Medicine and the Endocrine Society of the United States, recommend maintaining serum 25(OH)D levels at 20 ng/mL [30,31]; however, research about young women in Korea has been limited. In this study, the serum 25(OH)D level, which showed a sharp increase in blood iPTH, was 18.44 ng/mL or less. This was similar to or slightly higher than the 46.9 nmol/L (18.79 ng/mL) [16] for Croatian women and the 44 nmol/L (17.63 ng/mL) [17] for African American women. As renal function declines with age, higher 25(OH)D levels are required in old age to prevent serum PTH elevation [17,40]. Therefore, it is considered desirable to maintain the serum vitamin D levels of young Korean women slightly above the results of this study.

In order to maintain the optimal serum 25(OH)D level of 18.44 ng/mL based on the iPTH level in this study, at least 10.97 μg/day of vitamin D should be ingested. This was the same even based on the vitamin D deficiency threshold of 20 ng/mL suggested by the American Institute of Medicine [30] and the Endocrine Society of the USA [31]. The ROC curve showed that the 18.44 ng/mL serum 25(OH)D level was closer to the upper left and had a greater AUC; hence, it was a stronger predictor of vitamin D intake than the 20 ng/mL serum 25(OH)D level. The optimal intake level for summertime attendees was found to be 10.97 μg/day, but no significant results were obtained for wintertime attendees (data not shown). As most participants' serum 25(OH)D levels were very low in the wintertime, the ROC analysis had little meaning. In a study conducted on young Japanese women, it was found that the optimal vitamin D intake for maintaining a serum 25(OH)D level of 20 ng/mL was 11.6 μg/day, which was slightly higher than the level found in this study [41]. The survey period of this Japanese study was December-January and data from summer with higher UV irradiation were not included. As Japan is located in the southern part of the Northern Hemisphere than of Korea, the vitamin D requirement may have been lower if data from the summer survey were included. According to the 2020 KDRIs, the adequate intake of vitamin D for young adult women was set at 10 μg/day. As

results (10.97 μg/day) for the optimal vitamin D intake of young Korean women derived from our study depended on the summer survey data, it is thought that adequate annual intake of vitamin D should be higher, considering vitamin D intake in winter when sunlight is scarce.

In order to reduce the risk of age-related BMD loss, it is crucial to begin consumption of sufficient calcium and vitamin D at a young age [42,43]. Although the average intake of vitamin D in total participants of our study was only 3.14 μg/day, the average vitamin D intake from food and supplements was 21.1 μg/day for the 55 participants, which was more than 2-folds higher than the 2020 KDRIs of vitamin D (10 μg/day). A previous study that analyzed the KNHANES found that vitamin D intake in Korean women aged 19–29 years was 2.48 μg/day, which was lower than the vitamin D intake of the participants of this study [19]. The daily calcium intake of the subjects in this study was 554.6 mg, which was higher than the calcium intake of 439.14 mg/day in Korean women aged 19–29 years, according to the 2020 KNHANES [42], but far below the 2020 KDRIs (700 mg/day) [22]. In a study on 2011 KNHANES, the average lumbar BMD of 19–29-year-old women was 0.97 ± 0.01 g/cm^2, which was slightly higher than that of the subjects in this study [44]. In a study on the effect of combined calcium and vitamin D supplementation on BMD, it was reported that the increase in BMD was significantly higher than that of the control group when calcium and vitamin D were supplemented together in healthy young women aged 16–36 years [45]. In a study on 2009 KNHANES, the effect of vitamin D intake on BMD was better in the low calcium intake group [23]. The subjects in the previous study were Korean women over the age of 50. Several other studies have also shown that vitamin D intake becomes more crucial in groups with low calcium intake [46,47]. Given that young Korean women consume more dairy products than older women, their calcium intake can be increased easily, but not dietary vitamin D intake [48]. Vitamin D fortification in food is not yet mandatory in Korea; therefore, it must be implemented soon.

Higher NTX and iPTH concentrations in young Korean women in wintertime than in summertime suggest that bone remodeling occurs more in wintertime. Previous studies have reported that BTMs increase in winter when vitamin D nutritional status is poor [49,50]. As this study included young females in whom bone remodeling is continuously observed, the relationship with BMD may not have been observed. In this study, there was no statistically significant correlation between BTMs and BMD as in some previous studies on young adult women [51,52]. BTMs provide a measure of overall bone turnover, while BMD measures specific areas of the skeletal system. Elevated BTMs are associated with increased bone turnover, which cause deteriorated bone quality and increased risk of fracture [53]. BTMs are negatively correlated with BMD, which is known to increase with age [54,55].

This study is the first to determine the optimal serum 25(OH)D level for women in their twenties in Korea and the optimal vitamin D intake level in Korea. This study had the additional strength of identifying seasonal factors that influence serum 25(OH)D levels. However, there were some limitations to this study. First of all, as this study was conducted on a relatively small number of women living in a major Korean city, there may have been a problem with the representativeness of the sample. Approximately 70% of the study participants were college students, came from various provinces of Korea, and had a slightly higher economic level than that the average of young women in Korea. Secondly, due to the COVID-19 pandemic, a reduction in time spent outdoors may have affected the serum 25(OH)D level in the study participants. Therefore, the situation might have differed slightly from that at the time of a normal life pattern. Thirdly, because BMD was only measured at the lumbar spine, correlations with BMD measured at other locations could not be confirmed. However, one study showed that bone density in different regions are correlated with each other, and the correlation is greater with younger age [56].

5. Conclusions

It was confirmed that the serum 25(OH)D levels for the prevention of bone loss is 18.44 ng/mL or higher and vitamin D intake should be higher than 10.97 μg/day to prevent

vitamin D deficiency in young Korean women. It seems reasonable to use the current level of 25(OH)D > 20 ng/mL as the KDRI for vitamin D as a target for bone health in young Korean women. However, as the current intake of vitamin D in young Korean women was very low, the intake of vitamin D must be increased by at least three times the current levels. To do this, the Korean government should implement a mandatory fortification for vitamin D in food, which should be argued prior to vitamin D supplementation to improve Korea's national nutrition status. In countries with vitamin D food fortification policies, vitamin D fortification is approximately 22–200 IU per serving of food [57]. Based on this standard, if Korea sets the vitamin D fortification standard to 5 μg/serving or less in foods such as grains, dairy products, and fruit juice, even making allowance for supplement intake or duplicate intake of food, in is unlikely that the standard upper limit of 100 μg/day vitamin D intake for adults aged 19 years or older would be exceeded.

Author Contributions: Conceptualization, H.R.S. and S.Y.L.; methodology, H.R.S.; software, H.R.S. and H.J.P.; validation, H.R.S., H.J.P. and S.Y.L.; formal analysis, H.R.S.; investigation, H.R.S. and H.J.P.; resources, H.R.S. and S.Y.L.; data curation, H.R.S. and H.J.P.; writing—original draft preparation, H.R.S. and H.J.P.; writing—review and editing, H.R.S. and S.Y.L.; visualization, H.R.S.; supervision, S.Y.L.; project administration, S.Y.L.; funding acquisition, H.R.S. and S.Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation of Korea Grant funded by the Korean government (Ministry of Science and ICT), grant number NRF-2020R1A2C1009608 and WISET-2022-496.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Chungnam National University (approval no. 202005-BR-047-01, 14 July 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, Q.; Yu, D.; Wang, J.; Lin, S. Association between vitamin D deficiency and fragility fractures in Chinese elderly patients: A cross-sectional study. *Ann. Palliat. Med.* **2020**, *9*, 1660–1665. [CrossRef] [PubMed]
2. Bischoff-Ferrari, H.; Can, U.; Staehelin, H.; Platz, A.; Henschkowski, J.; Michel, B.; Dawson-Hughes, B.; Theiler, R. Severe vitamin D deficiency in Swiss hip fracture patients. *Bone* **2008**, *42*, 597–602. [CrossRef] [PubMed]
3. Zanker, J.; Duque, G. Osteoporosis in Older Persons: Old and New Players. *J. Am. Geriatr. Soc.* **2019**, *67*, 831–840. [CrossRef] [PubMed]
4. Rizzoli, R. Nutrition: Its role in bone health. *Best Pract. Res. Clin. Endocrinol. Metab.* **2008**, *22*, 813–829. [CrossRef] [PubMed]
5. Wu, H.; Pang, Q. The effect of vitamin D and calcium supplementation on falls in older adults. *Der Orthopäde* **2017**, *46*, 729–736. [CrossRef]
6. Amrein, K.; Scherkl, M.; Hoffmann, M.; Neuwersch-Sommeregger, S.; Köstenberger, M.; Berisha, A.T.; Martucci, G.; Pilz, S.; Malle, O. Vitamin D deficiency 2.0: An update on the current status worldwide. *Eur. J. Clin. Nutr.* **2020**, *74*, 1498–1513. [CrossRef]
7. You, H.; Shin, H.R.; Song, S.; Ly, S.Y. Vitamin D intake and bone mineral density in Korean adults: Analysis of the 2009–2011 Korea National Health and Nutrition Examination Survey. *Nutr. Res. Pract.* **2021**, *16*, e19.
8. Chew, C.K.; Clarke, B.L. Causes of low peak bone mass in women. *Maturitas* **2018**, *111*, 61–68. [CrossRef]
9. Matkovic, V. Calcium and peak bone mass. *J. Intern. Med.* **1992**, *231*, 151–160. [CrossRef]
10. Kelly, P.J.; Eisman, J.A.; Sambrook, P.N. Interaction of genetic and environmental influences on peak bone density. *Osteoporos. Int.* **1990**, *1*, 56–60. [CrossRef]
11. Richelson, L.S.; Wahner, H.W.; Melton III, L.; Riggs, B.L. Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. *New Engl. J. Med.* **1984**, *311*, 1273–1275. [CrossRef] [PubMed]
12. Qian, Y.; Wang, L.; Yu, L.; Huang, W. Pregnancy- and lactation-associated osteoporosis with vertebral fractures: A systematic review. *BMC Musculoskelet. Disord.* **2021**, *22*, 926. [CrossRef] [PubMed]
13. Woo, J.; Penckofer, S.; Fagan, M.; Giurgescu, C. Associations between Pregnancy-Related Symptoms, Serum 25(OH)D, and Physical Quality of Life in Pregnant Women. *Nutrients* **2022**, *14*, 482. [CrossRef] [PubMed]

14. Zhang, Y.; Jukic, A.M.Z.; Song, H.; Zhang, L.; Yang, F.; Wu, S.; Yin, D.; Jiang, H. Serum Vitamin D Concentrations, Time to Pregnancy, and Pregnancy Outcomes among Preconception Couples: A Cohort Study in Shanghai, China. *Nutrients* **2022**, *14*, 3058. [CrossRef]
15. Fischer, V.; Haffner-Luntzer, M.; Amling, M.; Ignatius, A. Calcium and vitamin D in bone fracture healing and post-traumatic bone turnover. *Eur. Cell. Mater.* **2018**, *35*, 365–385. [CrossRef]
16. Keser, I.; Cvijetić, S.; Bituh, M.; Rumora Samarin, I.; Ilich, J.Z.; Colić Barić, I.; Rumbak, I. Vitamin D and parathyroid hormone in relation to bone health in Croatian women. *Arch. Osteoporos.* **2018**, *13*, 69. [CrossRef] [PubMed]
17. Aloia, J.F.; Talwar, S.A.; Pollack, S.; Feuerman, M.; Yeh, J.K. Optimal vitamin D status and serum parathyroid hormone concentrations in African American women. *Am. J. Clin. Nutr.* **2006**, *84*, 602–609. [CrossRef]
18. AlQuaiz, A.M.; Mujammami, M.; Kazi, A.; Hasanato, R.M.; Alodhayani, A.; Shaik, S.A.; Al-Daghri, N.M. Vitamin D cutoff point in relation to parathyroid hormone: A population based study in Riyadh city, Saudi Arabia. *Arch. Osteoporos.* **2019**, *14*, 22. [CrossRef]
19. Shin, H.R.; Park, H.J.; Song, S.; Ly, S.Y. Dietary vitamin D intake in low ultraviolet irradiation seasons is associated with a better nutritional status of vitamin D in Korean adults according to the 2013–2014 National Health and Nutrition Examination Survey. *Nutr. Res.* **2022**, *105*, 53–65. [CrossRef]
20. Asakura, K.; Etoh, N.; Imamura, H.; Michikawa, T.; Nakamura, T.; Takeda, Y.; Mori, S.; Nishiwaki, Y. Vitamin D status in Japanese adults: Relationship of serum 25-hydroxyvitamin D with simultaneously measured dietary vitamin D intake and ultraviolet ray exposure. *Nutrients* **2020**, *12*, 743. [CrossRef]
21. Fayet-Moore, F.; Brock, K.E.; Wright, J.; Ridges, L.; Small, P.; Seibel, M.J.; Conigrave, A.D.; Mason, R.S. Determinants of vitamin D status of healthy office workers in Sydney, Australia. *J. Steroid Biochem. Mol. Biol.* **2019**, *189*, 127–134. [CrossRef] [PubMed]
22. The Korean Nutrition Society. *Dietary Reference Intakes for Koreans 2020*; Ministry of Health and Welfare; The Korean Nutrition Society: Sejong, Republic of Korea, 2020.
23. Yoo, K.-O.; Kim, M.-J.; Ly, S.Y. Association between vitamin D intake and bone mineral density in Koreans aged $\geq$ 50 years: Analysis of the 2009 Korea National Health and Nutrition Examination Survey using a newly established vitamin D database. *Nutr. Res. Pract.* **2019**, *13*, 115. [CrossRef] [PubMed]
24. Yoon, D.S.; Lee, Y.-K.; Ha, Y.-C.; Kim, H.-Y. Inadequate dietary calcium and vitamin D intake in patients with osteoporotic fracture. *J. Bone Metab.* **2016**, *23*, 55–61. [CrossRef] [PubMed]
25. Lee, Y.-K.; Chang, J.-S.; Min, Y.-K.; Byun, D.-W.; Park, Y.; Ha, Y.-C. Low calcium and vitamin D intake in Korean women over 50 years of age. *J. Bone Miner. Metab.* **2017**, *35*, 522–528. [CrossRef] [PubMed]
26. Yoo, K.; Cho, J.; Ly, S. Vitamin D intake and serum 25-hydroxyvitamin D levels in korean adults: Analysis of the 2009 Korea national health and nutrition examination survey (KNHANES IV-3) using a newly established vitamin D database. *Nutrients* **2016**, *8*, 610. [CrossRef]
27. Oh, J.Y.; Yang, Y.J.; Kim, B.S.; Kang, J.H. Validity and Reliability of Korean Version of International Physical Activity Questionnaire (IPAQ) Short Form. *Korean J. Fam. Med.* **2007**, *28*, 532–541.
28. The Korean Nutrition Society. Computer Aided Nutritional Analysis Program; CAN-Pro 5.0 (Web Ver.). Available online: http://canpro5.kns.or.kr/ (accessed on 25 August 2022).
29. Chen, Y.; Kinney, L.; Božović, A.; Smith, H.; Tarr, H.; Diamandis, E.P.; LeBlanc, A. Performance Evaluation of Siemens ADVIA Centaur and Roche MODULAR Analytics E170 Total 25-OH Vitamin D Assays. *Clin. Biochem.* **2012**, *45*, 1485–1490. [CrossRef]
30. Del Valle, H.B.; Yaktine, A.L.; Taylor, C.L.; Ross, A.C. Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium. In *Dietary Reference Intakes for Calcium and Vitamin D*; National Academies Press: Washington, DC, USA, 2011.
31. Holick, M.F.; Binkley, N.C.; Bischoff-Ferrari, H.A.; Gordon, C.M.; Hanley, D.A.; Heaney, R.P.; Murad, M.H.; Weaver, C.M.; Endocrine Society. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* **2011**, *96*, 1911–1930. [CrossRef]
32. Guillemant, J.; Taupin, P.; Le, H.T.; Taright, N.; Allemandou, A.; Pérès, G.; Guillemant, S. Vitamin D status during puberty in French healthy aale adolescents. *Osteoporos. Int.* **1999**, *10*, 222–225. [CrossRef]
33. Chapuy, M.C.; Preziosi, P.; Maamer, M.; Arnaud, S.; Galan, P.; Hercberg, S.; Meunier, P.J. Prevalence of Vitamin D Insufficiency in an Adult Normal Population. *Osteoporos. Int.* **1997**, *7*, 439–443. [CrossRef]
34. Holick, M.F. Vitamin D deficiency. *New Engl. J. Med.* **2007**, *357*, 266–281. [CrossRef] [PubMed]
35. Dharmshaktu, P.; Saha, S.; Kar, P.; Sreenivas, V.; Ramakrishnan, L.; Goswami, R. Absence of vitamin D deficiency among common outdoor workers in Delhi. *Clin. Endocrinol.* **2019**, *91*, 356–362. [CrossRef]
36. Kwak, M.K.; Kim, J.H. The Radiative Characteristics of EUV-B over the Korean Peninsula and Exposure Time for Synthesizing Adequate Vitamin D. *Atmosphere* **2011**, *21*, 123–130.
37. Park, S.S.; Lee, Y.G.; Kim, M.; Kim, J.; Koo, J.-H.; Kim, C.K.; Um, J.; Yoon, J. Simulation of threshold UV exposure time for vitamin D synthesis in South Korea. *Adv. Meteorol.* **2019**, *2019*, 4328151. [CrossRef]
38. Ekwaru, J.P.; Zwicker, J.D.; Holick, M.F.; Giovannucci, E.; Veugelers, P.J. The importance of body weight for the dose response relationship of oral vitamin D supplementation and serum 25-hydroxyvitamin D in healthy volunteers. *PLoS ONE* **2014**, *9*, e111265. [CrossRef] [PubMed]

39. Nikooyeh, B.; Neyestani, T.R. The effects of vitamin D-fortified foods on circulating 25(OH)D concentrations in adults: A systematic review and meta-analysis. *Br. J. Nutr.* **2022**, *127*, 1821–1838. [CrossRef]
40. Dawson-Hughes, B.; Heaney, R.P.; Holick, M.F.; Lips, P.; Meunier, P.J.; Vieth, R. Estimates of optimal vitamin D status. *Osteoporos. Int.* **2005**, *16*, 713–716. [CrossRef]
41. Ohta, H.; Kuroda, T.; Tsugawa, N.; Onoe, Y.; Okano, T.; Shiraki, M. Optimal vitamin D intake for preventing serum 25-hydroxyvitamin D insufficiency in young Japanese women. *J. Bone Miner. Metab.* **2018**, *36*, 620–625. [CrossRef]
42. Zareef, T.A.; Jackson, R.T.; Alkahtani, A.A. Vitamin D Intake among Premenopausal Women Living in Jeddah: Food Sources and Relationship to Demographic Factors and Bone Health. *Nutr. Metab.* **2018**, *2018*, 8570986. [CrossRef]
43. Holick, M.F. The role of vitamin D for bone health and fracture prevention. *Curr. Osteoporos. Rep.* **2006**, *4*, 96–102. [CrossRef]
44. Kim, M.-Y.; Kim, M.-J.; Ly, S.Y. Vitamin D intake, serum 25OHD, and bone mineral density of Korean adults: Based on the Korea national health and nutrition examination survey (KNHANES, 2011). *J. Nutr. Health* **2016**, *49*, 437. [CrossRef]
45. Islam, M.Z.; Shamim, A.A.; Viljakainen, H.T.; Akhtaruzzaman, M.; Jehan, A.H.; Khan, H.U.; Al-Arif, F.A.; Lamberg-Allardt, C. Effect of vitamin D, calcium and multiple micronutrient supplementation on vitamin D and bone status in Bangladeshi premenopausal garment factory workers with hypovitaminosis D: A double-blinded, randomised, placebo-controlled 1-year intervention. *Br. J. Nutr.* **2010**, *104*, 241–247. [CrossRef] [PubMed]
46. Aloia, J.F.; Dhaliwal, R.; Shieh, A.; Mikhail, M.; Fazzari, M.; Ragolia, L.; Abrams, S.A. Vitamin D supplementation increases calcium absorption without a threshold effect. *Am. J. Clin. Nutr.* **2014**, *99*, 624–631. [CrossRef]
47. Shapses, S.A.; Sukumar, D.; Schneider, S.H.; Schlussel, Y.; Sherrell, R.M.; Field, M.P.; Ambia-Sobhan, H. Vitamin D supplementation and calcium absorption during caloric restriction: A randomized double-blind trial. *Am. J. Clin. Nutr.* **2013**, *97*, 637–645. [CrossRef] [PubMed]
48. Korea Health Industry Development Institute. National Nutrition Statistics. Available online: https://www.khidi.or.kr/kps/dhraStat/result5?menuId=MENU01656&gubun=age3&year=2020 (accessed on 26 August 2022).
49. Brown, J.P.; Albert, C.; Nassar, B.A.; Adachi, J.D.; Cole, D.; Davison, K.S.; Dooley, K.C.; Don-Wauchope, A.; Douville, P.; Hanley, D.A.; et al. Bone turnover markers in the management of postmenopausal osteoporosis. *Clin. Biochem.* **2009**, *42*, 929–942. [CrossRef]
50. Macdonald, H.M.; Mavroeidi, A.; Barr, R.J.; Black, A.J.; Fraser, W.D.; Reid, D.M. Vitamin D status in postmenopausal women living at higher latitudes in the UK in relation to bone health, overweight, sunlight exposure and dietary vitamin D. *Bone* **2008**, *42*, 996–1003. [CrossRef]
51. Al-Yatama, F.I.; AlOtaibi, F.; Al-Bader, M.D.; Al-Shoumer, K.A. The effect of clothing on vitamin D status, bone turnover markers, and bone mineral density in young Kuwaiti females. *Int. J. Endocrinol.* **2019**, *2019*, 6794837. [CrossRef]
52. Orito, S.; Kuroda, T.; Onoe, Y.; Sato, Y.; Ohta, H. Age-related distribution of bone and skeletal parameters in 1,322 Japanese young women. *J. Bone Miner. Metab.* **2009**, *27*, 698–704. [CrossRef]
53. Williams, C.; Sapra, A. Osteoporosis Markers. Available online: https://www.ncbi.nlm.nih.gov/books/NBK559306/ (accessed on 19 October 2022).
54. Vasikaran, S.; Eastell, R.; Bruyère, O.; Foldes, A.J.; Garnero, P.; Griesmacher, A.; McClung, M.; Morris, H.A.; Silverman, S.; Trenti, T.; et al. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: A need for international reference standards. *Osteoporos. Int.* **2011**, *22*, 391–420. [CrossRef]
55. Eastell, R.; Pigott, T.; Gossiel, F.; Naylor, K.E.; Walsh, J.S.; Peel, N.F.A. DIAGNOSIS OF ENDOCRINE DISEASE: Bone turnover markers: Are they clinically useful? *Eur. J. Endocrinol.* **2018**, *178*, R19–R31. [CrossRef]
56. Kanis, J.A.; Melton III, L.J.; Christiansen, C.; Johnston, C.C.; Khaltaev, N. The diagnosis of osteoporosis. *J. Bone Miner. Res.* **1994**, *9*, 1137–1141. [CrossRef] [PubMed]
57. Whiting, S.J.; Calvo, M.S. Vitamin D fortification and supplementation policies to correct vitamin D insufficiency/deficiency globally. In *Vitamin D*; Elsevier Academic Press: London, UK, 2018; pp. 91–108.

nutrients

MDPI

Article

Bone Strength of the Calcaneus Is Associated with Dietary Calcium Intake in Older Japanese Men, but Not Women

Keita Suzuki [1,*], Hiromasa Tsujiguchi [1,2,3], Akinori Hara [1,2,3], Sakae Miyagi [3,4], Thao Thi Thu Nguyen [5], Yasuhiro Kambayashi [6], Yukari Shimizu [7], Fumihiko Suzuki [1,8], Chie Takazawa [1], Masaharu Nakamura [1], Hirohito Tsuboi [9], Takayuki Kannon [10], Atsushi Tajima [11] and Hiroyuki Nakamura [1,2,3]

[1] Department of Hygiene and Public Health, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-8640, Ishikawa, Japan
[2] Department of Public Health, Graduate School of Advanced Preventive Medical Sciences, Kanazawa University, Kanazawa 920-8640, Ishikawa, Japan
[3] Kanazawa University Advanced Preventive Medical Sciences Research Center, Kanazawa 920-8640, Ishikawa, Japan
[4] Innovative Clinical Research Center, Kanazawa University, Kanazawa 920-8640, Ishikawa, Japan
[5] Department of Epidemiology, Faculty of Public Health, Haiphong University of Medicine and Pharmacy, Hai Phong 04000-05000, Vietnam
[6] Department of Public Health, Faculty of Veterinary Medicine, Okayama University of Science, Imabari 794-0085, Ehime, Japan
[7] Department of Nursing, Faculty of Health Sciences, Komatsu University, Komatsu 923-8511, Ishikawa, Japan
[8] Community Medicine Support Dentistry, Ohu University Hospital, Koriyama 963-8611, Fukushima, Japan
[9] Graduate School of Human Nursing, The University of Shiga Prefecture, Hikone 522-8533, Shiga, Japan
[10] Department of Biomedical Data Science, School of Medicine, Fujita Health University, Toyoake 470-1101, Aichi, Japan
[11] Department of Bioinformatics and Genomics, Graduate School of Advanced Preventive Medical Sciences, Kanazawa University, Kanazawa 920-8640, Ishikawa, Japan
* Correspondence: keitasuzuk@gmail.com; Tel.: +81-76-265-2218

Citation: Suzuki, K.; Tsujiguchi, H.; Hara, A.; Miyagi, S.; Nguyen, T.T.T.; Kambayashi, Y.; Shimizu, Y.; Suzuki, F.; Takazawa, C.; Nakamura, M.; et al. Bone Strength of the Calcaneus Is Associated with Dietary Calcium Intake in Older Japanese Men, but Not Women. *Nutrients* **2022**, *14*, 5225. https://doi.org/10.3390/nu14245225

Academic Editors: Roberto Iacone and Connie Weaver

Received: 30 October 2022
Accepted: 6 December 2022
Published: 8 December 2022

Abstract: The relationship between calcium intake and bone strength in older Asian individuals, including Japanese, is controversial; therefore, we herein investigated this relationship in older Japanese populations. We performed a cross-sectional analysis of 314 participants older than 65 years who voluntarily participated in a medical examination and responded to questionnaires. The osteosono assessment index (OSI) measured at the right calcaneus using a quantitative ultrasonic device was used as an indicator of bone strength. The daily dietary intake of calcium was assessed using a brief-type self-administered diet history questionnaire. A two-way analysis of covariance revealed a significant interaction between sex and calcium intake on the OSI ($p < 0.01$). A multiple regression analysis showed a positive correlation between calcium intake and the OSI in males ($p < 0.01$), but not females ($p = 0.27$). In females, grip strength divided by body weight positively correlated with the OSI ($p = 0.04$). The present results suggest that a higher calcium intake contributes to bone strength in older Japanese males. Although a higher grip strength may contribute to bone strength in females, the potential of estrogen as a confounding factor needs to be considered.

Keywords: calcium intake; bone strength; grip strength; older people; cross-sectional study

1. Introduction

Health care providers and the general population consider a sufficient calcium intake to be an essential factor for maintaining or improving bone strength. The Food and Nutrition Board at the National Academies of Sciences, Engineering, and Medicine recommends a calcium intake of 1200 mg/day to promote bone maintenance and a neutral calcium balance in individuals aged 70 years and older [1].

However, not all studies support the beneficial effects of an adequate dietary calcium intake on bone. A systematic review that included longitudinal cohorts concluded that a

positive relationship was not demonstrated between calcium intake and changes in bone mineral density (BMD) in women aged 60 years and older [2]. Furthermore, a meta-analysis of randomized controlled trials revealed that an increased calcium intake from dietary sources or by taking calcium supplements achieved only small non-progressive increases in BMD in participants aged 50 years or older [3]. Moreover, a longitudinal cohort composed of community-based, multiethnic women across the menopause transition recently reported that the consumption of dairy products did not contribute to the preservation of BMD or prevention of fractures [4]. Therefore, the benefits of calcium intake for maintaining BMD appear to be unestablished.

Serum 25-hydroxyvitamin D (25(OH)D) concentrations influence the absorption efficiency of calcium in the intestines [5,6]. Previous studies demonstrated that calcium absorption varied with age [7,8] and race [9]. Therefore, the effects of calcium consumption on BMD may differ according to race, age, and the vitamin D status. However, the majority of studies in reviews [2,3] recruited Caucasians and Blacks. Moreover, the age range of participants in each study was broad and did not focus on the elderly. To the best of our knowledge, only a few studies have investigated the relationships between dietary calcium intake and bone strength parameters in an older community-based Asian population [10–12], and the relationships observed were neither significant nor consistently linear.

Therefore, we herein examined a general Japanese population aged 65 years and older in which calcium absorption is considered to be reduced due to aging. Since our cohort resides in a rural area in Japan, high ethnic homogeneity was expected due to the lack of immigrants from other countries in the region. The aim of this cross-sectional analysis was to investigate the relationship between dietary calcium intake and bone strength in an older Japanese population according to sex.

2. Methods

2.1. Study Design and Participants

We utilized cross-sectional data collected between 2011 and 2015 in the Shika study. We have conducted a longitudinal community-based observational study since 2011 among the residents of Shika town, which is located on the Noto peninsula in Ishikawa prefecture, Japan. The population of Shika town is almost 20,000, and more than 40% are older than 65 years. In the Shika study, self-administrated questionnaires were distributed to all adults older than 40 years in the four model districts by trained interviewers. The interviewers were instructed on the outline of the Shika study and how to fill in the questionnaires before their distribution. Moreover, an invitation letter for comprehensive medical examinations was distributed to the residents in the four districts. Participants voluntarily underwent a medical examination.

In the present study, we included participants older than 65 years who responded to the questionnaires and underwent the medical examination (n = 620). Participants with missing data on variables used in the analysis were excluded. To avoid a treatment bias, participants who received treatments for osteoporosis were omitted. Data collected from 314 participants who completed the medical examination and replied to the questionnaires were available for analysis in the present study.

The present study was approved by the Medical Ethics Committee of Kanazawa University (approval number 1491). All participants provided written informed consent for inclusion before participation. The present study was conducted in accordance with the Declaration of Helsinki.

2.2. Assessment of Nutrient Intake

A brief-type self-administered diet history questionnaire (BDHQ) was used to assess the daily dietary intakes of protein, lipids, carbohydrates, vitamin D, calcium, and total energy. Participants were asked about the consumption frequency of 58 food and beverage items during the previous month in BDHQ. These items are mainly from the food list used in the National Health and Nutrition Survey of Japan and are commonly consumed

in Japan. The validity of BDHQ and a satisfactory ranking ability for many nutrients in Japanese individuals were previously demonstrated [13,14]. We omitted participants who reported a total energy intake of less than 600 kcal/day (half of the energy intake required for the lowest physical activity category) or more than 4000 kcal/day (1.5-fold the energy intake required for the moderate physical activity category) from the analysis because of under-/overestimations of nutrient intake. Nutrient data were adjusted for daily energy intake using the density method. The participants were divided into two groups according to the median calcium intake calculated in males and females, respectively.

2.3. Measurement of Bone Strength

A previous study reported a strong correlation between the osteo-sono assessment index (OSI) measured at the calcaneus and BMD measured by dual-energy X-ray absorptiometry (DXA) at the radius [15]. To calculate the OSI, the speed of sound (SOS) and the transmission index (TI) were measured at the right calcaneus using a quantitative ultrasonic device (AOS-100NW-B, Hitachi Aloka Medical, Tokyo, Japan). The SOS reflects the ultrasound velocity that penetrated the calcaneus. The higher SOS indicates a higher bone density. The TI indicates the frequency-dependent attenuation of the ultrasound. The greater bone mass shows a higher TI [16]. The OSI was given by the following formula [17]:

$$\mathrm{OSI} = \mathrm{TI} \times \mathrm{SOS}^2 \quad (1)$$

In the present study, the participants were assigned into two groups according to the median of the OSI calculated for each sex.

2.4. Assessment of Other Variables

Age, height, weight, blood pressure, grip strength, and calf circumference were assessed in the medical examination. We calculated body mass index (BMI) as weight in kilograms divided by the square of height in meters. Calf circumference and grip strength were divided by body weight (GS/BW) to adjust for the influence of body size. Fasting plasma glucose (FPG), serum cystatin C, serum calcium, serum 25(OH)D concentrations, and intact parathyroid hormone (PTH) were measured using blood samples taken in the medical examination. Blood samples were drawn from the forearm vein in the morning after fasting for 12 h. Serum 25(OH)D concentrations were measured using a radioimmunoassay (25-hydroxyvitamin D 125I RIA Kit, DiaSorin Inc., Stillwater, MN, USA). Intact PTH was measured by an electro-chemiluminescence immunoassay (SRL, Inc., Tokyo, Japan). Estimated glomerular filtration rates (eGFR) based on cystatin C were calculated using the following equations modified for the Japanese population [18]:

$$\text{eGFR for males: } [104 \times (\text{serum cystatin C})^{-1.019} \times 0.996^{\text{age}}] - 8$$

$$\text{eGFR for females: } [104 \times (\text{serum cystatin C})^{-1.019} \times 0.996^{\text{age}} \times 0.929] - 8$$

Drinking habits and the smoking status were assessed using a self-administered questionnaire. To assess drinking habits, participants were asked "How much alcohol do you drink per day?". Based on their answer, participants were divided into the following four groups: non-drinker, less than 20 g/day, 20 to 40 g/day, and more than 40 g/day. Participants were assigned to non-smoker, ex-smoker, and current smoker groups according to their answer on their smoking status.

2.5. Statistical Analysis

Continuous variables were summarized as means and standard deviations (SD), and categorical variables as numbers (*n*) and percentages (%). The mean levels of continuous variables and categorical variables were compared between the low- and high-OSI groups using the Student's *t*-test or chi-square test. A two-way analysis of covariance (ANCOVA) was used to examine the interaction between sex and calcium intake levels on the OSI

with adjustments for age, BMI, GS/BW, eGFR, drinking habits, the smoking status, intact PTH, and the serum 25(OH)D concentrations. The relationship between the serum calcium concentrations and intact PTH was assessed using a multiple regression analysis with adjustments for age, BMI, drinking habits, and the smoking status. A multiple regression analysis was performed to examine the relationship between calcium intake and the OSI according to sex with adjustments for the following independent factors: age, BMI, GS/BW, eGFR, drinking habits, the smoking status, intact PTH, and the serum 25(OH)D concentrations.

All statistical analyses were performed using the Japanese version of IBM SPSS Statistics version 27.0 (IBM Japan, Tokyo, Japan). A two-sided p-value < 0.05 was considered to be significant.

3. Results

3.1. Participant Characteristics

Table 1 shows participant characteristics. Among 314 participants, 157 were males and 157 were females. The mean ages of male and female participants were 71.7 ± 5.8 and 72.1 ± 6.2 years, respectively. The OSI was significantly higher in males than in females ($p < 0.01$), whereas calcium intake was significantly higher in females ($p < 0.01$). Males had significantly higher 25(OH)D concentrations than females ($p < 0.01$), while intact PTH was lower in males than in females ($p = 0.01$).

Table 1. Participant characteristics.

	Total (*n* = 314)		**Male (*n* = 157)**		**Female (*n* = 157)**		
	Mean (*n*)	**SD (%)**	**Mean (*n*)**	**SD (%)**	**Mean (*n*)**	**SD (%)**	***p*-Value**
Age (y)	71.9	6.0	71.7	5.8	72.1	6.2	0.49
Height (cm)	155.2	14.8	162.5	13.1	147.9	12.8	**<0.01**
Weight (kg)	57.6	10.0	63.5	9.2	51.8	6.7	**<0.01**
BMI (kg/m^2)	23.4	2.9	23.7	3.1	23.2	2.6	**0.10**
Systolic blood pressure (mmHg)	143	19	143	18	142	19	0.92
Diastolic blood pressure (mmHg)	78	11	78	11	77	11	0.28
Fasting plasma glucose (mg/dL)	99.3	19.3	103.0	22.3	95.6	15.1	**<0.01**
Serum calcium concentrations (mg/dL)	9.31	0.32	9.29	0.31	9.33	0.33	0.20
Serum 25(OH)D concentrations (ng/mL)	25.8	7.8	28.0	7.8	23.6	7.2	**<0.01**
Intact PTH (pg/mL)	47.8	18.2	45.1	17.0	50.5	19.0	**0.01**
CC/BW (cm/kg)	0.58	0.07	0.54	0.05	0.63	0.05	**<0.01**
GS/BW (kgf/kg)	0.51	0.11	0.58	0.10	0.44	0.08	**<0.01**
eGFR (mL/min/1.73 m^2)	71.6	14.8	71.4	15.2	71.8	14.5	0.80
Osteo-sono assessment index	2.54	0.36	2.75	0.34	2.34	0.23	**<0.01**
Total energy intake (kcal/day)	1899	625	2083	627	1715	567	**<0.01**
Protein intake (g/1000 kcal)	15.6	3.5	14.9	3.2	16.3	3.6	**<0.01**
Fat intake (g/1000 kcal)	24.0	6.1	22.4	5.8	25.6	5.9	**<0.01**
Carbohydrate intake (g/1000 kcal)	55.2	8.5	54.3	8.3	56.2	8.5	**0.04**
Vitamin D intake (μg/1000 kcal)	9.4	5.9	8.8	5.6	10.0	6.1	0.09
Calcium intake (mg/1000 kcal)	321	119	297	113	346	119	**<0.01**
Smoking status (*n*, %)							**<0.01**
Non-smoker	168	54	27	17	141	90	
Ex-smoker	107	34	98	62	9	6	
Current smoker	39	12	32	20	7	4	
Drinking habit (*n*, %)							**<0.01**
Non-drinker	180	57	49	31	131	83	
Less than 20 mg/day	70	22	47	30	23	15	
20–40 mg/day	61	19	58	37	3	2	
More than 40 mg/day	3	1	3	2	0	0	

Note: p-values were from the Student's t-test for continuous variables and the chi-square test for categorical variables. p-values < 0.05 are highlighted in bold. Abbreviations: BMI, body mass index; GS, grip strength; CC, calf circumference; BW, body weight; eGFR, estimated glomerular filtration rates; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; SD, standard deviation.

3.2. Comparison of Characteristics between Low- and High-OSI Groups according to Sex

The results of comparisons between the low- and high-OSI groups are shown in Table 2. In males, the high-OSI group was significantly younger than the low-OSI group ($p < 0.01$). The high-OSI group had a significantly higher body weight ($p < 0.01$) and BMI ($p < 0.01$) than those in the low-OSI group, whereas CC/BW was significantly lower ($p < 0.01$).

Table 2. Comparison of characteristics between low- and high OSI groups according to sex.

	Male					**Female**				
	Low-OSI (*n* = 79)		**High-OSI (*n* = 78)**			**Low-OSI (*n* = 76)**		**High-OSI (*n* = 81)**		
	Mean (*n*)	**SD (%)**	**Mean (*n*)**	**SD (%)**	***p*-Value**	**Mean (*n*)**	**SD (%)**	**Mean (*n*)**	**SD (%)**	***p*-Value**
Age (y)	73.2	6.3	70.1	4.7	**<0.01**	73.2	6.9	71.1	5.3	**0.04**
Height (cm)	161.0	17.4	164.1	6.0	0.14	148.2	5.6	147.6	17.0	0.77
Weight (kg)	60.4	8.8	66.5	8.7	**<0.01**	49.9	6.1	53.5	6.8	**<0.01**
BMI (kg/m^2)	22.7	2.9	24.7	2.9	**<0.01**	22.7	2.6	23.6	2.5	**0.04**
Systolic blood pressure (mmHg)	144	18	142	18	0.50	143	21	142	17	0.64
Diastolic blood pressure (mmHg)	78	12	79	11	0.58	77	13	76	10	0.64
Fasting plasma glucose (mg/dL)	101.9	15.6	104.0	27.5	0.56	93.3	10.5	97.7	18.1	0.06
Serum calcium concentrations (mg/dL)	9.28	0.33	9.29	0.30	0.78	9.31	0.34	9.36	0.33	0.36
Serum 25(OH)D concentrations (ng/mL)	27.2	7.2	28.7	8.4	0.23	24.4	8.3	22.9	5.9	0.20
Intact PTH (pg/mL)	46.4	18.5	43.8	15.2	0.34	51.7	20.5	49.4	17.4	0.44
CC/BW (cm/kg)	0.55	0.05	0.53	0.05	**<0.01**	0.64	0.05	0.61	0.05	**<0.01**
GS/BW (kgf/kg)	0.58	0.10	0.58	0.10	0.87	0.43	0.08	0.45	0.08	0.09
eGFR (mL/min/1.73 m^2)	70.5	16.3	72.2	14.0	0.48	71.6	15.7	71.9	13.5	0.89
Osteo-sono assessment index	2.49	0.16	3.01	0.27	**<0.01**	2.15	0.10	2.51	0.18	**<0.01**
Total energy intake (kcal/day)	2016	590	2151	660	0.18	1745	598	1687	538	0.52
Protein intake (g/1000 kcal)	14.8	2.9	15.1	3.6	0.58	16.2	3.6	16.4	3.7	0.78
Fat intake (g/1000 kcal)	22.4	6.0	22.5	5.6	0.85	25.5	6.5	25.6	5.3	0.88
Carbohydrate intake (g/1000 kcal)	55.5	8.2	53	8.4	0.06	56.6	9.4	55.9	7.7	0.62
Vitamin D intake (μg/1000 kcal)	8.5	4.6	9.2	6.5	0.39	9.8	5.8	10.1	6.4	0.75
Calcium intake (mg/1000 kcal)	285	104	309	122	0.18	354	121	340	118	0.47
Smoking status (*n*, %)					0.83					0.52
Non-smoker	15	19	12	15		69	91	72	89	
Ex-smoker	48	61	50	64		5	7	4	5	
Current smoker	16	20	16	21		2	3	5	6	
Drinking habit (*n*, %)					0.27					0.63
Non-drinker	29	37	20	26		62	82	69	85	
Less than 20 mg/day	25	32	22	28		13	17	10	12	
20–40 mg/day	24	30	34	44		1	1	2	2	
More than 40 mg/day	1	1	2	3		0	0	0	0	

Note: *p*-values were from the Student's *t*-test for continuous variables and the chi-square test for categorical variables. *p*-values < 0.05 are highlighted in bold. Abbreviations: BMI, body mass index; GS, grip strength; CC, calf circumference; BW, body weight; eGFR, estimated glomerular filtration rates; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; SD, standard deviation.

In females, age ($p = 0.02$) and CC/BW ($p < 0.01$) were significantly lower in the high-OSI group than in the low-OSI group. The high-OSI group showed a significantly higher body weight ($p < 0.01$) and BMI ($p = 0.02$) than those in the low-OSI group.

3.3. Interaction between Sex and Calcium Intake on the OSI

Table 3 shows the results of a two-way ANCOVA, which was performed to examine the interaction between sex and calcium intake on the OSI. A significant main effect of sex on the OSI was observed after adjustments for age, BMI, eGFR, GS/BW, drinking habits, the smoking status, intact PTH, and the serum 25(OH)D concentrations ($p < 0.01$), whereas the main effect of calcium intake was not significant ($p = 0.28$). A two-way ANCOVA detected a significant interaction between sex and calcium intake on the OSI after adjustments for the covariates ($p < 0.01$).

Table 3. Interaction between sex and calcium intake levels on the OSI.

		Low Calcium Intake		High Calcium Intake				
Dependent Variable	**Sex**	**Mean**	**SE**	**Mean**	**SE**	***p* for Sex [a]**	***p* for Calcium Intake Levels [b]**	***p* for Interactions [c]**
OSI	Male	2.61	0.04	2.72	0.03	**<0.01**	0.28	**<0.01**
	Female	2.44	0.04	2.39	0.03			

Notes: Analyses were adjusted for age, body mass index, grip strength divided by body weight, estimated glomerular filtration rates, drinking habits, the smoking status, intact parathyroid hormone, and the serum 25-hydroxyvitamin D concentrations. *p*-values < 0.05 are highlighted in bold. [a]: *p*-value of the main effect of sex; [b]: *p*-value of the main effect of calcium intake levels; [c]: *p*-value of the interaction test between sex and calcium intake on the OSI; Abbreviations: OSI, the osteo-sono assessment index; SE, standard error.

3.4. Multiple Regression Analysis of the Serum Calcium Concentrations as a Dependent Variable according to Sex

The relationship between the serum calcium concentrations and intact PTH is shown in Table 4. Intact PTH negatively correlated with the serum calcium concentrations in all participants ($p < 0.01$), males ($p < 0.01$), and females ($p = 0.03$). A significant negative association between the serum calcium concentrations and age was observed in all participants ($p = 0.03$).

Table 4. Multiple regression analysis of the serum calcium concentrations as a dependent variable according to sex.

	Total			Male			Female		
Variables	**Non-Standardized β**	**Standardized β**	***p*-Value**	**Non-Standardized β**	**Standardized β**	***p*-Value**	**Non-Standardized β**	**Standardized β**	***p*-Value**
Age	−0.01	−0.12	**0.03**	−0.01	−0.10	0.23	−0.01	−0.13	0.10
BMI	0.00	−0.01	0.80	0.00	0.02	0.78	−0.01	−0.06	0.45
Drinking habits	−0.05	−0.12	0.05	−0.04	−0.11	0.19	−0.06	−0.08	0.32
Smoking status	−0.01	−0.01	0.86	−0.02	−0.03	0.71	0.05	0.08	0.36
Intact PTH	0.00	−0.20	**<0.01**	0.00	−0.23	**<0.01**	0.00	−0.17	**0.03**

Notes: β means a partial regression coefficient of each independent variable to the serum calcium concentrations. *p*-values < 0.05 are highlighted in bold. Abbreviations: BMI, body mass index; PTH, parathyroid hormone.

3.5. Multiple Regression Analysis of the OSI as a Dependent Variable according to Sex

The results of a multiple regression analysis are shown in Table 5. Among all participants, the relationship between calcium intake and the OSI was not significant ($p = 0.14$). The OSI positively correlated with BMI ($p < 0.01$), and GS/BW ($p < 0.01$), and negatively correlated with age ($p = 0.01$), and intact PTH ($p = 0.01$). Moreover, the OSI correlated with drinking habits ($p < 0.01$) and the smoking status ($p < 0.01$).

Table 5. Multiple regression analysis of the OSI as a dependent variable according to sex.

	Total			Male			Female		
Variables	**Non-Standardized β**	**Standardized β**	***p*-Value**	**Non-Standardized β**	**Standardized β**	***p*-Value**	**Non-Standardized β**	**Standardized β**	***p*-Value**
Age	−0.01	−0.14	**0.01**	−0.02	−0.26	**<0.01**	−0.01	−0.31	**<0.01**
BMI	0.04	0.35	**<0.01**	0.04	0.32	**<0.01**	0.02	0.28	**<0.01**
eGFR	0.00	−0.10	0.06	0.00	−0.03	0.73	0.00	−0.23	**0.02**
Drinking habits	0.07	0.16	**<0.01**	0.03	0.08	0.35	−0.01	−0.03	0.73
Smoking status	0.11	0.21	**<0.01**	0.04	0.07	0.33	0.09	0.18	**0.03**
GS/BW	0.76	0.25	**<0.01**	0.05	0.02	0.85	0.55	0.19	**0.04**
Intact PTH	0.00	−0.12	**0.01**	0.00	−0.09	0.22	0.00	−0.11	0.16
Serum 25(OH)D	0.00	0.09	0.06	0.01	0.16	**0.04**	0.00	−0.03	0.74
Calcium intake	0.00	0.07	0.14	0.00	0.24	**<0.01**	0.00	−0.08	0.27

Notes: β means a partial regression coefficient of each independent variable to the OSI. *p*-values < 0.05 are highlighted in bold. Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rates; GS/BW, grip strength divided by body weight; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

In males, the OSI positively correlated with calcium intake ($p < 0.01$), BMI ($p < 0.01$), and the serum 25(OH)D concentrations ($p = 0.04$), and negatively correlated with age ($p < 0.01$).

In females, calcium intake did not correlate with the OSI ($p = 0.27$). However, the OSI positively correlated with BMI ($p < 0.01$), eGFR ($p = 0.02$), and GS/BW ($p = 0.04$), and negatively correlated with age ($p < 0.01$). A significant association between the smoking status and OSI was observed ($p = 0.03$).

4. Discussion

The results of this cross-sectional study demonstrated that a higher dietary calcium intake was associated with a higher OSI in males even after adjustments for covariates. In females, the OSI did not correlate with calcium intake, but positively correlated with GS/BW and eGFR.

PTH regulates calcium homeostasis in the body [19]. The secretion of PTH from the parathyroid glands is altered to keep serum calcium levels within the appropriate range depending on extracellular calcium concentrations. In the present study, a negative correlation between the serum calcium concentrations and intact PTH was observed. It is considered that the secretion of PTH was upregulated in individuals with lower serum calcium concentrations. In addition, PTH regulates bone remodeling by stimulating osteoblasts and indirectly activating osteoclasts [20]. These studies indicate that PTH strongly influences calcium homeostasis and bone metabolism. Therefore, we adjusted the association between calcium intake and the OSI with intact PTH in the present study.

The relationship between the OSI and calcium intake differed between males and females in the present study. A discrepancy in this relationship between males and females was also reported in a previous study [21]. The sex difference observed in the effects of calcium intake on bone strength may be partially explained by differences in serum 25(OH)D concentrations between males and females. Previous studies reported that serum 25(OH)D concentrations were higher in males than in females [22,23]. In the present study, males had significantly higher 25(OH)D concentrations than females. Vitamin D regulates the intestinal absorption of dietary calcium [5,6]. Therefore, calcium absorption appeared to be more inefficient in females. Furthermore, aging has been shown to decrease calcium absorption due to vitamin D insufficiency and intestinal resistance to the effects of vitamin D [7,24]. Therefore, the lack of a relationship between calcium intake and the OSI was attributed to impaired calcium absorption in older females in the present study.

Several previous studies reported that the SOS and broadband ultrasound attenuation (BUA) measured at the calcaneus were closely associated with BMD assessed using DXA [25,26]. In addition, the SOS and BUA negatively correlated with age [19,26]. Therefore, we believe that the SOS and BUA measured by ultrasound reflect the age-related loss of bone density. However, the possibility that the OSI does not rigorously detect the loss of bone density should also be considered. The OSI depends not only on the bone density in the calcaneus but also on the amount of yellow marrow or fat [27]. Furthermore, the impact of marrow on the quantitative ultrasound transmission of cancellous bone is likely to be greatest in women with low BMD [27]. A previous study reported that the amount of marrow fat in the calcaneus does not vary with the development of osteoporosis [28]. Therefore, the lack of significant association of calcium intake with the OSI in females in the present study may be attributed to the inability of the OSI to detect the reduction of bone density particularly in females.

GS/BW correlated with the OSI in females in the present study. Previous studies reported that grip strength positively correlated with BMD in aged adults and postmenopausal women [29–31]. Mechanical loading generated by muscle is an essential mechanism for maintaining bone health [32,33]. Therefore, the present results appear to support previous findings and suggest that the maintenance of muscle strength contributes to bone strength in older Japanese females.

However, the relationship between GS/BW and the OSI in females needs to be interpreted with caution because it was not observed in males. Furthermore, the effects of estrogen on bone mass and muscle function need to be considered. An estrogen deficiency in women after menopause activates osteoclastic bone resorption, causing osteoporosis [34]. Furthermore, estrogen treatments ameliorate osteoporosis [35,36]. In addition, a decline in estrogen levels in menopausal women was previously reported to impair muscle strength via the inadequate preservation of skeletal muscle mass and decrements in the quality of the remaining skeletal muscle [37,38]. Therefore, estrogen levels may have mediated the relationship between GS/BW and the OSI in the present study. However, the effects of estrogen remain unclear because we did not measure estrogen levels. Therefore, the potential of estrogen as a confounding factor needs to be considered when interpreting the present results.

There are further limitations that need to be addressed. The causality of the relationship of the OSI with calcium intake and GS/BW remains unclear because of the nature of a cross-sectional study. Moreover, selection bias may limit the generalizability of the present results. The ratio of health-conscious individuals was likely to be high because only voluntary participants underwent the medical examination in the present study. The lack of assessment of the bone density at the femoral neck or lumbar may prevent our findings from providing robust suggestions on the prevention of fractures. However, the previous studies reported that the SOS and BUA measured at the calcaneus closely correlated with BMD of the femoral neck, spine, and total body measured by DXA [39–41]. Therefore, although further studies focused on the femoral neck and spine are needed, our findings observed at the calcaneus are likely to apply to the other sites of the body.

5. Conclusions

Dietary calcium intake was associated with the OSI in older Japanese males, but not females. On the other hand, GS/BW correlated with the OSI in females. The present results suggest that the effects of calcium intake on the preservation of bone strength may differ between the sexes in an older Japanese population.

Author Contributions: Conceptualization, K.S. and H.N.; Data curation, H.T. (Hiromasa Tsujiguchi), T.K. and A.T.; Formal analysis, K.S.; Funding acquisition, H.N.; Investigation, K.S., H.T. (Hiromasa Tsujiguchi), A.H., S.M., T.T.T.N., Y.K., Y.S., F.S., C.T., M.N., H.T. (Hirohito Tsuboi), T.K., A.T. and H.N.; Methodology, K.S. and H.N.; Project administration, H.T. (Hiromasa Tsujiguchi) and H.N.; Resources, H.N.; Software, K.S., H.T. (Hiromasa Tsujiguchi) and T.K.; Supervision, H.N.; Validation, K.S., H.T. (Hiromasa Tsujiguchi) and H.N.; Visualization, K.S.; Writing—original draft, K.S.; Writing—review and editing, H.T. (Hiromasa Tsujiguchi), A.H., S.M., T.T.T.N., Y.K., Y.S., F.S., C.T., M.N., H.T. (Hirohito Tsuboi), T.K., A.T. and H.N. All authors have read and agreed to the published version of the manuscript.

Funding: The present study was funded by a Grant-in-Aid for Scientific Research (B) (number: JP19H03882) and a Grant-in-Aid for Research Activity Start-up (number: JP21K21251) by the Japan Society for the Promotion of Science.

Institutional Review Board Statement: The present study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Kanazawa University (No. 1491; 28 December 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data in the present study are available upon reasonable request from the corresponding author. Data are not publicly available due to privacy and ethical policies.

Acknowledgments: The authors wish to thank all the field workers and laboratory personnel of the Shika study for their efforts.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. *Dietary Reference Intakes for Calcium and Vitamin D*; Ross, A.C.; Taylor, C.L.; Yaktine, A.L.; Del Valle, H.B. (Eds.) National Academies Press: Washington, DC, USA, 2011.
2. Bristow, S.M.; Bolland, M.J.; Gamble, G.D.; Leung, W.; Reid, I.R. Dietary Calcium Intake and Change in Bone Mineral Density in Older Adults: A Systematic Review of Longitudinal Cohort Studies. *Eur. J. Clin. Nutr.* **2022**, *76*, 196–205. [CrossRef]
3. Tai, V.; Leung, W.; Grey, A.; Reid, I.R.; Bolland, M.J. Calcium Intake and Bone Mineral Density: Systematic Review and Meta-Analysis. *BMJ* **2015**, *351*, h4183. [CrossRef]
4. Wallace, T.C.; Jun, S.; Zou, P.; McCabe, G.P.; Craig, B.A.; Cauley, J.A.; Weaver, C.M.; Bailey, R.L. Dairy Intake Is Not Associated with Improvements in Bone Mineral Density or Risk of Fractures across the Menopause Transition: Data from the Study of Women's Health Across the Nation. *Menopause* **2020**, *27*, 879–886. [CrossRef]
5. Holick, M.F. High Prevalence of Vitamin D Inadequacy and Implications for Health. *Mayo Clin. Proc.* **2006**, *81*, 353–373. [CrossRef]
6. Heaney, R.P.; Dowell, M.S.; Hale, C.A.; Bendich, A. Calcium Absorption Varies within the Reference Range for Serum 25-Hydroxyvitamin D. *J. Am. Coll. Nutr.* **2003**, *22*, 142–146. [CrossRef]
7. Bullamore, J.R.; Wilkinson, R.; Gallagher, J.C.; Nordin, B.E.; Marshall, D.H. Effect of Age on Calcium Absorption. *Lancet* **1970**, *296*, 535–537. [CrossRef]
8. Nordin, B.E.C.; Need, A.G.; Morris, H.A.; O'Loughlin, P.D.; Horowitz, M. Effect of Age on Calcium Absorption in Postmenopausal Women. *Am. J. Clin. Nutr.* **2004**, *80*, 998–1002. [CrossRef]
9. Abrams, S.A.; O'brien, K.O.; Liang, L.K.; Stuff, J.E. Differences in Calcium Absorption and Kinetics between Black and White Girls Aged 5–16 Years. *J. Bone Miner. Res.* **1995**, *10*, 829–833. [CrossRef]
10. Nakamura, K.; Tsugawa, N.; Saito, T.; Ishikawa, M.; Tsuchiya, Y.; Hyodo, K.; Maruyama, K.; Oshiki, R.; Kobayashi, R.; Nashimoto, M.; et al. Vitamin D Status, Bone Mass, and Bone Metabolism in Home-Dwelling Postmenopausal Japanese Women: Yokogoshi Study. *Bone* **2008**, *42*, 271–277. [CrossRef]
11. Kim, K.M.; Choi, S.H.; Lim, S.; Moon, J.H.; Kim, J.H.; Kim, S.W.; Jang, H.C.; Shin, C.S. Interactions between Dietary Calcium Intake and Bone Mineral Density or Bone Geometry in a Low Calcium Intake Population (KNHANES IV 2008–2010). *J. Clin. Endocrinol. Metab.* **2014**, *99*, 2409–2417. [CrossRef]
12. Nakamura, K.; Saito, T.; Nishiwaki, T.; Ueno, K.; Nashimoto, M.; Okuda, Y.; Tsuchiya, Y.; Oshiki, R.; Muto, K.; Yamamoto, M. Correlations between Bone Mineral Density and Demographic, Lifestyle, and Biochemical Variables in Community-Dwelling Japanese Women 69 Years of Age and Over. *Osteoporos. Int.* **2006**, *17*, 1202–1207. [CrossRef]
13. Kobayashi, S.; Murakami, K.; Sasaki, S.; Okubo, H.; Hirota, N.; Notsu, A.; Fukui, M.; Date, C. Comparison of Relative Validity of Food Group Intakes Estimated by Comprehensive and Brief-Type Self-Administered Diet History Questionnaires against 16 d Dietary Records in Japanese Adults. *Public Health Nutr.* **2011**, *14*, 1200–1211. [CrossRef]
14. Sasaki, S.; Yanagibori, R.; Amano, K. Self-Administered Diet History Questionnaire Developed for Health Education: A Relative Validation of the Test-Version by Comparison with 3-Day Diet Record in Women. *J. Epidemiol.* **1998**, *8*, 203–215. [CrossRef]
15. Sasaki, M.; Harata, S.; Kumazawa, Y.; Mita, R.; Kida, K.; Tsuge, M. Bone Mineral Density and Osteo Sono Assessment Index in Adolescents. *J. Orthop. Sci.* **2000**, *5*, 185–191. [CrossRef]
16. Suzuki, Y.; Maruyama-Nagao, A.; Sakuraba, K.; Kawai, S. Level of Serum Undercarboxylated Osteocalcin Correlates with Bone Quality Assessed by Calcaneal Quantitative Ultrasound Sonometry in Young Japanese Females. *Exp. Ther. Med.* **2017**, *13*, 1937–1943. [CrossRef]
17. Tsuda-Futami, E.; Hans, D.; Njeh, C.F.; Fuerst, T.; Fan, B.; Li, J.; He, Y.Q.; Genant, H.K. An Evaluation of a New Gel-Coupled Ultrasound Device for the Quantitative Assessment of Bone. *Br. J. Radiol.* **1999**, *72*, 691–700. [CrossRef]
18. Horio, M.; Imai, E.; Yasuda, Y.; Watanabe, T.; Matsuo, S. GFR Estimation Using Standardized Serum Cystatin C in Japan. *Am. J. Kidney Dis.* **2013**, *61*, 197–203. [CrossRef]
19. Moris, M.; Peretz, A.; Tjeka, R.; Negaban, N.; Wouters, M.; Bergmann, P. Quantitative Ultrasound Bone Measurements: Normal Values and Comparison with Bone Mineral Density by Dual X-Ray Absorptiometry. *Calcif. Tissue Int.* **1995**, *57*, 6–10. [CrossRef]
20. Morimoto, A.; Kikuta, J.; Nishikawa, K.; Sudo, T.; Uenaka, M.; Furuya, M.; Hasegawa, T.; Hashimoto, K.; Tsukazaki, H.; Seno, S.; et al. SLPI Is a Critical Mediator That Controls PTH-Induced Bone Formation. *Nat. Commun.* **2021**, *12*, 2136. [CrossRef]
21. Anderson, J.J.B.; Roggenkamp, K.J.; Suchindran, C.M. Calcium Intakes and Femoral and Lumbar Bone Density of Elderly U.S. Men and Women: National Health and Nutrition Examination Survey 2005–2006 Analysis. *J. Clin. Endocrinol. Metab.* **2012**, *97*, 4531–4539. [CrossRef]
22. Muscogiuri, G.; Barrea, L.; Di Somma, C.; Laudisio, D.; Salzano, C.; Pugliese, G.; de Alteriis, G.; Colao, A.; Savastano, S. Sex Differences of Vitamin D Status across BMI Classes: An Observational Prospective Cohort Study. *Nutrients* **2019**, *11*, 3034. [CrossRef]
23. Gocho, Y.; Tanaka, M.; Sugawara, H.; Furuhashi, M.; Moniwa, N.; Yamashita, T.; Takizawa, H.; Mukai, H.; Ohno, K.; Maeda, T.; et al. Seasonal Variation of Serum 25-Hydroxyvitamin D Level in Hemodialysis Patients in the Northernmost Island of Japan. *Clin. Exp. Nephrol.* **2021**, *25*, 1360–1366. [CrossRef]
24. Wood, R.J.; Fleet, J.C.; Cashman, K.; Bruns, M.E.; Deluca, H.F. Intestinal Calcium Absorption in the Aged Rat: Evidence of Intestinal Resistance to 1,25$(OH)_2$ Vitamin D. *Endocrinology* **1998**, *139*, 3843–3848. [CrossRef]
25. Brooke-Wavell, K.; Jones, P.R.; Pye, D.W. Ultrasound and Dual X-Ray Absorptiometry Measurement of the Calcaneus: Influence of Region of Interest Location. *Calcif. Tissue Int.* **1995**, *57*, 20–24. [CrossRef]

26. Waud, C.E.; Lew, R.; Baran, D.T. The Relationship between Ultrasound and Densitometric Measurements of Bone Mass at the Calcaneus in Women. *Calcif. Tissue Int.* **1992**, *51*, 415–418. [CrossRef]
27. Nicholson, P.H.F.; Bouxsein, M.L. Bone Marrow Influences Quantitative Ultrasound Measurements in Human Cancellous Bone. *Ultrasound Med. Biol.* **2002**, *28*, 369–375. [CrossRef]
28. Capuani, S. Water Diffusion in Cancellous Bone. *Microporous Mesoporous Mater.* **2013**, *178*, 34–38. [CrossRef]
29. Wu, N.; Li, X.; Mu, S.; Fu, Q.; Ba, G. Handgrip Strength Is Positively Associated with Bone Mineral Density in Middle and Aged Adults: Results from NHANES 2013–2014. *Arch. Osteoporos.* **2021**, *16*, 121. [CrossRef]
30. Tachiki, T.; Kouda, K.; Dongmei, N.; Tamaki, J.; Iki, M.; Kitagawa, J.; Takahira, N.; Sato, Y.; Kajita, E.; Fujita, Y.; et al. Muscle Strength Is Associated with Bone Health Independently of Muscle Mass in Postmenopausal Women: The Japanese Population-Based Osteoporosis Study. *J. Bone Miner. Metab.* **2019**, *37*, 53–59. [CrossRef]
31. Luo, Y.; Jiang, K.; He, M. Association between Grip Strength and Bone Mineral Density in General US Population of NHANES 2013–2014. *Arch. Osteoporos.* **2020**, *15*, 47. [CrossRef]
32. Schoenau, E.; Fricke, O. Mechanical Influences on Bone Development in Children. *Eur. J. Endocrinol.* **2008**, *159* (Suppl. S1), S27–S31. [CrossRef]
33. Ho-Pham, L.T.; Nguyen, U.D.T.; Nguyen, T.V. Association between Lean Mass, Fat Mass, and Bone Mineral Density: A Meta-Analysis. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 30–38. [CrossRef]
34. Miyauchi, Y.; Sato, Y.; Kobayashi, T.; Yoshida, S.; Mori, T.; Kanagawa, H.; Katsuyama, E.; Fujie, A.; Hao, W.; Miyamoto, K.; et al. HIF1α Is Required for Osteoclast Activation by Estrogen Deficiency in Postmenopausal Osteoporosis. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 16568–16573. [CrossRef]
35. Rodan, G.A.; Martin, T.J. Therapeutic Approaches to Bone Diseases. *Science* **2000**, *289*, 1508–1514. [CrossRef]
36. Delmas, P.D. Treatment of Postmenopausal Osteoporosis. *Lancet* **2002**, *359*, 2018–2026. [CrossRef]
37. Collins, B.C.; Laakkonen, E.K.; Lowe, D.A. Aging of the Musculoskeletal System: How the Loss of Estrogen Impacts Muscle Strength. *Bone* **2019**, *123*, 137–144. [CrossRef]
38. Ikeda, K.; Horie-Inoue, K.; Inoue, S. Functions of Estrogen and Estrogen Receptor Signaling on Skeletal Muscle. *J. Steroid Biochem. Mol. Biol.* **2019**, *191*, 105375. [CrossRef]
39. Faulkner, K.G.; McClung, M.R.; Coleman, L.J.; Kingston-Sandahl, E. Quantitative Ultrasound of the Heel: Correlation with Densitometric Measurements at Different Skeletal Sites. *Osteoporos. Int.* **1994**, *4*, 42–47. [CrossRef]
40. Yamazaki, K.; Kushida, K.; Ohmura, A.; Sano, M.; Inoue, T. Ultrasound Bone Densitometry of the Os Calcis in Japanese Women. *Osteoporos. Int.* **1994**, *4*, 220–225. [CrossRef]
41. Baran, D.T.; McCarthy, C.K.; Leahey, D.; Lew, R. Broadband Ultrasound Attenuation of the Calcaneus Predicts Lumbar and Femoral Neck Density in Caucasian Women: A Preliminary Study. *Osteoporos. Int.* **1991**, *1*, 110–113. [CrossRef]

nutrients

MDPI

Article

Urinary and Daily Assumption of Polyphenols and Hip-Fracture Risk: Results from the InCHIANTI Study

Raffaello Pellegrino [1,†], Roberto Paganelli [2,†], Stefania Bandinelli [3], Antonio Cherubini [4], Cristina Andrés-Lacueva [5], Angelo Di Iorio [6,*], Eleonora Sparvieri [7], Raul Zamora-Ros [8] and Luigi Ferrucci [9]

1 Department of Scientific Research, Campus Ludes, Off-Campus Semmelweis University, 6912 Lugano, Switzerland
2 Internal Medicine, UniCamillus, International Medical University, 00133 Rome, Italy
3 Geriatric Unit, Azienda Toscana Centro, 50125 Florence, Italy
4 Geriatrics and Geriatric Emergency Care, Italian National Research Center on Aging (IRCCS-INRCA), 60126 Ancona, Italy
5 Biomarkers and Nutrimetabolomics Laboratory, Nutrition, Food Science and Gastronomy Department, Faculty of Pharmacy and Food Science, University of Barcelona, 08035 Barcelona, Spain
6 Department of Innovative Technologies in Medicine & Dentistry, University "Gabriele d'Annunzio", 66100 Chieti, Italy
7 Internal Medicine Unit, Ospedale S. Liberatore, 64100 Teramo, Italy
8 Unit of Nutrition and Cancer, Epidemiology Research Program, Catalan Institute of Oncology, Bellvitge Biomedical Research Institute (IDIBELL), 08035 Barcelona, Spain
9 Longitudinal Studies Section, Translational Gerontology Branch, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA
* Correspondence: angelo.diiorio@unich.it; Tel.: +39-087-135-53333
† These authors contributed equally to this work.

Citation: Pellegrino, R.; Paganelli, R.; Bandinelli, S.; Cherubini, A.; Andrés-Lacueva, C.; Di Iorio, A.; Sparvieri, E.; Zamora-Ros, R.; Ferrucci, L. Urinary and Daily Assumption of Polyphenols and Hip-Fracture Risk: Results from the InCHIANTI Study. *Nutrients* **2022**, *14*, 4754. https://doi.org/10.3390/nu14224754

Academic Editor: Qian Zhang

Received: 9 October 2022
Accepted: 9 November 2022
Published: 10 November 2022

Abstract: A high polyphenol intake has been associated with higher bone-mineral density. In contrast, we recently demonstrated that the urinary levels of these micronutrients were associated with the long-term accelerated deterioration of the bone. To expand on the health consequences of these findings, we assessed the association between urinary level and dietary intake of polyphenols and the 9-year risk of hip fractures in the InCHIANTI study cohort. The InCHIANTI study enrolled representative samples from two towns in Tuscany, Italy. Baseline data were collected in 1998 and at follow-up visits in 2001, 2004, and 2007. Of the 1453 participants enrolled at baseline, we included 817 participants in this study who were 65 years or older at baseline, donated a 24 hour urine sample, and underwent a quantitative computerized tomography (pQCT) of the tibia. Fracture events were ascertained by self-report over 9 years of follow-up. Thirty-six hip fractures were reported over the 9-year follow-up. The participants who developed a hip fracture were slightly older, more frequently women, had a higher dietary intake of polyphenols, had higher 24-hour urinary polyphenols excretion, and had a lower fat area, muscle density, and cortical volumetric Bone Mineral Density (vBMD) in the pQCT of the tibia. In logistic regression analyses, the baseline urinary excretion of total polyphenols, expressed in mg as a gallic acid equivalent, was associated with a higher risk of developing a hip fracture. Dietary intake of polyphenols was not associated with a differential risk of fracture. In light of our findings, the recommendation of an increase in dietary polyphenols for osteoporosis prevention should be considered with caution.

Keywords: bone-pQCT; dietary polyphenols; longitudinal study; urinary polyphenols; femur fracture risk

1. Introduction

The aging of the population leads to the increased incidence and prevalence of chronic morbidity, which imposes high costs of care and challenges the quality of life of patients and their caregivers. The identification of risk factors for chronic diseases that can be

prevented through campaigns that promote positive health behaviors play a crucial role in managing this trend. A gradual loss of bone density and quality occurs with aging and results in progressive osteopenia and osteoporosis [1,2]. Osteoporosis substantially increases the risk of skeletal fractures and further morbidity and mortality [3]. The diagnostic criteria for osteoporosis include a DEXA bone-mineral density (BMD) measurement $\geq$2.5 (SD) below the average value for young healthy women (T-score ≤ -2.5 SD), while osteopenia is characterized by a borderline decrease in BMD and is defined by a BMD T-score between -1.0 and -2.5 [4]. Preventive measures, apart from pharmacologic agents, include lifestyle adjustments, nutritional support, fall prevention strategies, and exercise [5]. Most clinical practice guidelines suggest that diets rich in calcium and vitamin D may limit osteoporosis progression [6,7], and when dietary sources are insufficient or poorly tolerated, pharmacological supplementation could be useful. However, this recommendation has been challenged in recent systematic reviews and meta-analyses [8,9]. Another lipophilic vitamin, menaquinone-7 (vitamin-K2), has been also suggested as potentially useful in preventing osteoporosis [10]; however, its high cost and the lack of agreement on optimal dosages have somewhat limited its widespread use.

Polyphenols, bioactive compounds derived from plant-based foods, have antioxidant and anti-inflammatory properties. It has been proposed that an increased intake of polyphenols can effectively slow down the osteoporosis process because of their bone anabolic action [11,12]. Among those polyphenols, naringenin, kaempferol, luteolin, quercetin, epigallocatechin 3-gallate, and resveratrol were reported to be effective in bone metabolism, at least in animal model studies [13,14]. Epidemiological studies have shown that at least five cups/day of tea, rich in polyphenols as much as in caffeine, are positively associated with higher bone-mineral density (BMD) at multiple skeletal sites, while the relationship with fracture risk is less clear [15,16]. Studies in humans have shown either a reduction or no difference in the risk of fragility fracture with tea consumption [15,17].

Contrary to this view, in the large population-based InCHIANTI study, we recently reported that higher urinary polyphenols were associated with a long-term accelerated deterioration of bone health (bone mass, diaphyseal design, and material quality), measured by peripheral quantitative computed tomography (pQCT) [18]. At least two hypotheses must be considered to explain those results: polyphenols form robust, even reversible complexes with ions, and in particular with iron and calcium (a higher consumption of polyphenols is associated with a lower prevalence of kidney stones); and the decline in glomerular filtration rate (GFR) with aging that could reduce the bioavailability of serum calcium [18]. However, the described deterioration of bone mass, diaphyseal design, and material quality associated with a higher polyphenol intake does not necessarily imply an increase in bone fracture. Therefore, the aim of this study was to evaluate the association of incident femur fractures with urinary total polyphenols (UTPs) and total polyphenol intake/day, in a cohort of free-living subjects, representative of the over-65 Italian population.

2. Materials and Methods

The design of the InCHIANTI study has been described in detail elsewhere [19]. Briefly, the study was designed by the Laboratory of Clinical Epidemiology of the Italian National Institute of Research and Care on Aging (INRCA, Florence, Italy), and was performed in two small towns in Tuscany. The baseline data were collected in 1998–2000, the three-year follow-up took place in 2001–2003, the six-year follow-up took place in 2004–2006, and the nine-year follow-up took place in 2007–2009.

2.1. Samples

Of the 1453 participants enrolled at baseline in the InCHIANTI study, 817 subjects were included in this study because they were 65 years or older, had at least one follow-up, and all variables of interest for this study were available. Participants were all European subjects and of Caucasian race. The Ethical Committee of the Local Health Authority of

Florence, Tuscany Region, approved the study protocol, and written informed consent was obtained from each participant. From the InCHIANTI study population, we selected all participants who reported an incident hip fracture during the follow-up. The subjects that reported a hip fracture at baseline were excluded from the analysis. Moreover, the few cases who experienced a bilateral or repeated hip fracture were counted only once.

2.2. Dietary Assessment

At baseline, as in the subsequent follow-ups, usual food consumption and energy intake were estimated through personal interviews and by the Italian version of the European Prospective Study into Cancer and Nutrition (EPIC) [20,21]. For the purpose of this study, the following variables were used: the number of glasses of wine consumed daily; and the intake of total dietary polyphenols (TDPs) consumed (mg/day), which was calculated according to Zamora-Ros as the sum of flavonoids, phenolic acids, lignans, stilbenes, and other polyphenols expressed as aglycone equivalents (mg/day) [22].

2.3. Urinary Total Polyphenols (UTPs)

At baseline, 24 hour urine samples were obtained from the participants. Urine samples were aliquoted and stored at −80 °C until analysis. Samples were thawed on ice and analyzed using the Folin–Ciocalteau (F–C) assay after solid-phase extraction, which yields the elimination of interfering substances that could react with the F–C assay, as described previously [23]. Intra-batch and inter-batch coefficients of variation were less than 10.5% and less than 10.7%, respectively [24]. UTP concentrations were expressed as mg of gallic acid equivalents (GAE) per 24-hour versionurine sample.

2.4. Tibial pQCT

Peripheral quantitative computed tomography (pQCT) was performed by the XCT 2000 device (Stratec Medizintechnik, Pforzheim, Germany). The description of the pQCT examination in the InCHIANTI study has been published elsewhere [1]. The images obtained from the pQCT were analyzed using BonAlyse software version 1.2 (BonAlyse Oy, Jyvaskyla, Finland). The following bone parameters were derived [25]: cortical bone area (CBA): area of the cortical bone region of the tibia cross-section in cm^2; total volumetric mineral density (vBMDtot) assessed as the average density of the total bone area in mg/cm^3; cortical volumetric mineral density (vBMDc), a selective measure of the apparent volumetric density of cortical bone, in mg/cm^3; cortical thickness (CTh), the average thickness of the circular crown formed by the centered periosteal and endocortical circumferences, in mm; medullary area (MedA), which is the difference between total and cortical bone areas and includes the marrow space and areas of the inner cortex trabecularized by endocortical resorption that has a cortical apparent vBMD < 710 mg/cm^3 and is sensitive to endocortical resorption, in cm^2; tibia cross-section total bone area in cm^2. From the pQCT images measured at 4% tibia length, the following bone parameters were obtained: bone circumference (mm); total bone area (mm^2); and cortical vBMD (mg/cm^3). Using the pQCT images obtained at 66% tibia length from the distal tip of the tibia, we also estimated the calf muscle cross-sectional area (CMCSA; expressed in cm^2), muscle density, and the bone–muscle ratio, both expressed in mg/cm^3. Lastly, from a scan performed at 90% tibia length, the maximal fat area was calculated and expressed in mm^2.

2.5. Covariates

2.5.1. Laboratory Tests

Blood samples were collected in the morning after a 12-hour fast, centrifuged, and stored at −80 °C. Serum creatinine levels (mg/dL) were measured by the Laboratory of Clinical Chemistry and Microbiological Assays, SS., Annunziata Hospital, Azienda Sanitaria 10, Florence, Italy, using a colorimetric assay (TP, Roche Diagnostics, GmbH, Mannheim, Germany) and a Roche analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). At baseline, the

analyzer used was a Hitachi 917. For the follow-ups, it was a Modular P800 Hitachi. The glomerular filtration rate was calculated according to the Cockcroft–Gault formula [26].

2.5.2. Physical Performance and Strength

The short physical performance battery (SPPB), based on lower-extremity performance tests, was used to summarize lower-extremity performance [27]. The SPPB consisted of walking speed, ability to stand from a chair, and ability to maintain balance in progressively more challenging positions. Each physical performance measure was categorized into a five-level score, with 0 representing the inability to conduct the test and 4 representing the highest level of performance. The score registered in the three measures were then added to create a summary measure ranging from 0 (worst) to 12 (best).

Handgrip strength was measured using a handheld dynamometer (hydraulic hand "BASELINE"; Smith & Nephew, Agrate Brianza, Milan, Italy). Participants were asked to perform the task twice with each hand, and the results were averaged.

Information on smoking status was collected at home interviews. BMI (in kg/m^2) was calculated using measured weight (in kg) divided by height (in m^2), both of which were measured during the medical visit.

2.6. Statistical Analysis

The urinary level of gallic acid and the intake of total dietary polyphenols were not normally distributed and were therefore normalized. The urinary level of gallic acid was cubic-root normalized (Skewness *p*-value = 0.84 and Kurtosis *p*-value = 0.15), whereas total dietary polyphenols were natural-logarithm transformed (Skewness *p*-value = 0.10 and Kurtosis *p*-value = 0.32). The baseline characteristics were compared between groups for all the variables of interest, using analysis of variance for the continuous variables and χ^2 test analyses for the dichotomous or categorical variables; moreover, in the descriptive table, we reported the *p*-values adjusted for age and sex using linear and logistic regression models, respectively. In the descriptive analysis, the normalized gallic acid urinary eq/day *p*-value was also adjusted for 24-hour creatinine clearance. The logistic regression model was used to assess the risk of developing hip fractures associated with the baseline urinary level of gallic acid considered as a continuous variable, adjusted for potential confounders that were associated with the risk of fracture at univariate analysis with a *p*-value < 0.10. Creatine clearance at 24-hour was forced into the model to counteract its confounding effect on the gallic acid urinary level. The lower median values for muscle density at 66% of tibia length (mg/cm^3), volumetric bone density BDG at 4% of tibia length (mg/cm^3), and fat area at 90% of tibia length (mm^2) were considered in the logistic model as the reference group. Due to not normally distribution of naringenin, kaempferol, luteolin, quercetin, epigallocatechin 3-gallate, and total resveratrol, and due to the difficulty in their normalization, difference comparisons between the groups (hip fracture, sex, and alcohol consumption) were evaluated through quantile regression to model the effects of covariates on the conditional quantiles of a response variable. To assess which factors were associated with TDP variation across the time of the study, a linear mixed model was analyzed, in which UTPs, age, male sex compared to female, and history of hip fracture were independent variables. Analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Thirty-six hip fractures were detected over a 9-year follow-up (4.4%); subjects who had a femur fracture were slightly older, more likely to be women ($p = 0.01$), reported a higher dietary intake of polyphenols ($p = 0.01$), and had higher values of 24-hour urinary excretion of gallic acid, independent of sex or age ($p = 0.05$) (Table 1).

Table 1. Baseline characteristics of the population according to incident hip fractures. All *p*-values were age- and sex-adjusted. Normalized gallic acid urinary eq/day *p*-value was also adjusted for 24 h creatinine clearance.

	Hip Fracture		*p*-Value
	No	Yes	
	781	36	
Age (years)	75.38 ± 7.64	76.75 ± 7.35	0.20
Sex female (*n*, %)	426 (54.6)	27 (75.0)	0.01
Alcohol consumption			0.92
Teetotal	123 (15.6)	6 (16.7)	
Former	175 (22.4)	7 (19.4)	
Current	483 (61.8)	23 (63.9)	
Cigarette smoking			0.61
Never	462 (59.2)	22 (61.1)	
Former	210 (26.9)	11 (30.6)	
Current	109 (13.9)	3 (8.3)	
Summary Performance Score (0–12)	9.77 ± 3.28	8.70 ± 3.39	0.20
Body Mass Index (kg/m^2)	27.49 ± 4.07	26.74 ± 4.77	0.22
Muscle strength (kg)	28.74 ± 12.04	25.17 ± 9.48	0.71
Normalized gallic acid urinary eq/day [1]	5.28 ± 0.74	5.43 ± 0.70	0.05
Normalized Tot. polyphenols consumed (mg/day) [2]	443.84 ± 165.88	467.17 ± 166.55	0.01
Creatinine clearance, 24-hour (mL/min)	76.00 ± 25.71	69.99 ± 27.69	0.73

[1] Gallic acid was cubic-root normalized. [2] Total polyphenols consumed was natural-logarithm transformed.

During the pQCT, participants who developed a hip fracture, compared to those who did not, had a smaller fat area (90% of tibia length), lower muscle density (66% of tibia length) and lower cortical vBMD (4% of tibia length) (Table 2).

Table 2. Baseline pQCT results according to incident femur fractures. All *p*-values were age- and sex-adjusted.

	Hip Fracture		*p*-Value
	No	Yes	
	781	36	
Fat area 90% tibia (mm^2)	2631.46 ± 1521.81	2451.89 ± 1381.59	0.007
Muscle area 66% tibia (mm^2)	6233.66 ± 1255.49	5865.70 ± 1417.17	0.79
Muscle density 66% tibia (mg/cm^3)	70.59 ± 3.66	69.08 ± 5.88	0.05
Cortical bone area 38% tibia (mm^2)	295.73 ± 75.31	275.33 ± 78.27	0.79
Volumetric BMD 38% tibia (mg/cm^3)	474.88 ± 45.49	479.21 ± 41.78	0.40
Volumetric Cortical BMD 38% tibia (mg/cm^3)	1097.97 ± 78.68	1080.51 ± 81.67	0.52
Total bone area 38% tibia (mm^2)	380.09 ± 71.51	361.26 ± 76.18	0.94
Medullar area 38% tibia (mm^2)	86.06 ± 32.13	85.93 ± 29.03	0.61
Cortical bone thickness 38% tibia (mm)	4.62 ± 1.24	4.34 ± 1.70	0.67
Bone/muscle ratio 66% tibia	0.10 ± 0.03	0.11 ± 0.04	0.57
Total vBMD 4% tibia (mm^2)	1079.87 ± 382.20	1041.26 ± 425.54	0.37
Cortical vBMD 4% tibia (mg/cm^3)	256.93 ± 52.96	228.33 ± 38.39	0.01
Bone circumference BDG 4% tibia (mm)	135.59 ± 37.51	139.59 ± 38.87	0.21

The development of a hip fracture was not associated with the prevalence and incidence of other morbidities, nor in the number of prescribed drugs, including those relevant for osteoporosis (vitamin D, bisphosphonates, or teriparatide (Supplementary Materials Tables S1 and S2)).

Table 3 shows the results of the logistic regression analysis: in Model 1, we evaluated the role of the urinary concentration of gallic acid in predicting the risk of hip fractures, while in Model 2, the effect of total polyphenols consumed daily was evaluated. Urinary gallic acid values were associated with higher fracture risks OR = 2.10; 95% CI: 1.16–3.81. The effect of the polyphenols was independent from the effect of other potential confounders included in the models, such as age, sex, BMD at 4% of tibia length, the area of adipose tissue at 90% of tibia length, and muscle density at 66% of tibia length. A greater muscle density was associated with a lower fracture risk [OR = 0.91; 95% CI: 0.83–0.99]. On the contrary, the daily dietary intake of polyphenols estimated by the EPIC questionnaire was not associated with hip-fracture risk.

Table 3. Logistic regression analysis, factors predicting fracture risk. Urinary gallic acid was cubic-root normalized; total polyphenols consumed was natural-logarithm transformed. Fat area at 90% tibia length, volumetric bone density BDG at 4% tibia length, and muscle density at 66% tibia length were contrasted in the analysis according to median level of distribution.

	Model 1		**Model 2**	
	OR (95%CI)		**OR (95%CI)**	
Normalized Gallic acid eq/day [1]	2.06 (1.12–3.76)	0.01		
Total polyphenols consumed daily (mg/day) [1]			1.71 (0.49–5.96)	n.s.
Sex				
Female	5.04 (1.53–16.6)	0.008	4.92 (1.43–16.89)	0.02
Male	reference group		reference group	
Age (years)	0.99 (0.94–1.06)	n.s.	0.99 (0.93–1.06)	n.s.
Fat area 90% tibia (mm^2)				
Upper median	0.32 (0.13–0.77)	0.01	0.36 (0.15–0.85)	0.02
Lower median	reference group		reference group	
Volumetric bone density BDG 4% tibia (mg/cm^3)				
Upper median	0.05 (0.01–0.46)	0.009	0.06 (0.01–0.54)	0.01
Lower median	reference group		reference group	
Muscle density 66% tibia (mg/cm^3)				
Upper median	0.01 (0.01–0.64)	0.03	0.01 (0.01–0.76)	0.04
Lower median	reference group		reference group	
Creatinine Clearance 24-hour [1]	0.99 (0.98–1.02)	n.s.	1.01 (0.98–1.02)	n.s.

[1] risk of femur fracture for one-unit increase. N.S. p-value > 0.05

To assess beyond the total polyphenol daily intake, the possible association of specific polyphenol intake levels with hip-fracture risk and differences in the median and 95%CI were evaluated for naringenin, kaempferol, luteolin, quercetin, epigallocatechin 3-gallate, and total resveratrol, which were age- and sex-adjusted (Table 4). Only the daily intake resveratrol showed higher and statistically significant levels in subjects without a hip fracture compared to those with fractures [−0.08 (−0.1:−0.0); p-value = 0.005]. Looking at a gender effect, we conducted a stratified analysis for sex. Male subjects showed a statistically significant higher level of daily intake of resveratrol compared to female subjects [0.18 (0.11:0.21); p-value < 0.001] age-adjusted. Lastly, to assess the potential confounding effect of alcohol consumption between sex differences, we also stratified this voluptuary. After adjusting for age and sex, teetotal subjects showed statistically signif-

icant lower levels of resveratrol, compared to the prior or current alcohol users group [−0.12 (−0.15:−0.09) *p*-value < 0.001]. A multiplicative gender for alcohol effect could not be assessed, probably due to the limited male sample that experienced hip fractures. Lastly, to verify role of UTPs independent of resveratrol intake and alcohol consumption in predicting the risk of hip fractures, we introduced resveratrol and alcohol intake as covariates in the logistic model predicting hip fractures, but the strength of the predictive risk of hip fractures associated with UTPs was practically unchanged [OR = 2.11; 95%CI (1.15–3.89); *p*-value = 0.01].

Table 4. Quantile regression models. Polyphenols consumed daily at baseline according to follow-up incident hip fracture. Data were reported as differences of the median estimates and 95%CI. All *p*-values were age- and sex-adjusted. The reference group was represented by subjects without hip fracture.

	Estimate (95%CI)	***p*-Value**
Naringenin (mg/day)	−2.18 (−5.7:1.4)	0.23
Kaempferol (mg/day)	0.28 (−0.1:0.60)	0.09
Luteolin (mg/day)	0.02 (−0.3:0.3)	0.91
Quercetin (mg/day)	−0.10 (−0.2:0.1)	0.09
Epigallocatechin 3-gallate (mg/day)	−0.28 (−2.6:2.1)	0.81
Total resveratrol (mg/day)	−0.08 (−0.1:−0.0)	0.005

To assess factors associated with TDP variation through the times of the study, linear mixed model analyses were conducted (Table 5). TDPs consumed did not vary across the time of the study (*p*-value = 0.39); UTPs were strongly associated with TDPs (*p*-value = 0.004), and no multiplicative effect could be demonstrated for the interaction with time (*p* = 0.77); with increasing age, lower UTP levels were found (*p*-value = 0.002). Lastly, the male sex showed higher levels of TDPs compared to the female sex (*p*-value < 0.001).

Table 5. Linear mixed model. Factors associated with total dietary polyphenols (TDPs) assumption, variation through the times of the study assessed with European Prospective Study into Cancer and Nutrition s(EPIC) questionnaire. Urinary total polyphenol (UTP) concentrations were expressed as normalized mg of gallic acid equivalents (GAE) per 24-hour urine. Linear Mixed Model estimates are reported as β ± SE.

	β ± SE	***p*-Value**
Intercept	592.49 ± 80.53	<0.001
Time	−18.24 ± 21.13	0.39
UTP normalized gallic acid	24.53 ± 8.47	0.004
Time for UTP normalized gallic acid	−1.16 ± 3.92	0.77
No hip fracture	−17.22 ± 21.82	0.44
Age (years)	−2.27 ± 0.73	0.002
Sex (male)	57.09 ± 9.35	<0.001

4. Discussion

The main result of this study was that high urinary polyphenol levels in the InCHIANTI population aged 65 and over were associated with a higher risk of developing hip fractures. The risk appears to be almost double for subjects with higher UTPs compared to those with lower values. Moreover, consistent with the previous literature, greater muscle density, higher median vBMD, and larger median fat area, represented independent protective factors against the development of hip fractures.

To the best of our knowledge this is the first prospective study of a large cohort of free-living subjects that assesses the role of urinary polyphenols in the risk of incident hip fractures. Several previous works have examined the potential effect of polyphenols on bone quality [28]. Epidemiological studies on tea consumption (as a source of polyphenols) demonstrated that tea could be a promising approach for increasing BMD [16]. Additionally, diet habits, namely the Mediterranean diet [29] and a regular consumption of fruit [30], have been reported to positively affect bone quality [26]. Recently, findings from the InCHIANTI study provided evidence that higher UTP levels were associated with the long-term accelerated deterioration of bone health [18]. In order to assess the level of bone health, several bone markers can be used, such as bone mass, material quality, and diaphyseal design; however, a low bone quality does not necessarily imply a higher risk of fracture, but subjects with low bone quality (osteopenia) suffer more fractures compared to osteoporotic subjects [31]. Several factors may contribute to this apparent paradox; among them is the low specificity of BMD as a predictor of femur and vertebral frailty fractures. Therefore, a higher BMD may not correlate with a reduced incidence of fracture. The evidence for the role of polyphenols in modulating the risk of fracture is at best not conclusive. Most research has focused on experimental animal models, demonstrating that polyphenols increase bone quality and could reduce the time of recovery from a fracture [32]. Clinical trials involving the oral supplementation of polyphenols in human subjects showed an enhancement of bone quality but failed to provide solid proof of a reduction in incident fracture risk [33,34]. On the contrary, our study clearly demonstrates an increase in fracture risk for the urinary polyphenols analyzed, independent from all the confounders considered. However, the total polyphenol intake in the diet did not correlate with the risk of incident fractures. Only resveratrol among the many polyphenols assumed daily and considered in the analysis showed higher levels in those subjects with incident hip factures. Additionally, in those subjects who reported to drink alcohol, resveratrol could not modulate the strength of the association between the urinary level of gallic acid and fracture risk. All together, these results indicate that resveratrol assumed daily is an indirect marker of alcohol consumption, which is a well-known factor risk of osteoporosis and hip fractures. It is difficult to speculate on the mechanism(s) which may account for our results. We may consider at least two hypotheses: the high affinity of polyphenols for iron and calcium [35]; and the decline of the glomerular filtration rate with aging [36], which might interfere with bone homeostasis and could facilitate the urinary formation of polyphenol–calcium complexes, thus reducing the bioavailability of calcium [36]. This scenario is not in agreement with a recent meta-analysis showing a reduction in serum calcium levels after resveratrol supplementation [37].

The still incomplete understanding of polyphenol metabolism is most probably the reason for these apparently conflicting results. The role of the gut microbiome on the metabolic transformation and absorption of polyphenols has only recently been recognized [38]. Therefore, the divergent roles of TDPs and UTPs in fracture risk could also be due to a phenotypical predisposition to an increased urinary excretion of polyphenols, which may translate into an increased risk of osteoporosis. This may represent an important limitation of our study, i.e., the use of UTP values as markers of polyphenol exposure, which may not be consistent with blood levels and/or dietary intake. UTPs were only measured at baseline, and this single measurement might be insufficient to fully assess the role of polyphenols in predicting bone health and fracture risk. Moreover, subjects' dietetic style could vary during life, and consequently polyphenol assumption could also vary. Therefore, the hip-fracture risk assessed in our analysis could be somehow overestimated. However, in our results, as in previous work [22], demonstrated a strong correlation between UTPs and TDPs, also independently from time (Table 5) and we could consider the UTP level constant during the time of the study. However, these limitations can be dismissed because the urinary assessment of polyphenols is a reliable and validated method [22]. An additional limitation of our study is the lack of consideration for fractures other than the femur, and the risk might be underestimated in this case. Finally, in our

study, bone turnover markers were not considered, which have emerged as promising tools in the management of osteoporosis independently from bone-mineral density (BMD) measurements [39].

5. Conclusions

This study demonstrated that higher urinary polyphenol levels are predictive of a greater risk of incident fractures in a representative, free-living Italian population. More extensive information is needed on the metabolism and effects of polyphenols on bone homeostasis and confirmed by ad hoc intervention clinical trials. In the light of the present results, the recommendation of dietary polyphenol supplementation for osteoporosis prevention should be considered with caution.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14224754/s1, Table S1: New drug prescriptions in the follow-up and drugs prescribed at the baseline to patients with hip fracture compared to subjects without hip fracture. Table S2: Diagnosis reported at baseline and during follow-up in patients with hip fracture compared to those subjects without hip fracture.

Author Contributions: Conceptualization, A.D.I. and R.P. (Roberto Paganelli); methodology, C.A.-L., R.Z.-R. and A.C.; formal analysis, A.D.I. and R.P. (Raffaello Pellegrino); investigation, E.S.; resources, L.F. and S.B.; data curation, S.B. and A.D.I.; writing—original draft preparation, A.D.I., E.S. and R.P. (Raffaello Pellegrino); writing—review and editing, S.B., C.A.-L., R.Z.-R., A.C. and L.F.; supervision, L.F.; project administration, S.B. and L.F.; funding acquisition, S.B. and L.F. All authors have read and agreed to the published version of the manuscript.

Funding: The InCHIANTI study was supported as a "targeted project" (ICS 110.1/RS97.71) by the Italian Ministry of Health and by the U.S. National Institute on Aging (contracts N01-AG-916413, N01-AG-5-0002, and N01-AG-821336, and grant R01-AG-027012).

Institutional Review Board Statement: The InCHIANTI study baseline was approved by the Ethical committee at INRCA, Ancona (protocol 14/CE, 28 February 2000) as the FU1 (protocol 45/01, 16 January 2001). InCHIANTI study FU2 and FU3 were approved by the Local Ethical Committee at Azienda Sanitaria Firenze (protocol n° 5/04, 12 May 2004) The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of INRCA di Ancona (Italy). Clinical Trial Registration: NCT01331512.

Informed Consent Statement: Written informed consent was obtained from the patients to publish this paper.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the responsible authors for the InCHIANTI study (Luigi Ferrucci) on reasonable request. Data of the InCHIANTI study is available to all researchers upon justified request using the proposal form available on the InChianti website (http://inchiantistudy.net/wp/how-to-submit-a-proposal/, accessed on 8 October 2022).

Conflicts of Interest: The authors (Angelo Di Iorio, Roberto Paganelli, Stefania Bandinelli, Antonio Cherubini, Cristin Andres-Lacueva, Raffaello Pellegrino, Eleonora Sparvieri; Raul Zamora-Ros, and Luigi Ferrucci) declare no conflict of interest.

References

1. Russo, C.R.; Lauretani, F.; Bandinelli, S.; Bartali, B.; Di Iorio, A.; Volpato, S.; Guralnik, J.M.; Harris, T.; Ferrucci, L. Aging bone in men and women: Beyond changes in bone mineral density. *Osteoporos. Int.* **2003**, *14*, 531–538. [CrossRef] [PubMed]
2. Russo, C.R.; Lauretani, F.; Seeman, E.; Bartali, B.; Bandinelli, S.; Di Iorio, A.; Guralnik, J.; Ferrucci, L. Structural adaptations to bone loss in aging men and women. *Bone* **2006**, *38*, 112–118. [CrossRef] [PubMed]
3. Hauger, A.V.; Bergland, A.; Holvik, K.; Ståhle, A.; Emaus, N.; Strand, B.H. Osteoporosis and osteopenia in the distal forearm predict all-cause mortality independent of grip strength: 22-year follow-up in the population-based Tromsø Study. *Osteoporos. Int.* **2018**, *29*, 2447–2456. [CrossRef] [PubMed]
4. Iolascon, G.; de Sire, A.; Curci, C.; Paoletta, M.; Liguori, S.; Calafiore, D.; Gimigliano, F.; Moretti, A. Osteoporosis guidelines from a rehabilitation perspective: Systematic analysis and quality appraisal using AGREE II. *Eur. J. Phys. Rehabil. Med.* **2021**, *57*, 273–279. [CrossRef]

5. Kanis, J.A.; Cooper, C.; Rizzoli, R.; Reginster, J.Y. European guidance for the diagnosis and management of osteoporosis in postmenopausal women. *Osteoporos. Int.* **2019**, *30*, 3–44. [CrossRef]
6. Levis, S.; Lagari, V.S. The role of diet in osteoporosis prevention and management. *Curr. Osteoporos. Rep.* **2012**, *10*, 296–302. [CrossRef]
7. Tangestani, H.; Djafarian, K.; Emamat, H.; Arabzadegan, N.; Shab-Bidar, S. Efficacy of vitamin D fortified foods on bone mineral density and serum bone biomarkers: A systematic review and meta-analysis of interventional studies. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 1094–1103. [CrossRef]
8. Bolland, M.J.; Leung, W.; Tai, V.; Bastin, S.; Gamble, G.D.; Grey, A.; Reid, I.R. Calcium intake and risk of fracture: Systematic review. *BMJ* **2015**, *351*, h4580. [CrossRef]
9. Bolland, M.J.; Grey, A.; Avenell, A. Effects of vitamin D supplementation on musculoskeletal health: A systematic review, meta-analysis, and trial sequential analysis. *Lancet Diabetes Endocrinol.* **2018**, *6*, 847–858. [CrossRef]
10. Zhang, Y.; Liu, Z.; Duan, L.; Ji, Y.; Yang, S.; Zhang, Y.; Li, H.; Wang, Y.; Wang, P.; Chen, J.; et al. Effect of Low-Dose Vitamin K2 Supplementation on Bone Mineral Density in Middle-Aged and Elderly Chinese: A Randomized Controlled Study. *Calcif. Tissue Int.* **2020**, *106*, 476–485. [CrossRef]
11. Torre, E. Molecular signaling mechanisms behind polyphenol-induced bone anabolism. *Phytochem. Rev.* **2017**, *16*, 1183–1226. [CrossRef] [PubMed]
12. Qiu, R.; Cao, W.T.; Tian, H.Y.; He, J.; Chen, G.D.; Chen, Y.M. Greater intake of fruit and vegetables is associated with greater bone mineral density and lower osteoporosis risk in middle-aged and elderly adults. *PLoS ONE* **2017**, *12*, e0168906. [CrossRef]
13. Jamal, R.; LaCombe, J.; Patel, R.; Blackwell, M.; Thomas, J.R.; Sloan, K.; Wallace, J.M.; Roper, R.J. Increased dosage and treatment time of Epigallocatechin-3-gallate (EGCG) negatively affects skeletal parameters in normal mice and Down syndrome mouse models. *PLoS ONE* **2022**, *17*, e0264254. [CrossRef] [PubMed]
14. Martiniakova, M.; Babikova, M.; Mondockova, V.; Blahova, J.; Kovacova, V.; Omelka, R. The Role of Macronutrients, Micronutrients and Flavonoid Polyphenols in the Prevention and Treatment of Osteoporosis. *Nutrients* **2022**, *14*, 523. [CrossRef]
15. Nash, L.A.; Ward, W.E. Tea and bone health: Findings from human studies, potential mechanisms, and identification of knowledge gaps. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 1603–1617. [CrossRef]
16. Duan, P.; Zhang, J.; Chen, J.; Liu, Z.; Guo, P.; Li, X.; Li, L.; Zhang, Q. Oolong tea drinking boosts calcaneus bone mineral density in postmenopausal women: A population-based study in southern China. *Arch. Osteoporos.* **2020**, *15*, 1–10. [CrossRef] [PubMed]
17. Chen, B.; Shi, H.F.; Wu, S.C. Tea consumption didn't modify the risk of fracture: A dose-response meta-analysis of observational studies. *Diagn. Pathol.* **2014**, *9*, 44. [CrossRef] [PubMed]
18. Di Iorio, A.; Abate, M.; Bandinelli, S.; Barassi, G.; Cherubini, A.; Andres-Lacueva, C.; Zamora-Ros, R.; Paganelli, R.; Volpato, S.; Ferrucci, L.; et al. Total urinary polyphenols and longitudinal changes of bone properties. The InCHIANTI study. *Osteoporos. Int.* **2020**, *32*, 353–362. [CrossRef]
19. Ferrucci, L.; Bandinelli, S.; Benvenuti, E.; Di Iorio, A.; Macchi, C.; Harris, T.B.; Guralnik, J.M. Subsystems Contributing to the Decline in Ability to Walk: Bridging the Gap Between Epidemiology and Geriatric Practice in the InCHIANTI Study. *J. Am. Geriatr. Soc.* **2000**, *48*, 1618–1625. [CrossRef]
20. Pisani, P. Relative validity and reproducibility of a food frequency dietary questionnaire for use in the Italian EPIC centres. *Int. J. Epidemiol.* **1997**, *26*, 152–160. [CrossRef]
21. Bartali, B.; Turrini, A.; Salvini, S.; Lauretani, F.; Russo, C.R.; Corsi, A.M.; Bandinelli, S.; D'Amicis, A.; Palli, D.; Guralnik, J.M.; et al. Dietary intake estimated using different methods in two Italian older populations. *Arch. Gerontol. Geriatr.* **2003**, *38*, 51–60. [CrossRef]
22. Zamora-Ros, R.; Achaintre, D.; Rothwell, J.A.; Rinaldi, S.; Assi, N.; Ferrari, P.; Leitzmann, M.; Boutron-Ruault, M.C.; Fagherazzi, G.; Auffret, A.; et al. Urinary excretions of 34 dietary polyphenols and their associations with lifestyle factors in the EPIC cohort study. *Sci. Rep.* **2016**, *6*, 26905. [CrossRef] [PubMed]
23. Zamora-Ros, R.; Rabassa, M.; Cherubini, A.; Urpi-Sarda, M.; Llorach, R.; Bandinelli, S.; Ferrucci, L.; Andres-Lacueva, C. Comparison of 24-hour volume and creatinine-corrected total urinary polyphenol as a biomarker of total dietary polyphenols in the Invecchiare InCHIANTI study. *Anal. Chim. Acta* **2011**, *704*, 110–115. [CrossRef] [PubMed]
24. Semba, R.D.; Ferrucci, L.; Bartali, B.; Urpí-Sarda, M.; Zamora-Ros, R.; Sun, K.; Cherubini, A.; Bandinelli, S.; Andres-Lacueva, C. Resveratrol Levels and All-Cause Mortality in Older Community-Dwelling Adults. *JAMA Intern. Med.* **2014**, *174*, 1077–1084. [CrossRef] [PubMed]
25. Capozza, R.F.; Feldman, S.; Mortarino, P.; Reina, P.S.; Schiessl, H.; Rittweger, J.; Ferretti, J.L.; Cointry, G.R. Structural analysis of the human tibia by tomographic (pQCT) serial scans. *J. Anat.* **2010**, *216*, 470–481. [CrossRef]
26. Garasto, S.; Fusco, S.; Corica, F.; Rosignuolo, M.; Marino, A.; Montesanto, A.; De Rango, F.; Maggio, M.; Mari, V.; Corsonello, A.; et al. Estimating Glomerular Filtration Rate in Older People. *Biomed Res. Int.* **2014**, *2014*, 1–12. [CrossRef]
27. Guralnik, J.M.; Ferrucci, L.; Simonsick, E.M.; Salive, M.E.; Wallace, R.B. Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. *N. Engl. J. Med.* **1995**, *332*, 556–562. [CrossRef]
28. Sacco, S.M.; Horcajada, M.N.; Offord, E. Phytonutrients for bone health during ageing. *Br. J. Clin. Pharmacol.* **2013**, *75*, 697–707. [CrossRef]
29. Byberg, L.; Bellavia, A.; Larsson, S.C.; Orsini, N.; Wolk, A.; Michaëlsson, K. Mediterranean Diet and Hip Fracture in Swedish Men and Women. *J. Bone Miner. Res.* **2016**, *31*, 2098–2105. [CrossRef]

30. Hooshmand, S.; Kern, M.; Metti, D.; Shamloufard, P.; Chai, S.C.; Johnson, S.A.; Payton, M.E.; Arjmandi, B.H. The effect of two doses of dried plum on bone density and bone biomarkers in osteopenic postmenopausal women: A randomized, controlled trial. *Osteoporos. Int.* **2016**, *27*, 2271–2279. [CrossRef]
31. Schuit, S.; Van Der Klift, M.; Weel, A.; de Laet, C.; Burger, H.; Seeman, E.; Hofman, A.; Uitterlinden, A.; Van Leeuwen, J.; Pols, H. Fracture incidence and association with bone mineral density in elderly men and women: The Rotterdam Study. *Bone* **2004**, *34*, 195–202. [CrossRef] [PubMed]
32. Ornstrup, M.J.; Brüel, A.; Thomsen, J.S.; Harsløf, T.; Langdahl, B.L.; Pedersen, S.B. Long-Term High-Dose Resveratrol Supplementation Reduces Bone Mass and Fracture Strength in Rats. *Calcif. Tissue Int.* **2017**, *102*, 337–347. [CrossRef]
33. Arjmandi, B.H.; Johnson, S.A.; Pourafshar, S.; Navaei, N.; George, K.S.; Hooshmand, S.; Chai, S.C.; Akhavan, N.S. Bone-protective effects of dried plum in postmenopausal women: Efficacy and possible mechanisms. *Nutrients* **2017**, *9*, 496. [CrossRef] [PubMed]
34. Bo, S.; Gambino, R.; Ponzo, V.; Cioffi, I.; Goitre, I.; Evangelista, A.; Ciccone, G.; Cassader, M.; Procopio, M. Effects of resveratrol on bone health in type 2 diabetic patients. A double-blind randomized-controlled trial. *Nutr. Diabetes* **2018**, *106*, 1292–1299. [CrossRef] [PubMed]
35. Ahmed, S.; Hasan, M.M.; Khan, H.; Mahmood, Z.A.; Patel, S. The mechanistic insight of polyphenols in calcium oxalate urolithiasis mitigation. *Biomed. Pharmacother.* **2018**, *106*, 1292–1299. [CrossRef]
36. Kurajoh, M.; Inaba, M.; Nagata, Y.; Yamada, S.; Imanishi, Y.; Emoto, M. Association of cystatin C- and creatinine-based eGFR with osteoporotic fracture in Japanese postmenopausal women with osteoporosis: Sarcopenia as risk for fracture. *J. Bone Miner. Metab.* **2018**, *37*, 282–291. [CrossRef]
37. Asis, M.; Hemmati, N.; Moradi, S.; Nagulapalli Venkata, K.C.; Mohammadi, E.; Farzaei, M.H.; Bishayee, A. Effects of resveratrol supplementation on bone biomarkers: A systematic review and meta-analysis. *Ann. N. Y. Acad. Sci.* **2019**, *1457*, 92–103. [CrossRef]
38. Cladis, D.P.; Debelo, H.; Lachcik, P.J.; Ferruzzi, M.G.; Weaver, C.M. Increasing Doses of Blueberry Polyphenols Alters Colonic Metabolism and Calcium Absorption in Ovariectomized Rats. *Mol. Nutr. Food Res.* **2020**, *64*, e2000031. [CrossRef]
39. Bhattoa, H.P.; Cavalier, E.; Eastell, R.; Heijboer, A.C.; Jørgensen, N.R.; Makris, K.; Ulmer, C.Z.; Kanis, J.A.; Cooper, C.; Silverman, S.L.; et al. Analytical considerations and plans to standardize or harmonize assays for the reference bone turnover markers PINP and β-CTX in blood. *Clin. Chim. Acta.* **2021**, *515*, 16–20. [CrossRef]

nutrients

MDPI

Article

Vitamin K-Dependent Carboxylation of Osteocalcin in Bone—Ally or Adversary of Bone Mineral Status in Rats with Experimental Chronic Kidney Disease?

Marta Ziemińska [1], Dariusz Pawlak [2], Beata Sieklucka [2], Katarzyna Chilkiewicz [1] and Krystyna Pawlak [1,*]

[1] Department of Monitored Pharmacotherapy, Medical University of Bialystok, Mickiewicza 2C Str., 15-222 Bialystok, Poland
[2] Department of Pharmacodynamics, Medical University of Bialystok, Mickiewicza 2C Str., 15-222 Bialystok, Poland
* Correspondence: krystyna.pawlak@umb.edu.pl; Tel.: +48-85-7485600

Abstract: Chronic kidney disease (CKD) commonly occurs with vitamin K (VK) deficiency and impaired bone mineralization. However, there are no data explaining the metabolism of endogenous VK and its role in bone mineralization in CKD. In this study, we measured serum levels of phylloquinone (VK1), menaquinone 4 and 7 (MK4, MK7), and VK-dependent proteins: osteocalcin, undercarboxylated osteocalcin (Glu-OC), and undercarboxylated matrix Gla protein (ucMGP). The carboxylated osteocalcin (Gla-OC), Glu-OC, and the expression of genes involved in VK cycle were determined in bone. The obtained results were juxtaposed with the bone mineral status of rats with CKD. The obtained results suggest that the reduced VK1 level observed in CKD rats may be caused by the accelerated conversion of VK1 to the form of menaquinones. The bone tissue possesses all enzymes, enabling the conversion of VK1 to menaquinones and VK recycling. However, in the course of CKD with hyperparathyroidism, the intensified osteoblastogenesis causes the generation of immature osteoblasts with impaired mineralization. The particular clinical significance seems to have a finding that serum osteocalcin and Glu-OC, commonly used biomarkers of VK deficiency, could be inappropriate in CKD conditions, whereas Gla-OC synthesized in bone appears to have an adverse impact on bone mineral status in this model.

Keywords: bone mineral status; chronic kidney disease (CKD); genes of vitamin VK cycle; vitamin K (VK); VK-dependent proteins; 5/6 nephrectomy model

Citation: Ziemińska, M.; Pawlak, D.; Sieklucka, B.; Chilkiewicz, K.; Pawlak, K. Vitamin K-Dependent Carboxylation of Osteocalcin in Bone—Ally or Adversary of Bone Mineral Status in Rats with Experimental Chronic Kidney Disease? *Nutrients* **2022**, *14*, 4082. https://doi.org/10.3390/nu14194082

Academic Editor: Qian Zhang

Received: 8 September 2022
Accepted: 27 September 2022
Published: 1 October 2022

1. Introduction

Chronic Kidney Disease Mineral and Bone Disorder (CKD-MBD) is a common complication of CKD, associated with abnormalities in bone metabolism and impaired bone mineralization [1–3]. In the course of CKD-MBD, both quality and quantity of bone tissue have been compromised, and the development of osteoporosis has an impact on a substantial increased risk of fractures and mortality in those patients [4,5].

Throughout life, bone undergoes a remodeling process in which the amount of resorbed bone should be equivalent to the amount of new bone formation and appropriate mineralization [6,7]. In the regulation of the mineralization process in CKD, many factors play a key role, such as parathormon (PTH), vitamin D, and vitamin K (VK) [8,9]. Vitamin K occurs in two forms—K1 (phylloquinone) and K2 (menaquinones, MKs). The most common MK in humans is the short-chain menaquinone 4 (MK4), which can be produced by endogenous conversion of phylloquinone to menaquinones with the enzyme UbiA prenyltransferase containing 1 (UBIAD1) [10–12]. The long chain MKs, such as menaquinone 7 (MK7), are found in fermented foods [13]. The pivotal role of vitamin K acts as a cofactor for the enzyme γ-glutamyl carboxylase (GGCX) in the gamma-carboxylation reaction, which in proper course is closely associated with its recycling, defined as the

"vitamin K cycle". The vitamin K epoxide is converted into the quinone form by vitamin K epoxide reductase complex subunit 1 (VKORC1). The transformation of the quinone to VK hydroquinone form occurs through quinone reductase. This form of VK can be reused as a cofactor for GGCX in the gamma-carboxylation reaction [14]. In the course of that reaction, carboxyl groups are added to Glu residues in proteins and transformed to Gla domains. This process transforms inactive (undercarboxylated) proteins into active (carboxylated) vitamin K-dependent proteins (cVKDPs), such as matrix Gla protein (MGP) and osteocalcin (OC) [15]. The increased circulating levels of undercarboxylated VKDPs—Glu-OC and ucMGP—reflect VK deficiency, and the measurement of these proteins is used to determine VK status [16,17].

Patients with CKD frequently suffer from subclinical VK deficiency, which may result from dietary restrictions, poor nutrient intake, and using drugs such as proton-pump inhibitors, steroids, statins, antihypertensives drugs, or broad-spectrum antibiotics, which decrease vitamin K synthesis by the impairment of the natural intestinal microflora [18–22]. Subclinical VK deficiency was confirmed in hemodialysis (HD) patients on the grounds of the high levels of ucOC, dephosphorylated-uncarboxylated MGP (dp-ucMGP), and low levels of VK1 [21,23]. In CKD, VK metabolism and recycling may also be impaired. McCabe et al. [24] assessed the genes expression of the VK cycle in a rat model of adenine-induced CKD and observed the reduced GGCX and VKORC1 expression in the thoracic aorta and a decreased level of UBIAD1 in the kidney.

Despite a small amount of research on the relationship between VK and bone health in CKD patients, it has been observed that low VK status has been linked to an increased risk of bone fracture [25,26] or reduced bone mineral density (BMD) [27–30]. Studies on the effect of VK supplementation on BMD are limited and inconclusive. In some research, an increase in BMD [31–36] or bone mineral content (BMC) [37] was observed under the influence of VK supplementation. However, in other studies, the above relationship was not shown [38,39]. MK7 supplementation reduced the levels of ucOC and dp-ucMGP in hemodialysis patients [40–42], whereas MK4 supplementation prevented bone loss in steroid-treated glomerulonephritis patients [43].

In the available literature, there is a lack of research explaining the role of endogenous VK in the bone mineralization process in the course of CKD. Therefore, the aim of the present study was the comprehensive assessment of endogenous VK metabolism in rats with CKD through determination of VK1, MK4, MK7, and undercarboxylated VKDPs—Glu-OC and ucMGP levels in serum, and the measurement of Gla-OC, Glu-OC levels, and their ratios in trabecular and cortical region of femurs. Then, the expression of genes related to VK recycling in bone were investigated. The obtained results were juxtaposed with the bone mineral status of uremic rats.

2. Materials and Methods

2.1. Animals

Twenty-six 4-week-old male Wistar rats were randomly assigned into two groups: the control group (CON, $n = 10$) subjected to sham-operation by renal decapsulation, and the experimental group (CKD, $n = 16$) after a two-step surgical 5/6 nephrectomy. During the experiment, animals were kept in conventional cages in vivarium conditions (humidity of 50%, 24 °C, and 12-h/12-h light/dark cycle) with unlimited access to sterilized tap water. The rats were fed a standard diet (Ssniff R/M-H) composed of 19% protein, 1% calcium, 0.70% phosphorus, 1000 IU of vitamin D3 per kg, and 5 mg of vitamin K as menadione per kg. The experiment's overall time course lasted 24 weeks in order to monitor the development and progression of CKD. Finally, blood samples and femurs were obtained from the anesthetized animals and were secured for further studies. In the presented experiment, we employed materials from our previous research, where we gave a detailed description of animals' characteristics, tissue collection, and applied procedures [44]. Briefly, markers of kidney function—serum urea and creatinine concentrations—were increased in CKD rats compared to CON (both $p < 0.001$). The animals with CKD had significantly

higher levels of PTH ($p < 0.01$), whereas 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] levels were comparable to healthy animals [44].

2.2. Measurement of Vitamin K Concentrations in Rat Serum

Vitamins K (i.e., phylloquinone (VK1), Menaquinone 4 (MK4), and Menaquinone 7 (MK7) were determined by the Laboratory of Perlan Technologies Polska, based in Gdynia, Poland. The following reagents and solvents from Merck KGaA were used: LC-MS grade water, LC-MS grade methanol, acetonitrile, hexane, bovine serum albumin (BSA), ammonium formate solution, formic acid, and standards of: vitamin K1 (phylloquinone), vitamins K2: MK4 (Menaquinone-4), and MK7 (Menaquinone-7) from Supelco Analytical Products(Merck Life Science Sp.z.o.o., Darmstadt, Germany), Deuterium-labelled internal standard of the vitamin K1-[d7] (phytonadione) were obtained from IsoSciences LLC (Ambler, PA, USA). Serum samples (100 µL) were spiked with Vitamin K1-[d7] ISTD, vortexed briefly, and acetonitrile was added to each tube, vortexed for 1 min, followed by hexane and again vortexed for 1 min. The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature. The final dry extract was dissolved in acetonitrile and transferred to an MS vial. Aliquots of 10 µL were automatically injected into the HPLC system.

The Agilent Technologies 1260 LC system(Agilent Technologies, Santa Clara, CA, USA). was used for vitamins K analysis, including an autosampler, binary pump, and thermostated column compartment with G6470A Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA). The sample separation was carried out on a reversed phase column Zorbax SB-C8 RRHT, 3.0 × 50 mm, 1.8 µm, 600 bar (Agilent Technologies, Santa Clara, CA, USA). The column temperature was kept constant at 30 °C. A gradient elution system was used with a flow rate of 0.4 mL/min. The binary gradient system consisted of 0.1% formic acid and 5 mM ammonium formate in water mobile phase (eluent A) and methanol acidified with 0.1% formic acid (eluent B). Gradient elution was applied as follows: 0 min—70% A, 30% B; 2 min—10% A, 90% B; 3 min—0% A, 100% B; 15 min—0% A, 100% B; 20 min—70% A, 30% B. The AJS ESI ion source operated in positive ion mode with drying gas temperature of 330 °C and 8 L/min gas flow. The sheath gas temperature was set to 320 °C with a flow of 11 L/min. Nitrogen was used as a nebulizer gas (45 psi) and ultrahigh-purity nitrogen was used as collision gas. The capillary and nozzle voltages were 3000 V and 1000 V, respectively. Identification and quantification were based on MS/MS multiple reaction monitoring (MRM). An overview of the MRM transitions, collision energies, and retention time for the analytes is given in Table 1. All aspects of system operation and data acquisition were controlled using Agilent MassHunter Workstation Software (version B.10.00).

Table 1. LC-MS/MS parameters.

	Precursor ion (*m*/*z*)	Production (*m*/*z*)	Collision Energy (V)	Retention Time (min)
K1	451.4	187.2	26	6.02
K1-d7	458.4	194.3	26	6
MK4	445.3	187.3	18	5.42
MK7	649.5	187.2	38	7.35

K1—phylloquinone; K1-d7—deuterium-labelled internal standard of the vitamin K1; MK4—Menaquinone-4; MK7—Menaquinone-7.

2.3. Serum and Bone Levels of Vitamin K-Dependent Proteins

Undercarboxylated osteocalcin (Glu-OC), carboxylated osteocalcin (Gla-OC), and total osteocalcin were determined by ELISA kits: Rat Glu-Osteocalcin High Sensitive EIA, Rat Gla-Osteocalcin High Sensitive EIA (Takara Bio Inc., Shiga, Japan), and Rat Osteocalcin ELISA kit from Immutopics, Inc., San Clemente, CA, USA, respectively. Rat undercarboxylated Matrix Gla Protein (ucMGP) was quantified in serum by ELISA kit from

MyBioSource, Inc., San Diego, CA, USA, according to the manufacturer's recommendations. Trabecular and cortical concentrations of Gla-OC and Glu-OC were adjusted for protein concentration, and the ratio Gla-OC/Glu-OC was calculated.

2.4. Preparation of Bone Tissue Homogenates

The procedure of the preparation of 10% homogenates from bone tissue, using a high-performance homogenizer (Ultra-Turrax T25; IKA, Staufen, Germany) with a stainless-steel dispersing element (S25N-8G; IKA), has been described in detail previously [45]. Briefly, segments of bone tissue were taken from the femoral diaphysis and distal femoral epiphysis (from the cortical bone region and trabecular bone region, respectively). Collected materials were weighed, closely rinsed, and then homogenized in the cold potassium phosphate buffer (50 mM, pH = 7.4; POCh). The supernatant was obtained by the centrifugation of 10% homogenates for 10 min at 700× *g* at 4 °C and stored at −80 °C.

2.5. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from femoral tissue using Thermo Scientific GeneJET RNA Purification Kit (Thermo Scientific, Vilnius, Lithuania), and Quantitative RT-PCR was performed as previously described [46]. Briefly, using the Thermo Scientific NanoDrop 2000 spectrophotometer (Waltham, MA, USA), RNA was quantified, and its quality was confirmed. The RevertAid™ First Stand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to reverse-transcribe total RNA (1 µg). In order to perform quantitative real-time PCR, the SYBR Green Master Mix was used (EURx, Gdańsk, Poland), and relative quantification of gene expression was performed by the comparative CT method (ΔΔCT). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a housekeeping gene to normalize expression level [46]. Primers were designed using Primer-BLAST software(Primer3 program). The primer sequences were (5′-3′ forward-reverse): VKORC1 (5′-TCCCGCGTCTTCTCCTCTC-3′; 5′-CCAACGTCCCCTCAAGCAAC-3′), GGCX (5′-GGATGCTGACTGGGTTGAGG-3′; 5′-GCTCCTCCGACAACACTAGC-3′) and UBIAD1 (5′-GCTGTGTGTGTGCTGCTTAC-5′; CCCAGTGCCACGTACTTGAA-3′).

2.6. Genes of Osteoblastogenesis

The expression of key genes involved in osteoblastogenesis, such as: Forkhead Box Transcription Factor 1 (FOXO1), Activating Transcription Factor 4 (ATF4), Runt-Related Transcription Factor 2 (RUNX2), Alkaline Phosphatase (ALP), and Bone Gamma-Carboxyglutamate Protein (BGLAP) were determined previously [46].

2.7. The Mineral Status of Femurs

Using the appropriate small animal software, bone densitometry scans were carried out on a Horizon QDR Series X-ray Bone Densitometer (Hologic Inc., Bedford, MA, USA). For all rats were conducted whole-femur measurements—bone mineral area, bone mineral density (BMD), and bone mineral content (BMC). Moreover, at the distal metaphysis (R1 region) and midshaft (R2 region), the subregional BMC and BMD of small uniform areas were quantified. The R1 region is constituted of mixed trabecular and cortical bone, whereas the R2 region represents cortical bone. The results of these densitometric measurements have been shown elsewhere [44].

2.8. Statistical Analysis

Shapiro–Wilk tests were performed to determine if continuous variables were normally distributed. Normally distributed data were expressed as mean ± SD. Non-Gaussian data were presented as median (25th to 75th percentiles). The two groups (CON and CKD) were compared by using an unpaired *t*-test with Welch correction or nonparametric Mann–Whitney Test. $p < 0.05$ was the accepted level of significance. The correlations between study variables were calculated using Spearman's rank correlation analysis. Computations were performed using Statistica ver.13 software (StatSoft, Tulsa, OK, USA), and the graphic

design presentation of the results was performed using GraphPad Prism 6.0 software (San Diego, USA).

3. Results

3.1. The Status of Endogenous Vitamin K in Rats with CKD

As has been shown in Figure 1, the levels of vitamin K were changed in chronic kidney disease. The concentration of VK1 in CKD was lower than in controls (Figure 1A). In contrast, we observed a considerable increase in MK7 (Figure 1B), especially in MK4 levels (Figure 1C) in uremic rats. The ratio of MK7/VK1 and MK4/VK1 indicates how efficiently phylloquinone is converted to menaquinone (VK2). In the present study, a significant rise in the above ratios was shown, particularly in MK4/VK1 ratio in CKD compared with the controls (Figure 1D,E).

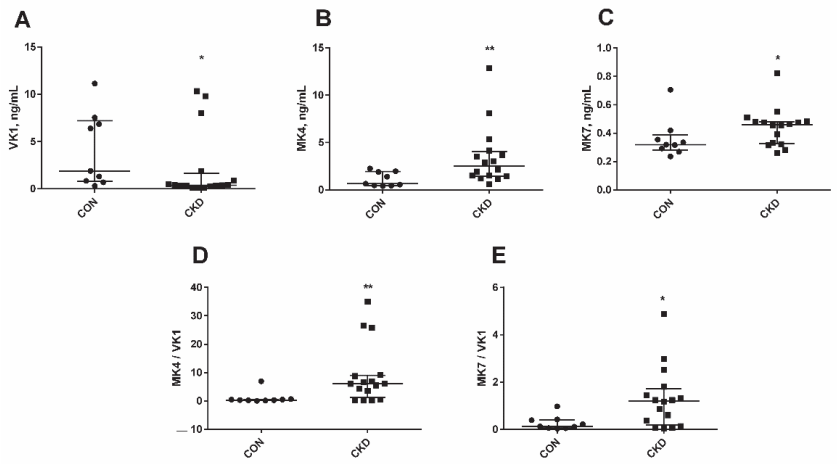


Figure 1. Vitamin K1 (**A**), MK4 (**B**), MK7 (**C**) concentrations, and the ratios of MK4/VK1 (**D**) and MK7/VK1 (**E**) in serum rats with chronic kidney disease (CKD) and healthy controls (CON) fed with a standard rodent diet. The lines correspond to the 25th and 75th percentiles and the median. * $p < 0.05$, ** $p < 0.01$ CON versus CKD rats; VK1—phylloquinone; MK4—Menaquinone 4; MK7—Menaquinone 7; the circles represent results in controls; the squares represent results in CKD.

3.2. Serum Levels of Vitamin K-Dependent Proteins in Rats with CKD, and the Impact of Kidney Function and PTH on Their Concentrations

The concentrations of total osteocalcin, its undercarboxylated form (Glu-OC), and uncarboxylated matrix Gla protein (ucMGP) are shown in Figure 2. These proteins reflect VK status and are used as markers of VK deficiency. A significant increase was observed in both total OC and Glu-OC in rats with CKD in comparison with healthy animals (Figure 2A,B). However, there was no difference in the ucMGP concentration between uremic rats and controls (Figure 2C).

Both total osteocalcin and its undercarboxylated form (Glu-OC) in serum were positively correlated with kidney function markers: creatinine and urea concentrations in CKD rats (Figure 3A,B,D,E). The inverse relationship was observed between total osteocalcin and creatinine clearance (Figure 3C), whereas circulating Glu-OC was related to PTH concentrations (Figure 3F).

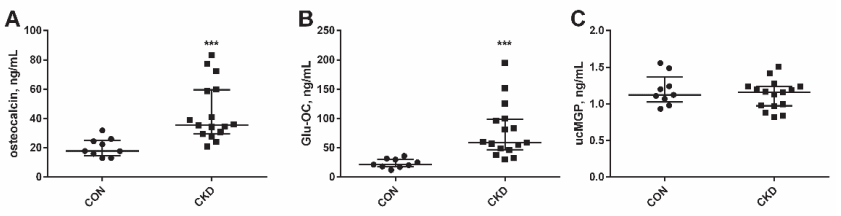


Figure 2. Serum levels of vitamin K-dependent proteins: osteocalcin (**A**), Glu-OC (**B**) and ucMGP (**C**) in rats with chronic kidney disease (CKD) and healthy controls (CON). The lines correspond to the 25th and 75th percentiles and the median. *** $p < 0.001$ CON versus CKD rats; Glu-OC—undercarboxylated osteocalcin; ucMGP—undercarboxylated Matrix Gla Protein.

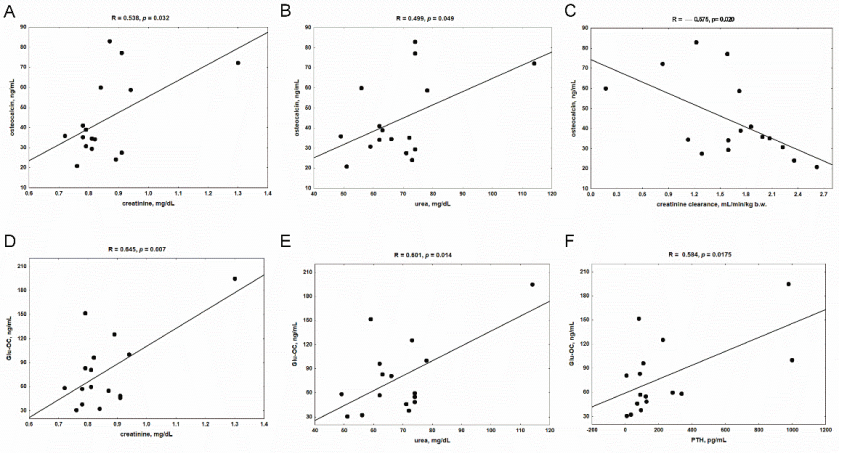


Figure 3. The associations between serum levels of osteocalcin and its undercarboxylated form (Glu-OC), and markers of kidney function: creatinine (**A**,**D**), urea (**B**,**E**), creatinine clearance (**C**) and parathyroid hormone (PTH) concentration (**F**) in rats with chronic kidney disease (CKD).

3.3. The Levels of Glu-OC, Gla-OC, and Gla-OC/Glu-OC Ratios in Femoral Bone Tissue of Rats with CKD, and Their Relations with Serum Glu-OC

In the current study, Gla-OC and Glu-OC concentrations and the Gla-OC/Glu-OC ratio were measured in trabecular (Figure 4A–C) and cortical (Figure 4D–F) bone tissue. Gla-OC levels in trabecular bone did not differ between the studied groups (Figure 4A), whereas Glu-OC level was considerably lower in CKD than in controls (Figure 4B). It is interesting to note that in CKD, the Gla-OC level in cortical bone tissue significantly increased (Figure 4D), while the Glu-OC concentration was reduced when compared to healthy animals (Figure 4E). Moreover, in both bone areas, the Gla-OC/Glu-OC ratios in uremic rats were considerably higher than in CON (Figure 4C,F).

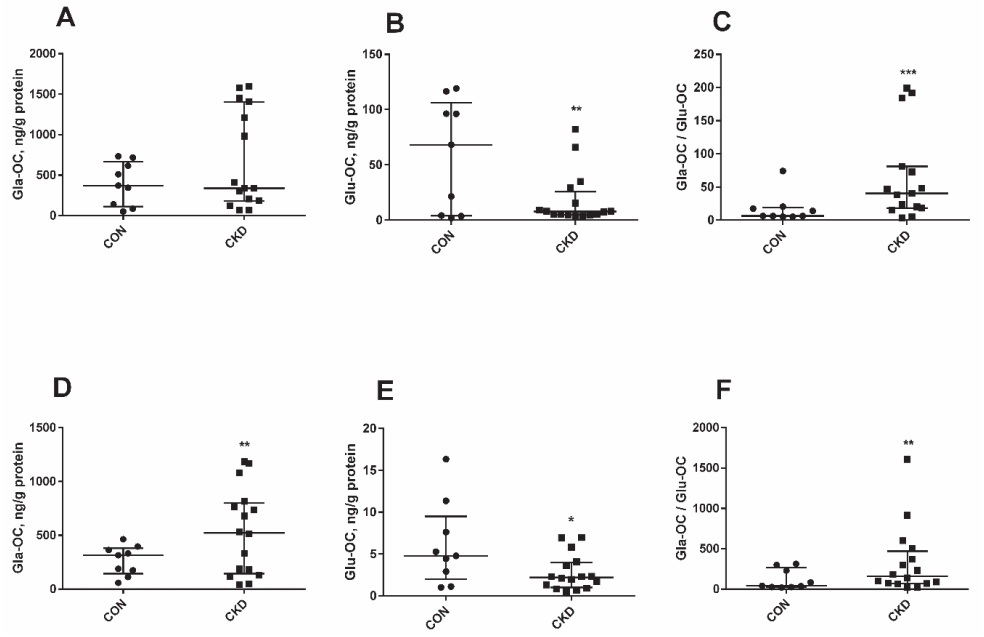


Figure 4. The levels of carboxylated osteocalcin (Gla-OC), undercarboxylated osteocalcin (Glu-OC) and their ratios (Gla-OC/Glu-OC) in trabecular (**A**–**C**) and cortical (**D**–**F**) femoral bone tissue. The lines correspond to the 25th and 75th percentiles and the median. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CON versus CKD rats; CKD—chronic kidney disease; CON—controls.

In CKD group, we observed a positive association between Gla-OC concentrations and Glu-OC/Gla-OC ratios in trabecular (Figure 5A) and in cortical (Figure 5B) bone regions. The weak correlation was also noticed between Glu-OC and Gla-OC levels in trabecular (R = 0.512, $p = 0.045$), but not in cortical bone tissue (R = 0.105, NS). Among the analyzed parameters, only the Gla-OC/Glu-OC ratio in the trabecular bone region was inversely correlated with the concentration of Glu-OC in the serum (Figure 5C).

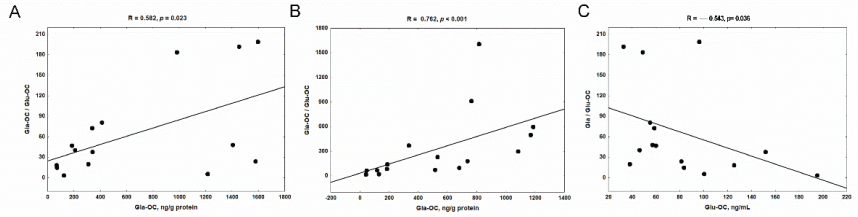
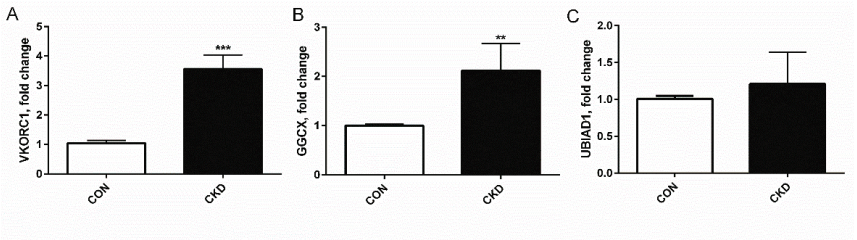


Figure 5. The associations between carboxylated osteocalcin (Gla-OC) and Gla-OC/Glu-OC ratio in trabecular (**A**) and cortical (**B**) bone tissue; the relations between trabecular Gla-OC/Glu-OC ratio and serum undercarboxylated osteocalcin (Glu-OC) levels; (**C**) in rats with chronic kidney disease (CKD).

3.4. The Expression of Genes Coding Vitamin K Cycle Enzymes in Femurs of Rats with CKD, and Their Associations with Genes Participating in Osteoblastogenesis

The expression of VKORC1 and GGCX gene was significantly higher in uremic rats compared to the control group (Figure 6A,B), whereas the increase in the UBIAD1 gene did not achieve a statistically significant level (Figure 6C).



D

	FOXO1	ATF4	RUNX2	ALP	BGLAP
VKORC1	**R = 0.632**	**R = 0.649**	*R = 0.430*	*R = 0.462*	R = 0.195
	p = 0.009	**p = 0.006**	*p = 0.096*	*p = 0.071*	NS
GGCX	**R = 0.538**	**R = 0.568**	R = 0.259	R = 0.361	R = 0.340
	p = 0.031	**p = 0.022**	NS	NS	NS
UBIAD1	**R = 0.839**	**R = 0.556**	*R = 0.445*	R = 0.440	R = 0.154
	p < 0.0001	**p = 0.031**	*p = 0.087*	NS	NS
Glu-OC	R = 0.361	**R = 0.628**	**R = 0.552**	*R = 0.464*	R = 0.013
	NS	**p = 0.012**	**p = 0.033**	*p = 0.081*	NS

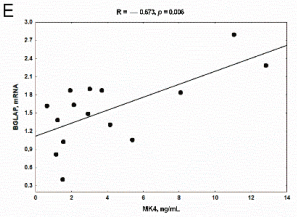


Figure 6. The expression of genes of vitamin K cycle enzymes in femurs of rats with chronic kidney disease (CKD) (**A**–**C**), and their associations with the expression of genes of osteoblastogenesis and trabecular Glu-OC levels (**D**), as well as the relationship between serum MK4 level and BGLAP expression in bone of rats with CKD (**E**). ** $p < 0.01$, *** $p < 0.001$ CON versus CKD rats; CON—controls; VKORC1—vitamin K epoxide reductase complex subunit 1; GGCX—the gamma-glutamyl carboxylase; UBIAD1—UbiA prenyltransferase domain-containing protein 1; FOXO1—Forkhead box transcription factor 1; ATF4—activating transcription factor 4; RUNX2—Runt-related transcription factor 2; ALP—alkaline phosphatase; BGLAP—bone gamma-carboxyglutamate protein; Glu-OC—undercarboxylated osteocalcin; MK4—menaquinone 4; the bold text indicates statistically significant correlations; italic text indicates tendency to correlation.

Our previous research performed on the bones of rats with CKD established that the expression of FOXO1, ATF4, RUNX2, and ALP, which are the key genes responsible for the initial phases of osteoblast development, was significantly increased, whereas the expression of BGLAP, which is linked to the late stage of osteoblast differentiation, was only slightly elevated [46]. As has been shown in Figure 6D, the expression of the genes involving in VK cycle (especially VKORC1 and UBIAD1) was strongly and positively correlated with the expression of the genes of the early stages of osteoblast development. The tendency of these positive relationships was also seen between VK cycle genes expression and the markers of osteoblast differentiation, such as RUNX2 and ALP. Interestingly, we also observed the association between trabecular Glu-OC levels and the expression of genes reflecting the osteoblast differentiation (ATF4, RUNX2, ALP). Other interesting observations were the lack of the correlation between BGLAP expression and the genes belonging to VK cycle and trabecular Glu-OC levels (Figure 6D), as well as the strong positive association between BGLAP mRNA and circulating MK4 level (Figure 6E).

3.5. The Relationship between the Expression of VKORC1 and the Concentration of Gla-OC in Trabecular Bone Tissue and Serum Glu-OC

Next, we analyzed the relations between the expression of VK cycle enzymes and both forms of osteocalcin in femoral bone of rats with CKD. There was no correlation between GGCX and the levels of Gla-OC and Glu-OC. Surprisingly, we found a positive relationship between VKORC1 expression and Gla-OC concentration in trabecular bone tissue (Figure 7A), as well as a strong inverse relationship between VKORC1 expression and serum Glu-OC concentration (Figure 7B).

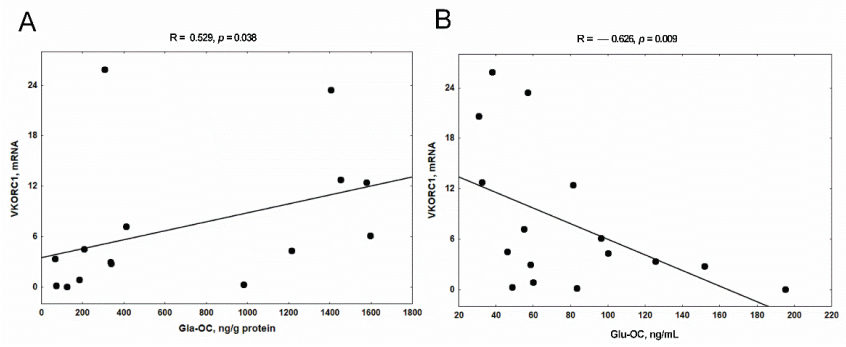


Figure 7. The association between bone VKORC1 expression and Gla-OC levels in trabecular bone (**A**), and VKORC1 expression and Glu-OC levels in serum of rats with CKD (**B**). MK4—menaquinone 4; BGLAP—bone gamma-carboxyglutamate protein; VKORC1—vitamin K epoxide reductase complex subunit 1; Gla-OC—carboxylated osteocalcin; Glu-OC—undercarboxylated osteocalcin; CKD—chronic kidney disease.

3.6. The Associations between Bone Glu-OC, Gla-OC, and Bone Mineral Status in Rats with CKD

Rats with CKD had significantly decreased densitometric parameters compared with controls in the whole-femur measurements, namely in the bone mineral area ($p < 0.05$), BMC, and BMD (both $p < 0.01$). These differences were particularly seen in the metaphyseal area (R1) of femurs, where the values of BMC and BMD were significantly decreased in rats with CKD compared to controls (both $p < 0.001$). In contrast, mineral status measured in the diaphyseal area (R2) was only slightly impaired—the values of BMC were lower in CKD than in controls ($p < 0.01$), whereas BMD did not differ between the studied groups (for details please see our previous study [44]).

In the present study, we analyzed the associations between both forms of osteocalcin and their ratios measured in trabecular and cortical bone tissue, with these densitometric parameters of femurs. At the level of the trabecular bone, we have demonstrated that only Gla-OC/Glu-OC ratio was strongly and inversely related to bone mineral area (Figure 8A) and BMC (Figure 8B) in uremic rats.

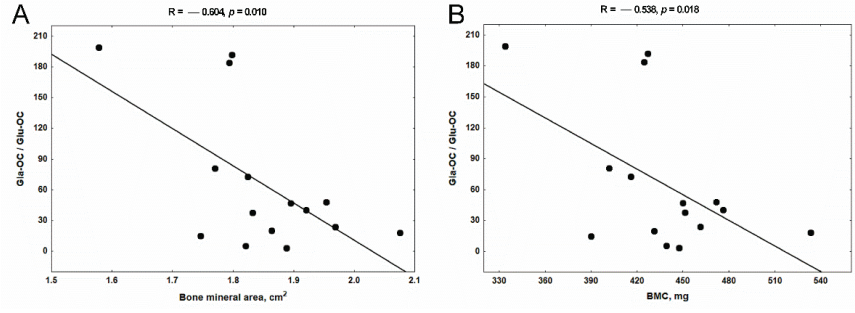


Figure 8. The associations between Gla-OC/Glu-OC ratio in trabecular bone tissue and the parameters of mineral status: bone mineral area (**A**) and BMC (**B**) of femoral bone of rats with chronic kidney disease (CKD). Gla-OC—carboxylated osteocalcin; Glu-OC—undercarboxylated osteocalcin; BMC—bone mineral content.

Surprisingly, at the level of the cortical bone, we observed that only Glu-OC values were positively associated with the whole-femur values of BMC (Figure 9A) and BMD (Figure 9D). These relations were particularly strong in the diaphyseal area (R2), which is

rich in cortical bone (Figure 9B,E), but they also were noticeable in the metaphyseal area (R1) of femurs (Figure 9C,F).

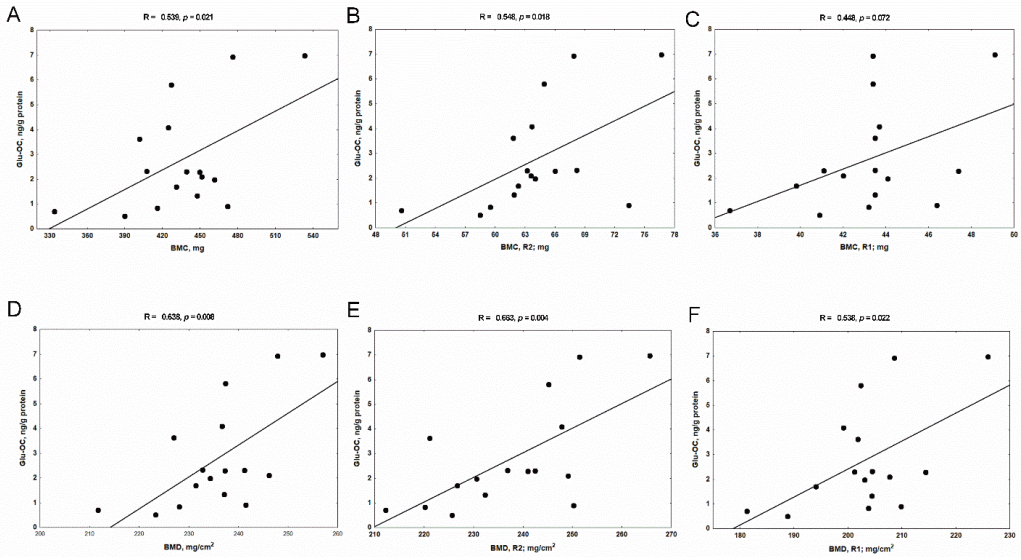


Figure 9. The associations between undercarboxylated osteocalcin (Glu-OC) levels in cortical bone tissue and whole BMC, BMD (**A**,**D**), diaphyseal BMC, BMD (**B**,**E**) and metaphyseal BMC, BMD (**C**,**F**) of femurs of rats with chronic kidney disease (CKD). BMC—bone mineral content; BMD—bone mineral density; R1—metaphyseal area; R2—diaphyseal area of femur.

4. Discussion

Although a high prevalence of subclinical vitamin K (VK) deficiency has been described in patients with CKD [18–22] and there are suggestions that this condition can impact bone quality [47,48], there are a lack of experimental data on this issue. To the best of our knowledge, this is the first comprehensive study in which we determined the endogenous VK metabolism, the bone levels of VK-dependent proteins (VKDPs), and the expression of VK cycle enzymes in femoral bone of rats with 5/6 nephrectomy. For determination of the impact of endogenous vitamins K on bone mineralization in this CKD model, the obtained results were juxtaposed with the parameters of the mineral status of femurs.

Among the K vitamins, the concentrations of phylloquinone (VK1), menaquinone 4 (MK4), and menaquinone 7 (MK7) were measured in rats' serum. Despite the fact that all rats have received the same standard diet supplemented with menadione, the VK status of uremic rats was altered, with a decrease in VK1 concentration and a significant increase in MK7, particularly in MK4 levels. VK1 is the major dietary form of VK, and the primary form found in circulation [49]. VK1 is endogenously converted to MK4 via a menadione intermediary [50–52]. Hirota et al. [52] were the first to show that the release of menadione from VK1 and its conversion to MK4 occurred in the intestine of rats, therefrom MK4 entered the blood circulation through the mesenteric lymphatic system. On the other hand, this same group proposed that menadione from intestine is delivered via the mesenteric lymphatics into the blood and then transported to peripheral tissues, where it is transformed into MK4 by UBIAD1. Details about MK7 distribution and metabolism in rats are still limited. Ikeda et al. [53] proposed that MK7 can also be a precursor of MK4 and may be converted to MK4 in the body. Regardless of how MK4 comes to be formed, it is believed that rodents consuming commercially available rodent chow, fortified with menadione (a precursor for MK4), have sufficient vitamin K for all tissue functions [54].

VK1 deficiency has been observed in CKD patients and animals with experimental CKD [24,48,55], and in this respect our results are in line with these earlier observations. However, the ratios of MK7/VK1 and MK4/VK1, which provide an index of the efficiency of conversion of VK1 to vitamins K2 [56], were increased in uremic animals compared to controls. This suggests that lower VK1 levels observed in CKD rats could be a result of greater tissue utilization of this vitamin, possibly reflecting an increased physiological demand, especially for MK4, during uremia. The similar phenomenon was previously described by Ferland et al. [57] in rats fed different VK1 diets during aging.

The measurement of serum levels of VKDPs, like total osteocalcin and its undercarboxylated form, Glu-OC, are sensitive indirect tests in detecting subclinical VK deficiency [16]. Both total osteocalcin and Glu-OC were markedly increased in the serum of CKD rats in this experiment, and similar findings have been consistently observed by others in human and experimental CKD [19,24,48]. It is generally believed that the high percentage of Glu-OC reflects insufficient VK intake, low VK status, and impaired bone mineralization [28–30]. In the present study, we observed the direct associations between kidney function markers and total osteocalcin, indicating that this parameter can be accumulated in the blood of CKD animals independently of its release from bone. This observation is in accordance with the study of Price et al. [58], who demonstrated that nephrectomy can block the serum clearance of osteocalcin. In turn, renal insufficiency combined with an increase in PTH levels seems to affect serum Glu-OC concentrations in our rats with CKD. It is possible that PTH may enhance the synthesis of Glu-OC by the stimulation of osteoblastogenesis, as has been reported previously [59–62]. Thus, the increased serum Glu-OC in the condition of uremia and hyperparathyroidism, observed in this study, does not necessarily reflect VK deficiency. A good alternative to determine VK status is measurement serum ucMGP, which is a recognized marker of VK status in nephrectomized rats [63]. In contrast to Glu-OC, ucMGP concentration did not differ between the studied groups in this study. All these data indicate that VK status was sufficient in CKD animals.

Osteocalcin is synthesized in osteoblasts, and its three-glutamate residues (Glu-OC) are posttranslationally converted into γ-carboxyglutamate (Gla-OC) by VK, which is a cofactor of GGCX [64,65]. Gla-OC strongly binds to hydroxyapatite in bone, whereas Glu-OC, which does not have this ability, is released into the blood [58]. We measured the Gla-OC, Glu-OC, and Gla-OC/Glu-OC ratio, being an index of the availability of VK at the bone level, in trabecular (Figure 4A–C) and cortical (Figure 4D–F) bone tissue. In both bone regions the Gla-OC/Glu-OC ratios were increased, and Glu-OC concentrations were reduced in CKD rats compared to controls. These results indicate the accelerated Glu-OC to Gla-OC transformation in bone of CKD rats, which increased Gla-OC while consuming Glu-OC [66], reflecting sufficient or even elevated levels of VK in their bones. However, in trabecular bone of uremic rats, Gla-OC level was comparable with that of controls, and the only weak relationship existed between Gla-OC and Gla-OC/Glu-OC ratio. In contrast, the Gla-OC level rose significantly, and it was strongly correlated with Gla-OC/Glu-OC ratio in the cortical bone region of these animals. These data support our new observation: that the generation of Gla-OC in trabecular bone, in which a more rapid bone turnover rate occurs, was not as effective as in cortical bone. Interestingly, serum Glu-OC levels were inversely related to trabecular Gla-OC/Glu-OC ratio, indicating that the Gla-OC generation process in trabecular bone region is an important factor influencing the serum Glu-OC level.

Vitamin K undergoes a cycle of oxidation followed by reduction through the "vitamin K cycle", which includes VKORC1, GGCX, and UBIAD1 enzymes [65,67]. During γ-carboxylation of Glu-OC, VK is converted into VK epoxide by GGCX [65], and VK epoxide can then be reduced by the enzyme VKORC1 to VK-hydroquinone, which can be used once again for the carboxylation reaction [65,67]. The UBIAD1 gene encodes an MK4 biosynthetic enzyme, which is responsible for VK1 to MK4 bioconversion in extrahepatic tissues [68].

The expressions of VK cycle enzymes were previously determined in different tissues of rats with adenine-induced CKD, and in general, the decreased recycling and utilization of VK was found [24,69,70]. To the best of our knowledge, this is the first study that measured the expression of genes involved in VK recycling at the bone level of uremic rats. There was a significant increase in the expression of VKORC1 and GGCX in the femurs of CKD rats compared to controls, whereas there was no difference in the expression of UBIAD1. These results indicated that VK1 to MK4 bioconversion, as well as MK4 recycling and utilization, can occur in the bones of CKD rats even more effectively than in healthy animals. Surprisingly, regarding Gla-OC levels in the studied bone regions, it seems that the enzymes of VK cycle should act better in a cortical, less metabolically active part of the bone than in the trabecular, which is characterized by more active bone turnover.

Previously, we observed the alterations in osteoblastogenesis process in bone of our rats with CKD, with the significantly increased expression of genes involved in the early stages of osteoblastogenesis, like FOXO1, ATF4, RUNX2, and ALP, whereas the expression of marker of the late stage of osteoblast differentiation, BGLAP, was only slightly increased. This phenomenon resulted in impaired osteoblast maturation and decreased bone mineral status [46]. Our findings were in line with clinical observation of Pereira et al. [71], who also showed that the increase of osteoblastic markers occurred simultaneously with low osteocytic gene expression in cells obtained from dialyzed patients. In the present study, we analyzed the associations between the expression of VK cycle enzymes and mentioned the above genes involved in the individual stages of osteoblastogenesis. As has been presented in Figure 6D, the expression of enzymes of VK cycles occurred in the early stages of osteoblastogenesis. What is more, Glu-OC levels in trabecular bone tissue were also associated with the early markers of osteoblast proliferation/differentiation, but not with BGLAP. Consistent with previous reports, PTH may accelerate differentiation of osteoprogenitor cells into osteoblasts [72]. Because PTH level was increased in serum of our CKD rats [44], and previously we showed that endogenous PTH may influence the expression of genes engaged in the early stage of osteoblastogenesis in young uremic rats [73], we speculate that this hormone could also contribute to the increased expression of VK cycle enzymes in this study. However, BGLAP expression increases significantly at the late stage of osteoblast differentiation [74], and this fact could explain the lack of association between BGLAP and the expression of VK cycle enzymes and bone Glu-OC generation. Interestingly, we noticed that circulating MK4 was positively associated with BGLAP expression in bone (Figure 6E). This is in accordance with the findings of Weng et al. [66], who evaluated the effect of MK4 on osteocalcin expression in calvarial bone defect repair in osteopenic rats. These and the above-presented results (Figure 6) suggest that in CKD conditions, the VK recycling, expression of BGLAP, and generation of different forms of osteocalcin may occur in the distinct stages of osteoblastogenesis under the control of endogenous PTH and MK4. Thus, the lack of difference in trabecular Gla-OC levels observed in the present study could be a result of the individual effects of PTH, which stimulates the synthesis of osteocalcin [60]; and MK4, which increased Gla-OC while consuming Glu-OC. In agreement with this hypothesis, the combined MK4 and PTH_{1-34} treatment increased serum Gla-OC/Glu-OC ratio in osteopenic rats, however the highest Gla-OC/Glu-OC ratio was observed not in the combined group, but in the MK4-treated group [66].

Another interesting finding of the present study is the association between VKORC1 mRNA levels and Gla-OC concentration in the trabecular bone region of uremic rats. In contrast, such relations were not noticed between GGCX and both forms of osteocalcin in the bone regions analyzed by us. The posttranslational modification of Glu-OC to Gla-OC inside osteoblasts generally involves two enzymes, GGCX and VKORC1, which together constitute the VK cycle. GGCX requires a reduced form of VK as an obligate cofactor, and γ-carboxylation is dependent on the reduction of VK epoxide by a VKORC1 [65]. Ferron et al. [75], using cell-specific gene inactivation models, demonstrated that VKORC1 is required for γ-carboxylation of osteocalcin in osteoblasts. They also showed that animals

lacking GGCX or VKORC1 presented almost no osteocalcin in their bones, providing the first in vivo evidence that the generation of Gla-OC is necessary for its accumulation in the mineral component of bone. This is consistent with the inverse relationship between bone VKORC1 gene expression and serum Glu-OC levels observed in our uremic rats (Figure 7B), confirming the significance of VKORC1 in the process of Gla-OC formation in conditions of CKD.

The second aim of the present study was to establish the potential consequence of VK-dependent mechanisms for the bone mineral status of uremic rats. Previously, we showed that CKD development resulted in significantly decreased densitometric parameters in our uremic animals compared with controls in whole-femur measurements, particularly in the metaphyseal area (R1). In contrast, mineral status measured in the diaphyseal area (R2) was damaged to a lesser extent [44]. In the present study, we juxtaposed the parameters of bone mineral status obtained in this previous study [44] with the concentrations of VK-dependent proteins: Gla-OC and Glu-OC in the corresponding regions of the femur. Unexpectedly, the Gla-OC/Glu-OC ratio in trabecular bone tissue was inversely associated with the mineral status of femurs (Figure 8). In turn, the positive relations were observed only between Glu-OC levels in cortical bone tissue and the parameters of bone mineral status, which were strongest in the diaphyseal area (R2) of femurs (Figure 9). Although Gla-OC, due to its specific interaction with hydroxyapatite, is thought to be associated with bone mineralization [76–78], there is also evidence showing that Gla-OC is not critical for bone mineralization or even that it functions as a "negative regulator of skeleton" [79]. Genetic osteocalcin depletion does not change the mineral content of bone matrix in mice [80]. Complete loss of osteocalcin resulted in bones with significantly increased trabecular thickness, density, and volume, whereas cortical bone volume and density were not increased in osteocalcin-null male rats [79]. The treatment with warfarin, despite significantly lowering Gla-OC levels, did not reduce the mineral content of fracture calluses [59], and is not directly linked with BMD of rat bone [81]. Amizuka et al. [82] demonstrated that osteocalcin is not related to mineral deposition but does participate in the growth and maturation of hydroxyapatite. A recent study by Simon et al. [83] demonstrates that osteocalcin takes on the functions of Ca-ion transport and suppression of hydroxyapatite expansion. An interesting study by Uchida et al. [84] revealed that the commensal microbiota prevents excessive bone mineralization by stimulating osteocalcin expression in osteoblasts, enhancing both osteoblast and osteoclast activity.

Assuming that Gla-OC is the marker of mature osteoblast [84], the relations observed in this study between bone mineral status and Glu-OC to Gla-OC transformation are compatible with the results of these teams, which demonstrated that Gla-OC may act as an inhibitor of bone mineralization [79–84]. We believe that in the trabecular bone region, PTH-dependent acceleration of osteoblastogenesis resulted in the generation of immature osteoblasts with insufficient Gla-OC production, which may lead to decreased bone mineral status [46]. In cortical bone, where Gla-OC level was approximately 1.5 times that in trabecular bone, the osteoblasts should be more mature. However, the mineral status in this bone region was directly associated with Glu-OC, representing the part of osteocalcin, which was not transformed to Gla-OC. The above results indicate an unfavorable role of Gla-OC in the mineralization of long bones in CKD conditions.

Some limitations should be considered in the interpretation our results. Firstly, the cross-sectional design of this study does not determine whether a causal relationship exists between VK metabolism in bone and the parameters of bone mineral status. Secondly, we cannot exclude the possibility that the observed associations could be partially attributed to other factors not considered in this study that may affect osteoblast proliferation/differentiation and bone mineral status, such as the metabolites of tryptophan [44,85]. Thirdly, gonadectomy was not performed in this study, so contributions to mineral and bone metabolism of sex hormones, especially androgen in the rats, were not examined.

A major strength of our study is the measurement of circulating vitamins K together with the expression of VK recycling enzyme in bone, and Glu-OC, Gla-OC directly in

the appropriate bone tissue, as the determination of these compounds in blood may not accurately represent the bone microenvironment [81]. Moreover, the studied groups were homogeneous with regard to age, sex, diet, and the absence of medication.

5. Conclusions

This study presents a comprehensive assessment of the metabolism of endogenous vitamin K, VK recycling in bone, VKDPs at serum and bone levels, and their impact on bone mineral status of rats with CKD. The obtained results indicate that the reduced level of VK1 observed in rats with CKD may be caused by the accelerated conversion of this vitamin to the form of menaquinones. The measurement of serum osteocalcin and Glu-OC, commonly used as the indicators of VK deficiency, seems to be ineffective in CKD conditions, especially in the presence of hyperparathyroidism. For the first time, we showed that bone tissue possesses a set of enzymes that allows for the conversion of VK1 to the form of menaquinone, as well as to recycling of VK. However, in the course of CKD with hyperparathyroidism, despite the appropriate conditions for the formation of active forms of VK, the intensified process of osteoblastogenesis causes the generation of immature osteoblasts with impaired mineralization capacity. Of particular clinical significance seems to be the fact that Gla-OC formed at the bone level turned out to be inversely correlated with bone mineral status in this model. This sheds new light on the metabolism of endogenous VK and its importance in the process of bone mineralization in CKD, particularly in patients with hyperparathyroidism.

Author Contributions: Conceptualization—M.Z., D.P. and K.P.; participation in animal experiments and data collection—B.S. and K.C.; statistical analysis—D.P. and K.P.; writing—original draft preparation, M.Z. and K.P.; visualization, B.S.; founding acquisition—D.P.; writing—review & editing, M.Z. and K.P. All authors have discussed the results. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Medical University of Bialystok, Poland, grant no. SUB/1/DN/21/001/2221. The APC was funded by Medical University of Bialystok, Poland.

Institutional Review Board Statement: The study was conducted according to the guidelines of the EU Directive 2010/63/EU for animal experiments and was approved by the local Bioethics Committee of Medical University of Bialystok No 29/2013.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is stored by corresponding author and may be share upon request.

Acknowledgments: We are grateful to the team of Laboratory of Perlan Technologies Polska, based in Gdynia, Poland for measurements of vitamin K status.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

1,25 $(OH)_2D$	1,2-dihydroxyvitamin D
ALP	alkaline phosphatase
ATF4	activating transcription factor 4
BGLAP	bone gamma carboxyglutamate protein; osteocalcin
BMC	bone mineral content
BMD	bone mineral density
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease–mineral bone disorders
CON	control group

dp-ucMGP	desphospho-uncarboxylated matrix Gla protein
FOXO1	forkhead box transcription factor 1
GGCX	γ-glutamyl carboxylase
Gla	gamma carboxyglutamic acid
Gla-OC	carboxylated osteocalcin
Glu	glutamic acid
Glu-OC	undercarboxylated osteocalcin
HD	hemodialysis
MGP	matrix gla protein
MKs	menaquinones
MK4	menaquinone 4
MK7	menaquinone 7
OC	osteocalcin
PTH	parathyroid hormone
R1	metaphyseal area of femur
R2	diaphyseal area of femur
RUNX2	Runt-related transcription factor 2
UBIAD1	UbiA prenyltransferase domain-containing 1 protein
ucMGP	uncarboxylated matrix gla protein
ucOC	uncarboxylated osteocalcin
VK	vitamin K
VK1	vitamin K1; phylloquinone
VK2	vitamin K2; menaquinones
VKDPs	vitamin K-dependent proteins
VKORC1	vitamin K epoxide reductase complex subunit 1

References

1. Waziri, B.; Duarte, R.; Naicker, S. Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD): Current perspectives. *Int. J. Nephrol. Renovasc. Dis.* **2019**, *12*, 263–276. [CrossRef] [PubMed]
2. Lewis, R. Mineral and bone disorders in chronic kidney disease: New insights into mechanism and management. *Ann. Clin. Biochem.* **2012**, *49*, 432–440. [CrossRef] [PubMed]
3. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Update Work Group. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int. Suppl.* **2017**, *7*, 1–59. [CrossRef] [PubMed]
4. Pimentel, A.; Ureña-Torres, P.; Zillikens, M.C.; Bover, J.; Cohen-Solal, M. Fractures in patients with CKD-diagnosis, treatment, and prevention: A review by members of the European Calcified Tissue Society and the European Renal Association of Nephrology Dialysis and Transplantation. *Kidney Int.* **2017**, *92*, 1343–1355. [CrossRef] [PubMed]
5. Miller, P.D. Chronic kidney disease and osteoporosis: Evaluation and management. *Bonekey Rep.* **2014**, *3*, 542. [CrossRef]
6. Zaidi, M. Skeletal remodeling in health and disease. *Nat. Med.* **2007**, *13*, 791–801. [CrossRef]
7. Katsimbri, P. The biology of normal bone remodelling. *Eur. J. Cancer Care (Engl.)* **2017**, *26*. [CrossRef]
8. Akbari, S.; Rasouli-Ghahroudi, A.A. Vitamin K and Bone Metabolism: A Review of the Latest Evidence in Preclinical Studies. *Biomed Res. Int.* **2018**, *2018*, 4629383. [CrossRef]
9. Napoli, N.; Strollo, R.; Sprini, D.; Maddaloni, E.; Rini, G.B.; Carmina, E. Serum 25-OH vitamin D in relation to bone mineral density and bone turnover. *Int. J. Endocrinol.* **2014**, *2014*, 487463. [CrossRef]
10. Booth, S.L. Vitamin K: Food composition and dietary intakes. *Food Nutr. Res.* **2012**, *56*, 5505. [CrossRef]
11. Thijssen, H.H.; Drittij-Reijnders, M.J. Vitamin K distribution in rat tissues: Dietary phylloquinone is a source of tissue menaquinone-4. *Br. J. Nutr.* **1994**, *72*, 415–425. [CrossRef] [PubMed]
12. Nakagawa, K.; Hirota, Y.; Sawada, N.; Yuge, N.; Watanabe, M.; Uchino, Y.; Okuda, N.; Shimomura, Y.; Suhara, Y.; Okano, T. Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme. *Nature.* **2010**, *468*, 117–121. [CrossRef]
13. Schurgers, L.J.; Vermeer, C. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. *Haemostasis.* **2000**, *30*, 298–307. [CrossRef] [PubMed]
14. Tie, J.K.; Stafford, D.W. Structural and functional insights into enzymes of the vitamin K cycle. *J. Thromb. Haemost.* **2016**, *14*, 236–247. [CrossRef] [PubMed]
15. Silaghi, C.N.; Ilyés, T.; Filip, V.P.; Farcaș, M.; van Ballegooijen, A.J.; Crăciun, A.M. Vitamin K Dependent Proteins in Kidney Disease. *Int. J. Mol. Sci.* **2019**, *20*, 1571. [CrossRef]
16. Fusaro, M.; Gallieni, M.; Rizzo, M.A.; Stucchi, A.; Delanaye, P.; Cavalier, E.; Moysés, R.; Jorgetti, V.; Iervasi, G.; Giannini, S.; et al. Vitamin K plasma levels determination in human health. *Clin. Chem. Lab. Med.* **2017**, *55*, 789–799. [CrossRef]
17. Caluwé, R.; Verbeke, F.; De Vriese, A.S. Evaluation of vitamin K status and rationale for vitamin K supplementation in dialysis patients. *Nephrol. Dial. Transplant.* **2020**, *35*, 23–33. [CrossRef]

18. Cranenburg, E.C.; Schurgers, L.J.; Uiterwijk, H.H.; Beulens, J.W.; Dalmeijer, G.W.; Westerhuis, R.; Magdeleyns, E.J.; Herfs, M.; Vermeer, C.; Laverman, G.D. Vitamin K intake and status are low in hemodialysis patients. *Kidney Int.* **2012**, *82*, 605–610. [CrossRef]
19. Holden, R.M.; Morton, A.R.; Garland, J.S.; Pavlov, A.; Day, A.G.; Booth, S.L. Vitamins K and D status in stages 3-5 chronic kidney disease. *Clin. J. Am. Soc. Nephrol.* **2010**, *5*, 590–597. [CrossRef]
20. McCabe, K.M.; Adams, M.A.; Holden, R.M. Vitamin K status in chronic kidney disease. *Nutrients* **2013**, *5*, 4390–4398. [CrossRef]
21. Voong, K.; Harrington, D.; Goldsmith, D. Vitamin K status in chronic kidney disease: A report of a study and a mini-review. *Int. Urol. Nephrol.* **2013**, *45*, 1339–1344. [CrossRef] [PubMed]
22. Stankowiak-Kulpa, H.; Krzyżanowska, P.; Kozioł, L.; Grzymisławski, M.; Wanic-Kossowska, M.; Moczko, J.; Walkowiak, J. Vitamin K status in peritoneally dialyzed patients with chronic kidney disease. *Acta Biochim. Pol.* **2011**, *58*, 617–620. [CrossRef] [PubMed]
23. Westenfeld, R.; Krueger, T.; Schlieper, G.; Cranenburg, E.C.; Magdeleyns, E.J.; Heidenreich, S.; Holzmann, S.; Vermeer, C.; Jahnen-Dechent, W.; Ketteler, M.; et al. Effect of vitamin K2 supplementation on functional vitamin K deficiency in hemodialysis patients: A randomized trial. *Am. J. Kidney Dis.* **2012**, *59*, 186–195. [CrossRef] [PubMed]
24. McCabe, K.M.; Booth, S.L.; Fu, X.; Ward, E.; Adams, M.A.; Holden, R.M. Vitamin K Metabolism in a Rat Model of Chronic Kidney Disease. *Am. J. Nephrol.* **2017**, *45*, 4–13. [CrossRef]
25. Pilkey, R.M.; Morton, A.R.; Boffa, M.B.; Noordhof, C.; Day, A.G.; Su, Y.; Miller, L.M.; Koschinsky, M.L.; Booth, S.L. Subclinical Vitamin K Deficiency in Hemodialysis Patients. *Am. J. Kidney Dis.* **2007**, *49*, 432–439. [CrossRef]
26. Kohlmeier, M.; Saupe, J.; Shearer, M.J.; Schaefer, K.; Asmus, G. Bone health of adult hemodialysis patients is related to vitamin K status. *Kidney Int.* **1997**, *51*, 1218–1221. [CrossRef]
27. Evenepoel, P.; Claes, K.; Meijers, B.; Laurent, M.; Bammens, B.; Naesens, M.; Sprangers, B.; Pottel, H.; Cavalier, E.; Kuypers, D. Poor Vitamin K Status Is Associated With Low Bone Mineral Density and Increased Fracture Risk in End-Stage Renal Disease. *J. Bone Miner. Res.* **2019**, *34*, 262–269. [CrossRef]
28. Kim, S.M.; Kim, K.M.; Kim, B.T.; Joo, N.S.; Kim, K.N.; Lee, D.J. Correlation of undercarboxylated osteocalcin (ucOC) concentration and bone density with age in healthy Korean women. *J. Korean Med. Sci.* **2010**, *25*, 1171–1175. [CrossRef]
29. Bügel, S. Vitamin K and bone health in adult humans. *Vitam. Horm.* **2008**, *78*, 393–416. [CrossRef]
30. Rodríguez-Olleros Rodríguez, C.; Díaz Curiel, M. Vitamin K and Bone Health: A Review on the Effects of Vitamin K Deficiency and Supplementation and the Effect of Non-Vitamin K Antagonist Oral Anticoagulants on Different Bone Parameters. *J. Osteoporos.* **2019**, *2019*, 2069176. [CrossRef]
31. Braam, L.A.; Knapen, M.H.; Geusens, P.; Brouns, F.; Hamulyák, K.; Gerichhausen, M.J.; Vermeer, C. Vitamin K1 supplementation retards bone loss in postmenopausal women between 50 and 60 years of age. *Calcif. Tissue Int.* **2003**, *73*, 21–26. [CrossRef] [PubMed]
32. Knapen, M.H.; Drummen, N.E.; Smit, E.; Vermeer, C.; Theuwissen, E. Three-year low-dose menaquinone-7 supplementation helps decrease bone loss in healthy postmenopausal women. *Osteoporos. Int.* **2013**, *24*, 2499–2507. [CrossRef] [PubMed]
33. Shiraki, M.; Shiraki, Y.; Aoki, C.; Miura, M. Vitamin K2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. *J. Bone Miner. Res.* **2000**, *15*, 515–521. [CrossRef] [PubMed]
34. Cockayne, S.; Adamson, J.; Lanham-New, S.; Shearer, M.J.; Gilbody, S.; Torgerson, D.J. Vitamin K and the prevention of fractures: Systematic review and meta-analysis of randomized controlled trials. *Arch. Intern. Med.* **2006**, *166*, 1256–1261. [CrossRef]
35. Iwamoto, J.; Takeda, T.; Ichimura, S. Effect of combined administration of vitamin D3 and vitamin K2 on bone mineral density of the lumbar spine in postmenopausal women with osteoporosis. *J. Orthop. Sci.* **2000**, *5*, 546–551. [CrossRef]
36. Huang, Z.B.; Wan, S.L.; Lu, Y.J.; Ning, L.; Liu, C.; Fan, S.W. Does vitamin K2 play a role in the prevention and treatment of osteoporosis for postmenopausal women: A meta-analysis of randomized controlled trials. *Osteoporos. Int.* **2015**, *26*, 1175–1186. [CrossRef]
37. Knapen, M.H.; Schurgers, L.J.; Vermeer, C. Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. *Osteoporos. Int.* **2007**, *18*, 963–972. [CrossRef]
38. Emaus, N.; Gjesdal, C.G.; Almås, B.; Christensen, M.; Grimsgaard, A.S.; Berntsen, G.K.; Salomonsen, L.; Fønnebø, V. Vitamin K2 supplementation does not influence bone loss in early menopausal women: A randomised double-blind placebo-controlled trial. *Osteoporos. Int.* **2010**, *21*, 1731–1740. [CrossRef]
39. Binkley, N.; Harke, J.; Krueger, D.; Engelke, J.; Vallarta-Ast, N.; Gemar, D.; Checovich, M.; Chappell, R.; Suttie, J. Vitamin K treatment reduces undercarboxylated osteocalcin but does not alter bone turnover, density, or geometry in healthy postmenopausal North American women. *J. Bone Miner. Res.* **2009**, *24*, 983–991. [CrossRef]
40. Booth, S.L.; Pennington, J.A.; Sadowski, J.A. Food sources and dietary intakes of vitamin K-1 (phylloquinone) in the American diet: Data from the FDA Total Diet Study. *J. Am. Diet. Assoc.* **1996**, *96*, 149–154. [CrossRef]
41. Caluwé, R.; Vandecasteele, S.; Van Vlem, B.; Vermeer, C.; De Vriese, A.S. Vitamin K2 supplementation in haemodialysis patients: A randomized dose-finding study. *Nephrol. Dial. Transplant.* **2014**, *29*, 1385–1390. [CrossRef] [PubMed]
42. Aoun, M.; Makki, M.; Azar, H.; Matta, H.; Chelala, D.N. High Dephosphorylated-Uncarboxylated MGP in Hemodialysis patients: Risk factors and response to vitamin K2, A pre-post intervention clinical trial. *BMC Nephrol.* **2017**, *18*, 191. [CrossRef] [PubMed]
43. Sasaki, N.; Kusano, E.; Takahashi, H.; Ando, Y.; Yano, K.; Tsuda, E.; Asano, Y. Vitamin K2 inhibits glucocorticoid-induced bone loss partly by preventing the reduction of osteoprotegerin (OPG). *J. Bone Miner. Metab.* **2005**, *23*, 41–47. [CrossRef] [PubMed]

44. Pawlak, D.; Znorko, B.; Kalaska, B.; Domaniewski, T.; Zawadzki, R.; Lipowicz, P.; Doroszko, M.; Łebkowska, U.; Grabowski, P.; Pawlak, K. LP533401 restores bone health in 5/6 nephrectomized rats by a decrease of gut-derived serotonin and regulation of serum phosphate through the inhibition of phosphate co-transporters expression in the kidneys. *Bone* **2018**, *113*, 124–136. [CrossRef] [PubMed]
45. Znorko, B.; Pawlak, D.; Oksztulska-Kolanek, E.; Domaniewski, T.; Pryczynicz, A.; Roszczenko, A.; Rogalska, J.; Lipowicz, P.; Doroszko, M.; Brzoska, M.M.; et al. RANKL/OPG system regulation by endogenous PTH and PTH1R/ATF4 axis in bone: Implications for bone accrual and strength in growing rats with mild uremia. *Cytokine* **2018**, *106*, 19–28. [CrossRef] [PubMed]
46. Pawlak, D.; Domaniewski, T.; Sieklucka, B.; Jakuc, M.; Pawlak, K. Inhibition of peripheral serotonin synthesis by LP533401 and disturbances in calciotropic hormones attenuated excessive osteoblastogenesis with simultaneous improvement of bone mineral status in 5/6 nephrectomized rats. *Biochim. Biophys. Acta Mol. Basis. Dis.* **2019**, *1865*, 165528. [CrossRef]
47. de Oliveira, R.B.; Stinghen, A.E.M.; Massy, Z.A. Vitamin K role in mineral and bone disorder of chronic kidney disease. *Clin. Chim. Acta.* **2020**, *502*, 66–72. [CrossRef]
48. Bellone, F.; Cinquegrani, M.; Nicotera, R.; Carullo, N.; Casarella, A.; Presta, P.; Andreucci, M.; Squadrito, G.; Mandraffino, G.; Prunestì, M.; et al. Role of Vitamin K in Chronic Kidney Disease: A Focus on Bone and Cardiovascular Health. *Int. J. Mol. Sci.* **2022**, *23*, 5282. [CrossRef]
49. Shearer, M.J.; Bach, A.; Kohlmeier, M. Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *J. Nutr.* **1996**, *126*, 1181S–1186S. [CrossRef]
50. Okano, T.; Shimomura, Y.; Yamane, M.; Suhara, Y.; Kamao, M.; Sugiura, M.; Nakagawa, K. Conversion of phylloquinone (Vitamin K1) into menaquinone-4 (Vitamin K2) in mice: Two possible routes for menaquinone-4 accumulation in cerebra of mice. *J. Biol. Chem.* **2008**, *283*, 11270–11279. [CrossRef]
51. Thijssen, H.H.; Drittij-Reijnders, M.J.; Fischer, M.A. Phylloquinone and menaquinone-4 distribution in rats: Synthesis rather than uptake determines menaquinone-4 organ concentrations. *J. Nutr.* **1996**, *126*, 537–543. [CrossRef] [PubMed]
52. Hirota, Y.; Tsugawa, N.; Nakagawa, K.; Suhara, Y.; Tanaka, K.; Uchino, Y.; Takeuchi, A.; Sawada, N.; Kamao, M.; Wada, A.; et al. Menadione (vitamin K3) is a catabolic product of oral phylloquinone (vitamin K1) in the intestine and a circulating precursor of tissue menaquinone-4 (vitamin K2) in rats. *J. Biol. Chem.* **2013**, *288*, 33071–33080. [CrossRef] [PubMed]
53. Ikeda, S.; Hanzawa, F.; Takahashi, S.; Suzuki, N.; Sano, K.; Oda, H.; Uchida, T. Tissue Distribution of Menaquinone-7 and the Effect of α-Tocopherol Intake on Menaquinone-7 Concentration in Rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2018**, *64*, 391–398. [CrossRef] [PubMed]
54. Fu, X.; Booth, S.L.; Smith, D.E. Vitamin K contents of rodent diets: A review. *J. Am. Assoc. Lab. Anim. Sci.* **2007**, *46*, 8–12.
55. Fusaro, M.; Cozzolino, M.; Plebani, M.; Iervasi, G.; Ketteler, M.; Gallieni, M.; Aghi, A.; Locatelli, F.; Cunningham, J.; Salam, S.; et al. Sevelamer Use, Vitamin K Levels, Vascular Calcifications, and Vertebral Fractures in Hemodialysis Patients: Results from the VIKI Study. *J. Bone Miner. Res.* **2021**, *36*, 500–509. [CrossRef] [PubMed]
56. McCabe, K.M.; Booth, S.L.; Fu, X.; Shobeiri, N.; Pang, J.J.; Adams, M.A.; Holden, R.M. Dietary vitamin K and therapeutic warfarin alter the susceptibility to vascular calcification in experimental chronic kidney disease. *Kidney Int.* **2013**, *83*, 835–844. [CrossRef] [PubMed]
57. Ferland, G.; Doucet, I.; Mainville, D. Phylloquinone and Menaquinone-4 Tissue Distribution at Different Life Stages in Male and Female Sprague-Dawley Rats Fed Different VK Levels Since Weaning or Subjected to a 40% Calorie Restriction since Adulthood. *Nutrients* **2016**, *8*, 141. [CrossRef]
58. Price, P.A.; Williamson, M.K.; Lothringer, J.W. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J. Biol. Chem.* **1981**, *256*, 12760–12766. [CrossRef]
59. Shimizu, T.; Takahata, M.; Kameda, Y.; Hamano, H.; Ito, T.; Kimura-Suda, H.; Todoh, M.; Tadano, S.; Iwasaki, N. Vitamin K-dependent carboxylation of osteocalcin affects the efficacy of teriparatide (PTH(1-34)) for skeletal repair. *Bone* **2014**, *64*, 95–101. [CrossRef]
60. Nagura, N.; Komatsu, J.; Iwase, H.; Hosoda, H.; Ohbayashi, O.; Nagaoka, I.; Kaneko, K. Effects of the combination of vitamin K and teriparatide on the bone metabolism in ovariectomized rats. *Biomed. Rep.* **2015**, *3*, 295–300. [CrossRef]
61. Harsløf, T.; Sikjær, T.; Sørensen, L.; Pedersen, S.B.; Mosekilde, L.; Langdahl, B.L.; Rejnmark, L. The Effect of Treatment With PTH on Undercarboxylated Osteocalcin and Energy Metabolism in Hypoparathyroidism. *J. Clin. Endocrinol. Metab.* **2015**, *100*, 2758–2762. [CrossRef] [PubMed]
62. Maser, R.E.; Lenhard, M.J.; Pohlig, R.T.; Balagopal, P.B.; Abdel-Misih, R. Effect of parathyroidectomy on osteopontin and undercarboxylated osteocalcin in patients with primary hyperparathyroidism. *Endocr. Res.* **2018**, *43*, 21–28. [CrossRef] [PubMed]
63. Neradova, A.; Wasilewski, G.; Prisco, S.; Leenders, P.; Caron, M.; Welting, T.; van Rietbergen, B.; Kramann, R.; Floege, J.; Vervloet, M.G.; et al. Combining phosphate binder therapy with vitamin K2 inhibits vascular calcification in an experimental animal model of kidney failure. *Nephrol. Dial. Transplant.* **2022**, *37*, 652–662. [CrossRef] [PubMed]
64. Vermeer, C.; Jie, K.S.; Knapen, M.H. Role of vitamin K in bone metabolism. *Annu. Rev. Nutr.* **1995**, *15*, 1–22. [CrossRef]
65. Stafford, D.W. The vitamin K cycle. *J. Thromb. Haemost.* **2005**, *3*, 1873–1878. [CrossRef]
66. Weng, S.J.; Xie, Z.J.; Wu, Z.Y.; Yan, D.Y.; Tang, J.H.; Shen, Z.J.; Li, H.; Bai, B.L.; Boodhun, V.; Dong, X.D.E.; et al. Effects of combined menaquinone-4 and PTH1-34 treatment on osetogenesis and angiogenesis in calvarial defect in osteopenic rats. *Endocrine* **2019**, *63*, 376–384. [CrossRef]

67. Shearer, M.J.; Newman, P. Recent trends in the metabolism and cell biology of vitamin K with special reference to vitamin K cycling and MK-4 biosynthesis. *J. Lipid Res.* **2014**, *55*, 345–362. [CrossRef]
68. Hirota, Y.; Nakagawa, K.; Sawada, N.; Okuda, N.; Suhara, Y.; Uchino, Y.; Kimoto, T.; Funahashi, N.; Kamao, M.; Tsugawa, N.; et al. Functional characterization of the vitamin K2 biosynthetic enzyme UBIAD1. *PLoS ONE* **2015**, *10*, e0125737. [CrossRef]
69. Kaesler, N.; Magdeleyns, E.; Herfs, M.; Schettgen, T.; Brandenburg, V.; Fliser, D.; Vermeer, C.; Floege, J.; Schlieper, G.; Krüger, T. Impaired vitamin K recycling in uremia is rescued by vitamin K supplementation. *Kidney Int.* **2014**, *86*, 286–293. [CrossRef]
70. Kaesler, N.; Schreibing, F.; Speer, T.; Puente-Secades, S.; Rapp, N.; Drechsler, C.; Kabgani, N.; Kuppe, C.; Boor, P.; Jankowski, V.; et al. Altered vitamin K biodistribution and metabolism in experimental and human chronic kidney disease. *Kidney Int.* **2022**, *101*, 338–348. [CrossRef]
71. Pereira, R.C.; Delany, A.M.; Khouzam, N.M.; Bowen, R.E.; Freymiller, E.G.; Salusky, I.B.; Wesseling-Perry, K. Primary osteoblast-like cells from patients with end-stage kidney disease reflect gene expression, proliferation, and mineralization characteristics ex vivo. *Kidney Int.* **2015**, *87*, 593–601. [CrossRef] [PubMed]
72. Balani, D.H.; Ono, N.; Kronenberg, H.M. Parathyroid hormone regulates fates of murine osteoblast precursors in vivo. *J. Clin. Investig.* **2017**, *127*, 3327–3338. [CrossRef]
73. Pawlak, K.; Sieklucka, B.; Pawlak, D. Paracrine Kynurenic Pathway Activation in the Bone of Young Uremic Rats Can Antagonize Anabolic Effects of PTH on Bone Turnover and Strength through the Disruption of PTH-Dependent Molecular Signaling. *Int. J. Mol. Sci.* **2021**, *22*, 6563. [CrossRef] [PubMed]
74. Kalajzic, I.; Staal, A.; Yang, W.P.; Wu, Y.; Johnson, S.E.; Feyen, J.H.; Krueger, W.; Maye, P.; Yu, F.; Zhao, Y.; et al. Expression profile of osteoblast lineage at defined stages of differentiation. *J. Biol. Chem.* **2005**, *280*, 24618–24626. [CrossRef] [PubMed]
75. Ferron, M.; Lacombe, J.; Germain, A.; Oury, F.; Karsenty, G. GGCX and VKORC1 inhibit osteocalcin endocrine functions. *J. Cell. Biol.* **2019**, *218*, 2071. [CrossRef] [PubMed]
76. Atkins, G.J.; Welldon, K.J.; Wijenayaka, A.R.; Bonewald, L.F.; Findlay, D.M. Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticatabolic phenotype by {gamma}-carboxylation-dependent and -independent mechanisms. *Am. J. Physiol. Cell Physiol.* **2009**, *297*, C1358–C1367. [CrossRef]
77. Boskey, A.L.; Gadaleta, S.; Gundberg, C.; Doty, S.B.; Ducy, P.; Karsenty, G. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. *Bone* **1998**, *23*, 187–196. [CrossRef]
78. Roy, M.E.; Nishimoto, S.K.; Rho, J.Y.; Bhattacharya, S.K.; Lin, J.S.; Pharr, G.M. Correlations between osteocalcin content, degree of mineralization, and mechanical properties of C. carpio rib bone. *J. Biomed. Mater. Res.* **2001**, *54*, 547–553. [CrossRef]
79. Lambert, L.J.; Challa, A.K.; Niu, A.; Zhou, L.; Tucholski, J.; Johnson, M.S.; Nagy, T.R.; Eberhardt, A.W.; Estep, P.N.; Kesterson, R.A.; et al. Increased trabecular bone and improved biomechanics in an osteocalcin-null rat model created by CRISPR/Cas9 technology. *Dis. Model. Mech.* **2016**, *9*, 1169–1179. [CrossRef]
80. Ducy, P.; Desbois, C.; Boyce, B.; Pinero, G.; Story, B.; Dunstan, C.; Smith, E.; Bonadio, J.; Goldstein, S.; Gundberg, C.; et al. Increased bone formation in osteocalcin-deficient mice. *Nature* **1996**, *382*, 448–452. [CrossRef]
81. Hara, K.; Kobayashi, M.; Akiyama, Y. Comparison of inhibitory effects of warfarin on gamma-carboxylation between bone and liver in rats. *J. Bone Miner. Metab.* **2005**, *23*, 366–372l. [CrossRef] [PubMed]
82. Amizuka, N.; Li, M.; Hara, K.; Kobayashi, M.; de Freitas, P.H.; Ubaidus, S.; Oda, K.; Akiyama, Y. Warfarin administration disrupts the assembly of mineralized nodules in the osteoid. *J. Electron. Microsc. (Tokyo)* **2009**, *58*, 55–65. [CrossRef] [PubMed]
83. Simon, P.; Grüner, D.; Worch, H.; Pompe, W.; Lichte, H.; El Khassawna, T.; Heiss, C.; Wenisch, S.; Kniep, R. First evidence of octacalcium phosphate@osteocalcin nanocomplex as skeletal bone component directing collagen triple-helix nanofibril mineralization. *Sci. Rep.* **2018**, *8*, 13696. [CrossRef]
84. Uchida, Y.; Irie, K.; Fukuhara, D.; Kataoka, K.; Hattori, T.; Ono, M.; Ekuni, D.; Kubota, S.; Morita, M. Commensal Microbiota Enhance Both Osteoclast and Osteoblast Activities. *Molecules* **2018**, *23*, 1517. [CrossRef]
85. Mor, A.; Pawlak, K.; Kalaska, B.; Domaniewski, T.; Sieklucka, B.; Zieminska, M.; Cylwik, B.; Pawlak, D. Modulation of the Paracrine Kynurenic System in Bone as a New Regulator of Osteoblastogenesis and Bone Mineral Status in an Animal Model of Chronic Kidney Disease Treated with LP533401. *Int. J. Mol. Sci.* **2020**, *21*, 5979. [CrossRef] [PubMed]

nutrients

MDPI

Review

A Scoping Review of the Skeletal Effects of Naringenin

Muhamed Lahtif Nor Muhamad, Sophia Ogechi Ekeuku *, Sok-Kuan Wong and Kok-Yong Chin *

Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Kuala Lumpur 56000, Malaysia

* Correspondence: sogechie@ukm.edu.my (S.O.E.); chinkokyong@ppukm.ukm.edu.my (K.-Y.C.); Tel.: +60-3-9145-9573 (S.O.E. & K.-Y.C.)

Abstract: Background: Osteoporosis is caused by the deterioration of bone density and microstructure, resulting in increased fracture risk. It transpires due to an imbalanced skeletal remodelling process favouring bone resorption. Various natural compounds can positively influence the skeletal remodelling process, of which naringenin is a candidate. Naringenin is an anti-inflammatory and antioxidant compound found in citrus fruits and grapefruit. This systematic review aims to present an overview of the available evidence on the skeletal protective effects of naringenin. Method: A systematic literature search was conducted using the PubMed and Scopus databases in August 2022. Original research articles using cells, animals, or humans to investigate the bone protective effects of naringenin were included. Results: Sixteen eligible articles were included in this review. The existing evidence suggested that naringenin enhanced osteoblastogenesis and bone formation through BMP-2/p38MAPK/Runx2/Osx, SDF-1/CXCR4, and PI3K/Akt/*c*-Fos/*c*-Jun/AP-1 signalling pathways. Naringenin also inhibited osteoclastogenesis and bone resorption by inhibiting inflammation and the RANKL pathway. Conclusions: Naringenin enhances bone formation while suppressing bone resorption, thus achieving its skeletal protective effects. It could be incorporated into the diet through fruit intake or supplements to prevent bone loss.

Keywords: naringenin; osteoblasts; osteoclasts; osteocytes; osteoporosis

Citation: Nor Muhamad, M.L.; Ekeuku, S.O.; Wong, S.-K.; Chin, K.-Y. A Scoping Review of the Skeletal Effects of Naringenin. *Nutrients* **2022**, *14*, 4851. https://doi.org/10.3390/nu14224851

Academic Editor: Qian Zhang

Received: 5 September 2022
Accepted: 13 November 2022
Published: 16 November 2022

1. Introduction

Osteoporosis is a degenerative skeletal condition characterised by reduced bone mass and microstructural deterioration, which subsequently lead to decreased bone strength and an increased fragility fracture risk [1]. Osteoporosis is asymptomatic until it presents as low-trauma fractures of the hip, spinal, proximal humerus, pelvis, and/or wrist [2]. Osteoporosis is more prominent in postmenopausal women because estrogen insufficiency accelerates bone loss [3]. The use of antiresorptive (e.g., bisphosphonates, denosumab, and selective estrogen receptor modulators) and anabolic medications (e.g., teriparatide and abaloparatide) can improve bone mineral density (BMD) and reduce the fracture risk of patients with osteoporosis [4,5]. However, they come with various side effects [6,7].

Bone loss occurs when the rate of osteoblastic bone formation is lower than the rate of osteoclastic bone resorption [8]. Various factors could influence the bone turnover process. Inflammation, known to promote bone resorption, is a risk factor for osteoporosis [9]. Proinflammatory cytokines stimulate the expression of receptor activators of nuclear factor-B (RANK) and its functional ligand (RANKL), along with macrophage colony-stimulating factor (M-CSF), which enhance osteoclast formation and function [8]. Furthermore, modifications in redox systems have been linked to the pathogenesis of osteoporosis. Reactive oxygen species (ROS) inhibit osteoblast formation, stimulate apoptosis in osteoblasts and osteocytes, and encourage the formation of osteoclasts [10], all of which result in bone loss and osteoporosis.

Apart from calcium and vitamin D routinely used in osteoporosis prevention [11], dietary antioxidants and anti-inflammatory compounds may slow the progression of osteoporosis [10,12]. Naringenin (Figure 1) is a flavanone present in citrus fruits, grapes,

and tomato skin [13]. Naringenin has been investigated for its antioxidant [14] and anti-inflammatory properties [15]. Previous research found that naringenin suppressed nuclear factor-kappa B (NF-kB) p65 activity and expression in streptozotocin (STZ)-induced diabetic mice [16] and carrageenan-induced paw oedema in rats [17]. In STZ and nicotinamide-induced diabetic rats, naringenin significantly increased the activities of pancreatic enzymatic antioxidants, plasma non-enzymatic antioxidant levels and decreased pancreatic tissue malondialdehyde levels [18]. Meanwhile, another report indicated that naringenin lowered lipid peroxidation and enhanced the activity of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione-s-transferase, glutathione peroxidase and reduced glutathione in the liver of STZ-induced diabetic mice [19]. The studies mentioned above point to the potential of naringenin as an antioxidant and anti-inflammatory agent, which could help to suppress bone loss.

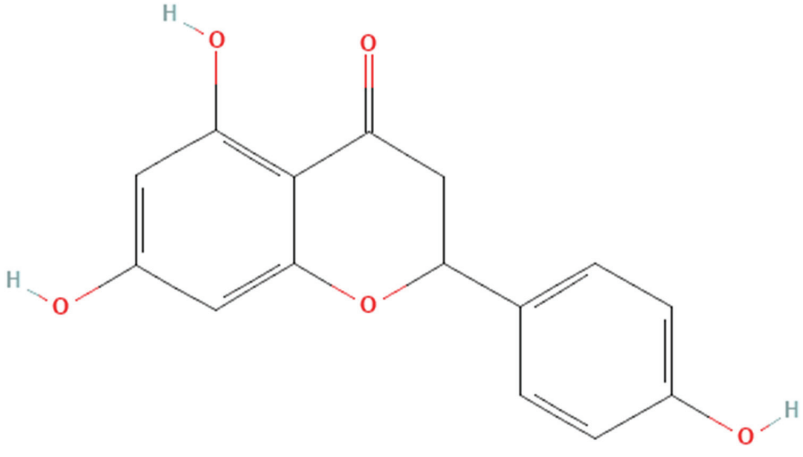


Figure 1. The molecular structure of naringenin.

Considering the effects of inflammation and antioxidants in the pathogenesis of osteoporosis, naringenin may be positioned as a functional food component in preventing bone loss. The objective of this review is to encapsulate the protective effects of naringenin on bone as evidenced by currently available studies. Furthermore, naringenin may also exert specific mechanisms to enhance bone health, which would be discussed in the current review.

2. Materials and Methods

2.1. Literature Review

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for scoping review (Table S1). To discern studies on the potential benefits of naringenin on bone, a systematic literature search was conducted using the PubMed and Scopus databases in August 2021. The keywords used in the search were (1) naringenin AND (2) (bone OR osteoporosis OR osteoblasts OR osteoclasts OR osteocytes).

2.2. Article Selection

Articles with the following features were included: (1) original research articles investigating the skeletal effects of naringenin; (2) studies conducted using cell cultures, animal models, or human subjects. Articles with the following characteristics were rejected: (1) conference abstracts, reviews, letters or commentary, editorial and opinion articles lacking original data; (2) studies not using pure naringenin; (3) articles written in a language

other than English. The search was executed by two authors (MLNM and SOE), using both databases and the keywords listed. The inclusion of an article was based on consensus by both authors. If no consensus could be obtained, the opinions of other authors were sought after (SKW and KYC) to determine the outcome of the article.

2.3. Data Extraction

The list of articles was organised using Mendeley (Elsevier, Amsterdam, The Netherlands). Identification of duplicated items was performed using Mendeley and manually. The authors' names, publication year, study design, dose, treatment period, outcomes, and study limitations were all extracted using a standardised table by two authors (MLNM and SOE).

3. Results

3.1. Article Selection

The literature search resulted in 210 unique articles, which were from PubMed and Scopus. Following the removal of duplicates (n = 51), 159 articles were screened. One hundred and forty-three articles were excluded because they did not fit the inclusion criteria (not original article = 63, out of scope = 60, not written in English = 4, raw extract/mixture/formulation = 16). Eventually, the review included 16 articles that met all the criteria (Table 1). The selection process is depicted in Figure 2.

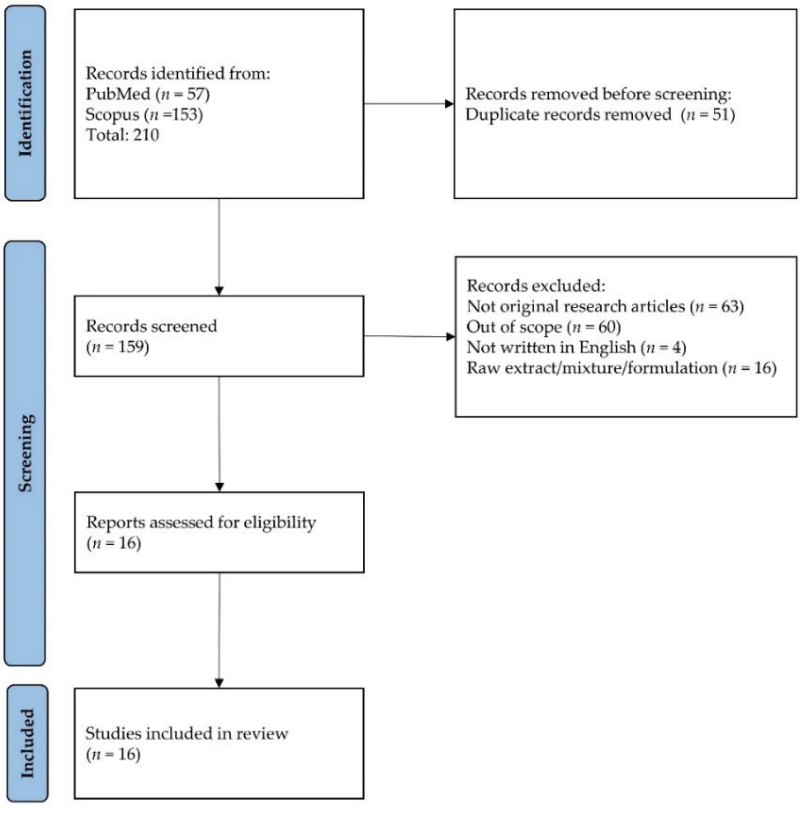


Figure 2. Flowchart showing selection of articles.

Table 1. Effects of naringenin on bone health.

Cell Culture Studies		Major Findings (Changes vs. Negative Control)			Conclusion
Reference	**Study Design**	**Indices Increased**	**Indices Decreased**	**Indices Unchanged**	
[20]	Cell: Human primary osteoclast precursor cells Induction: RANKL- and M-CSF-induced osteoclastogenesis Treatment: 36.7, 91.8, and 183.6 μM of naringenin (2 μL per well) for 2 and 6 days. Control: Negative: No treatment Positive: No	• IL-8 and TNF-α secretion	• Osteoclastogenesis • IL-1α, IL-23 and monocyte chemoattractant protein-1 secretion • Helical peptide 620–633 release	• IL-6, IL-1b, IL-17, and OPG secretion	Naringenin inhibits osteoclast formation and bone resorption.
[21]	Cell: rBMSCs from femur and tibia of Wistar rats Induction: Dexamethasone -induced osteogenesis Treatment: 10 μM of naringenin or 8-prenylnaringenin for 16 days Control: Negative: No treatment Positive: n.a	• ALP activity • Osteocalcin level • Calcium level • BMP-2, OPG, Runx-2 and Osx expression • p38 MAPK expression at 12 and 24 h • Effects of prenylnaringenin > naringenin			Both prenylnaringenin and naringenin promote osteoblast differentiation and mineralization. Effects of prenylnaringenin are better than naringenin.
[22]	Cell: Calvarial osteoblasts from newborn Wistar rats and osteoclasts cells from femur and tibia of rabbit Induction: n.a Treatment: 10 μM of naringenin and 8-prenylnaringenin for 3, 6, 9, and 12 days Control: Negative: No treatment Positive: n.a	• ALP activity at all time points Osteocalcin expression (day 3–6 and day 6–9) • Calcium content (day 6) • numbers and areas of mineralized nodules (day 12) • Runx-2, Osx, BMP-2 and Col1α2 expression • OPG expression, OPG/RANKL ratio • Apoptotic osteoclast at 24 h	• Osteoclast number, number of pits, and total area of pits • TRAP-positive multinuclear cells and expression of TRAP and cathepsin-K	• RANKL expression	Both prenylnaringenin and naringenin suppress osteoclast formation and survival and promote osteoblast differentiation. Effects of prenylnaringenin are better than naringenin.

Table 1. *Cont.*

	Cell Culture Studies	Major Findings (Changes vs. Negative Control)			Conclusion
[23]	Cell: Mouse calvarial osteoblasts from Balb/cByJ mice Induction: n.a Treatment: Naringenin or naringenin-6-C-glucoside (0.001,0.01 and 0.1, 1, 10, 25, and 50 μM) for 21 days Control: Negative: No treatment Positive: 17β-oestradiol (1 nM) for 21 days	• ALP activity (25 and 50 μM for naringenin, 1–100 nM for naringenin-6-C-glucoside) • Nodule formation (50 μM for naringenin, 100 nM for naringenin-6-C-glucoside)			Both naringenin or naringenin-6-C-glucoside promote osteoblast differentiation and bone formation. Naringenin-6-C-glucoside is more potent than naringenin.
[24]	Cell: co-culture of T-cells from male BALB/c mice and BMMs Induction: M-CSF- and RANKL-induced osteoclastogenesis Treatment: Naringenin (0–800 μM) for 1, 2, and 10 days. Control: Negative: no treatment Positive: n. a	• IL-4 release by T-cells	• TRAP-positive multinucleated osteoclast • Size and number of F-actin rings • mRNA and protein expression of cathepsin K, *c*-Fos, DC-STAMP, NFATc1, TRAP, and V-ATPase d2		The anti-osteoclastogenesis effects of naringenin are exerted through IL-4 release by T-cells.
[25]	Cell: BMMs from femur and tibia of C-57/BL6 mice and RAW 264.7 cells. Induction: M-CSF- and RANKL-induced osteoclastogenesis. Treatment: Naringenin (0–800 μM) for 2 days. Control: Negative: no treatment Positive: n. a		• Size and number of F-actin ring • Resorption area • RANKL-induced cathepsin K, *c*-Fos, DC-STAMP, NFATc1, TRAP, and V-ATPase d2 • RANKL-induced phosphorylation of p38 signalling	• NF-κB, ERK, JNK, IκBα, and *p*-IκBα expression	Naringenin inhibits osteoclast formation through suppression of p38 signaling.

Table 1. *Cont.*

	Cell Culture Studies	Major Findings (Changes vs. Negative Control)			Conclusion
[26]	Cell: BMSCs from femur and tibia of Sprague Dawley rats Induction: dexamethasone-induced osteogenesis Treatment: 734.6 μM of naringenin for 14 days Control: Negative: No treatment Positive: n. a	• mRNA and protein expression of ALP, Runx-2, CXCR4, and SDF-1a		• Cell viability and proliferation rates	Naringenin stimulates bone formation via upregulation of the SDF-1a through the SDF-1/CXCR4 signaling pathway
[27]	Cell: MC3T3-E1, hOB and pOB cell line Induction: n.a Treatment: 0.3, 1, 3, and 10 μM of naringenin for 1–7 days Control: Negative: no treatment Positive: n.a	• ALP activity • Osteocalcin and osteopontin expression • BMP-2 via PI3K and Akt-dependent signaling • proliferation of MC3T3-E1	• BMP-3 expression • Etoposide- and TNF-α-induced cell apoptosis		The osteogenic effects of naringin are exerted through upregulation of BMP-2 expression via the PI3K, Akt, *c*-Fos/*c*-Jun and AP-1-dependent signaling pathway
[28]	Cell: hPDLSCs Induction: osteogenic differentiation Treatment: 0.1, 1 and 10 mM of naringenin for 0–72 h Control: Negative: no treatment Positive: n.a	• ALP activity (day 3 and 7) • SDF-1 mRNA expression • SDF-1 and CXCR4 protein expression			Naringenin increases the osteogenic potential of hPDLSCs.
[29]	Cell: MG-63 cell lines Induction: n.a. Treatment: 100 μL of 0.15–10 μg/mL naringenin nanosuspension for 48 h Control: Negative: no treatment Positive: n.a			• OCN protein expression	Naringenin nanosuspension may have pro-osteogenic effects.

Table 1. *Cont.*

Cell Culture Studies		Major Findings (Changes vs. Negative Control)			Conclusion
[30]	Cell: mouse MBMMφ and RAW 264.7 cells Induction: M-CSF- and RANKL-induced osteoclastogenesis Treatment: 2.5, 5, and 10 μg/mL naringenin for 72 h Control: Negative: no treatment Positive: n.a			• RANKL-induced osteoclastogenesis and resorption area	Naringenin suppresses osteoclast formation and resorption activity.
Animal Studies		**Major Findings (Changes vs. Negative Control)**			
Researchers (Year)	**Study Design**	**Indices Increased**	**Indices Decreased**	**Indices Unchanged**	
[31]	Animal: 28 Female Wistar rats (3 months old) Induction: OVX-induced osteoporosis Treatment: 50 mg/kg of naringenin for 4 weeks Control: Negative: no treatment Positive: 0.2 mg/kg of estradiol oestrogen for 4 weeks	• Trabecular width at epiphysis and metaphysis	• Organic substances in tibia • Periosteal transverse growth of the diaphysis • Ratio of the transverse cross-sectional area of the marrow cavity / diaphysis	• Load, displacement, fracture load, and young modulus in femoral diaphysis • Maximal load in femoral neck • Displacement, maximal load fracture load, displacement for fracture load and • Calcium and phosphorus content in femur, tibia, and L4 vertebrae young modulus in tibia metaphysis • Transverse cross-sectional area of cortical bone in diaphysis and width of cartilage	Naringenin is safe for the skeleton and may have marginal skeletal beneficial effects on the bone.

Table 1. *Cont.*

Animal Studies		Major Findings (Changes vs. Negative Control)			
Researchers (Year)	**Study Design**	**Indices Increased**	**Indices Decreased**	**Indices Unchanged**	
[32]	Animal: 100 Y59 Female rats (3 months old, 200–250 g) Induction: Retinoic acid-induced bone loss Treatment: 100 mg/kg of naringenin for 14 days Control: Negative: no treatment Positive: 40 mg/kg of alendronate for 14 days	• Calcium and phosphorus content in femur • BMD in proximal and distal femur • Femur length • Glutathione content in liver and kidney	• MDA in kidney and liver		Naringenin protects against retinoic acid-induced bone loss via antioxidant effects.
[23]	Animal: 15 Balb/cByJ mice Induction: OVX-induced osteoporosis Treatment: Preliminary studies—1 and 5 mg/kg/day of naringenin (25 μL) for 3 days in newborn mice Preventive studies—5 mg/kg/day of naringenin for 5 weeks in OVX mice Therapeutic studies—5 mg/kg/day of naringenin (i.p. injection) for 6 weeks in OVX mice Control: Negative: no treatment Positive: Preventive studies—17β estradiol (5 μg/kg/day for 5 weeks Therapeutic studies—40 μg/kg/day of human 1-34PTH (i.p. injection) for 6 weeks	• Expression of Erα, Erβ, and BMP-2 in calvaria from newborn mice. • BV/TV, Tb.N, CD, Tb.Sp, Tb.pf, and SMI at femur epiphysis in the preliminary study • Expression of Runx-2 and type I collagen from bone marrow cells harvested from animals • MAR and BFR/BS in preventive and therapeutic studies		• BV/TV, Tb.N, Tb.Th, Tb.Sp, Tb.pf, and SMI in tibia trabecular preventive studies (Naringenin-6-*C*-glucoside could reverse these changes) • BV/TV, Tb. N, Tb. Sp, SMI, and CD in distal femoral epiphysis and tibial proximal metaphysis (Naringenin-6-*C*-glucoside could reverse these changes)	Naringenin is less effective than Naringenin-6-*C*-glucoside in preventing bone loss.

Table 1. *Cont.*

Animal Studies		Major Findings (Changes vs. Negative Control)			
Researchers (Year)	**Study Design**	**Indices Increased**	**Indices Decreased**	**Indices Unchanged**	
[25]	Animals: 20 C-57/BL6 mice (8 weeks old Induction: Ti-particle-induced osteolysis. Treatment: Naringenin (10 mg/kg and 25 mg/kg) for 2 weeks Control: Negative: no treatment Positive: n.a	• BV/TV in the ROI of the calvaria	• Number of pores and percent porosity in the ROI of the calvaria • TRAP-positive multinucleated osteoclasts		Naringenin prevents titanium particle-induced osteolysis caused by excessive osteoclast formation and activity.
[33]	Animal: 40 male Sprague-Dawley rats (46–54 g, 21-day-old) Induction: Soft diet-induced periodontal hypofunction. Treatment: 0.09%, 0.18%, 0.36%, and 0.72% of naringenin for 42 days Control: Negative: no treatment Positive: n.a		• CAB-CEJ distance in buccal maxilla and mandible and lingua maxilla and mandible		Naringenin decreases the molar CAB-CEJ distance during alveolar development in young male rats.
[27]	Animal: Female ICR mice (4 weeks old, 23–29 g) Induction: OVX-induced osteoporosis Treatment: 3 and 10 mg/mL of naringenin every 2 days for 4 weeks Control: Negative: no treatment Positive: 17β-estradiol (0.1 mg/mL every 2 days for 4 weeks)	• BMD and BMC • ALP activity, BMP-2 expression in group treated with 10 mg/mL naringenin			Naringenin prevents bone loss due to ovariectomy.

Table 1. *Cont.*

Animal Studies		Major Findings (Changes vs. Negative Control)			
Researchers (Year)	**Study Design**	**Indices Increased**	**Indices Decreased**	**Indices Unchanged**	
[29]	Animal: 48 adult female adult Wistar rats (200–220 g) Induction: OVX-induced osteoporosis Treatment: 20 mg/kg naringenin for 60 days Control: Negative: no treatment Positive: 5.4 mg/kg raloxifene for 60 days	• Serum ALP levels Improved cortical and trabecular bone architecture • Well-organised bone matrix	• Serum ACP levels		Oral naringenin nanosuspension prevents bone loss due to ovariectomy.
[34]	Animal: 50 Y59 Female rats (3 months old) Induction: Retinoic acid-induced bone loss Treatment: 100 mg/kg of naringenin for 14 days Control: Negative: no treatment Positive: 40 mg/kg of alendronate for 14 days		• Serum β-CTx level • Serum IL-1β, IL-6, TNFα and RANTES • MDA in kidney and ovary • GSH and CAT in kidney	• Serum OCN level Calcium and phosphorus content in femur and serum • BMD and BMC in proximal and distal femur • Eroded endosteal bone surface, cortical bone thickness, and porosity	Naringenin prevents bone loss through antioxidant and anti-inflammatory effects.

Abbreviations: ↑, increase or upregulate; ↓, decrease or downregulate; ↔, no change; RANKL, receptor activator nuclear factor-B; M-CSF, macrophage colony-stimulating factor; IL-1α, -1β, -23, -8, -6, -4, -17, interleukin 1 alpha, 1 beta, -23, -8, -6, -4, -17; TNF-α, tumor necrosis factor alpha; OPG, osteoprotegerin; BMP-2, bone morphogenetic protein-2; Runx2,runt related transcription factor2; Osx, Osterix; p38MAPK, p38 mitogen activated protein kinase; Col1α2, collagen-type I-alpha 2; TRAP, tartrate-resistant acid phosphatase; sTRAP, secretory TRAP; cTRAP, cellular TRAP; mRNA, messenger ribonucleic acid; DC-STAMP, dendritic cell-specific transmembrane protein; NFATc1, nuclear factor of activated T-cell 1; V-ATPase, vacuolar type proton-translocating ATPase; NF-κB, nuclear factor-κB; ERK, extracellular signal-regulated kinase; JNK, *c*-Jun *N*-terminal kinase; IκBα, nuclear factor kappa light polypeptide gene enhancer B-cells inhibitor alpha; *p*-IκBα, phosphorylated nuclear factor kappa light polypeptide gene enhancer B-cells inhibitor alpha; BMSC, bone marrow-derived mesenchymal stem cells; hOB, human osteoblastic cells; pOB, primary osteoblastic cells; *c*-fos, proto-oncogene;CXCR4, chemokine receptor type 4; SDF-1a, stromal cell-derived factor; ALP, alkaline phosphatase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; OVX, ovariectomy; BMD, bone mineral density; BMC, bone mineral content; OP, osteoporosis; MDA, malondialdehyde; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; BV/TV, bone volume/total volume; Tb.N, trabecular number; CD, connectivity density; Tb.sp, trabecular seperation; Tb.pf; SMI; Tb.Th., trabecular thickness; MAR, mineral apposition rate; BFR/BS, bone forming rate/bone surface; ROI, region of interest; CAB-CEJ, crestal alveolar bone-cemento-enamel junction; Ti, titanium; rBMSCs, rats bone marrow stroma cells; ACP, acid phosphatase; RANTES, regulated on Activation, Normal T Expressed and Secreted; β-CTx, β-CrossLaps; OCN, osteocalcin; GSH, reduced glutathione; CAT, catalase; hPDLSCs, human periodontal ligament stem cells.

3.2. Study Characteristics

The studies selected for this review were published from 2005 to 2021. There were eleven in vitro studies included in this review. The studies were either osteoblastogenesis/osteogenesis or osteoclastogenesis. For osteoblastogenesis/osteogenesis studies, cells used were rat bone marrow stroma cells (rBMSCs), mouse calvarial osteoblasts, bone marrow-derived mesenchymal stem cells (BMSCs), rat/mouse calvarial osteoblasts, MC3T3-E1, human foetal osteoblasts (hOB), primary osteoblast cells (pOB) and human periodontal ligament stem cells (hPDLSCs) [21–23,26–29]. The osteogenic effect of naringenin was evaluated through dexamethasone-induced osteogenesis [21,26].

For osteoclastogenesis studies, cells used were rabbit osteoclasts, human primary osteoclasts precursor cells, co-culture of T-cells with bone marrow macrophages (BMMs), bone marrow monocytes (BMM), mouse MBMMφ and RAW 264.7 cell line [20,22,24,25,30]. Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) were used to induce the conversion of osteoclast precursor cells into mature osteoclasts to investigate the effects of naringenin on osteoclast differentiation [20,22,24,25,30]. Doses of naringenin used in the in vitro studies ranged from 0 to 800 μM. The treatment duration for osteoclastogenesis differentiation was about 2–6 days, while osteogenesis differentiation lasted 14–16 days.

Meanwhile, eight in vivo studies involved using rats (Wistar, Sprague Dawley, Y59) and mice (Balb/cByJ, C-57/BL6, and ICR) [23,25,27,29,31–34]. In animal studies, naringenin doses ranged from 0.005–25 mg/kg, 3–10 mg/mL, and 0.09–0.72% weight of diet [23,25,27,29,31–34]. The disease models used in these studies were ovariectomy (OVX)/oestrogen deficiency, retinoic acid-induced bone loss, titanium (Ti) particle-induced osteolysis, and soft diet-induced periodontal hypofunction. In these studies, the bone structure was determined using micro-computed tomography and histomorphometry. The bone remodelling process was investigated using circulating bone remodelling markers. The treatment duration for in vivo studies was between 2–6 weeks. There was no finding from human studies on this topic. The effects of naringenin on bone health are summed up in Table 1.

3.3. Findings from In Vitro Studies

Osteoblasts are mesenchymal cells that support bone formation by producing a bone matrix and subsequently mineralising it [35]. Naringenin was found to inhibit etoposide- and tumour necrosis factor-alpha (TNF-α)-induced osteoblast cell apoptosis in murine primary osteoblastic cells [27]. Naringenin improved osteoblast differentiation, mineralization, and osteogenic function in cultured rat calvarial osteoblasts [22,23,26,27], rBMSCs [21], and hPDLSCs [28] through the bone morphogenetic protein-2 (BMP-2)/p38 mitogen-activated protein kinase (p38 MAPK)/runt-related transcription factor2 (Runx2)/Osterix (Osx)/alkaline phosphatase (ALP) signalling pathway. Enhanced expressions of C-X-C chemokine receptor type 4 (CXCR4) and stromal cell-derived factor 1 (SDF-1a) levels were observed in naringenin-treated BMSCs [26] and hPDLSCs [28], suggesting that naringenin regulated osteogenic differentiation through the SDF-1/CXCR4 signalling pathway. Naringenin also exerted BMP-dependent osteogenic effects via the phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), *c*-Fos/*c*-Jun and activator protein 1 (AP-1) dependent signalling pathways [27]. Another study also found that naringenin increased OPG/RANKL ratio based on mRNA and protein expression from osteoblasts [22]. This alteration can potentially alter osteoclast formation. However, no changes in osteocalcin (OCN) were observed in MG-63 cell lines treated with naringenin. The possible molecular mechanisms of naringenin's effect on osteoblasts are summarized in Figure 3.

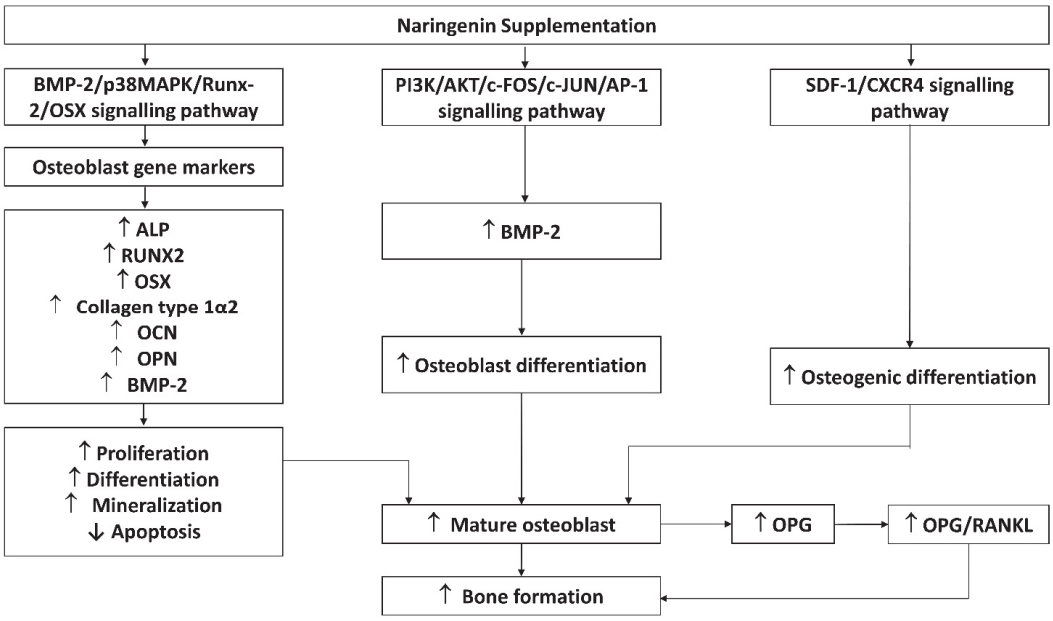


Figure 3. Possible mechanism of naringenin effect on osteoblasts. Abbreviations: ALP, alkaline phosphatase; IL-1β, OPG, osteoprotegerin; OSX, osterix; RANK, receptor activator of nuclear factor-kappa B ligand; BMP-2; p38MAPK, phosphoinositide 38 mitogen-activated protein kinase; Runx2, runt-related transcription factor2; OCN, osteocalcin; OPN, osteopontin; P13K, phosphoinositide 3-kinase (PI3K); AKT, protein kinase B; *c*-FOS; *c*-JUN; AP-1, activator protein 1; SDF-1, stromal cell-derived factor 1; CXCR4, C-X-C chemokine receptor type 4.

Osteoclasts are multinucleated hematopoietic cells that serve as the main bone-resorbing cells and play an important role in bone remodelling [36]. Naringenin significantly inhibited osteoclastogenesis and secretion of interleukin (IL)-1α, IL-23 as well monocyte chemoattractant protein-1 in pre-osteoclast cultures. A significant decrease in helical peptide 620–633 release indicating bone resorption activity by naringenin, and an increase in tumour necrosis factor-α (TNF-α), IL-8, and IL-4 [24] levels were observed in human pre-osteoclasts, but no changes were found on IL-6, IL-1b, IL-17, and osteoprotegerin (OPG) levels [20]. Besides, naringenin also was found to inhibit osteoclast formation and bone resorption activity, indicated by reduced numbers of osteoclasts, bone resorption pits, and area, as well as markers of osteoclast maturation, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin-K [22,24,25]. Naringenin was proven to minimize the number and size of F-actin rings, lower the expressions of cathepsin K, *c*-Fos, dendritic cell-specific transmembrane protein (DC-STAMP), nuclear factor of activated T-cell 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP) and vacuolar type proton-translocating ATPase (V-ATPase d2) of osteoclasts at mRNA and protein levels [24,25]. Furthermore, naringenin has been shown to reduce the RANKL-induced p38 phosphorylation signalling in pre-osteoclasts and cause no changes in gene expression of nuclear factor-κB (NF-κB), extracellular signal-regulated kinase (ERK), *c*-Jun *N*-terminal kinase (JNK), nuclear factor kappa light polypeptide gene enhancer B-cells inhibitor alpha (IκBα) and phosphorylated nuclear factor kappa light polypeptide gene enhancer B-cells inhibitor alpha (*p*-IκBα) [25]. The possible mechanism of actions of naringenin on osteoclasts are summarised in Figure 4.

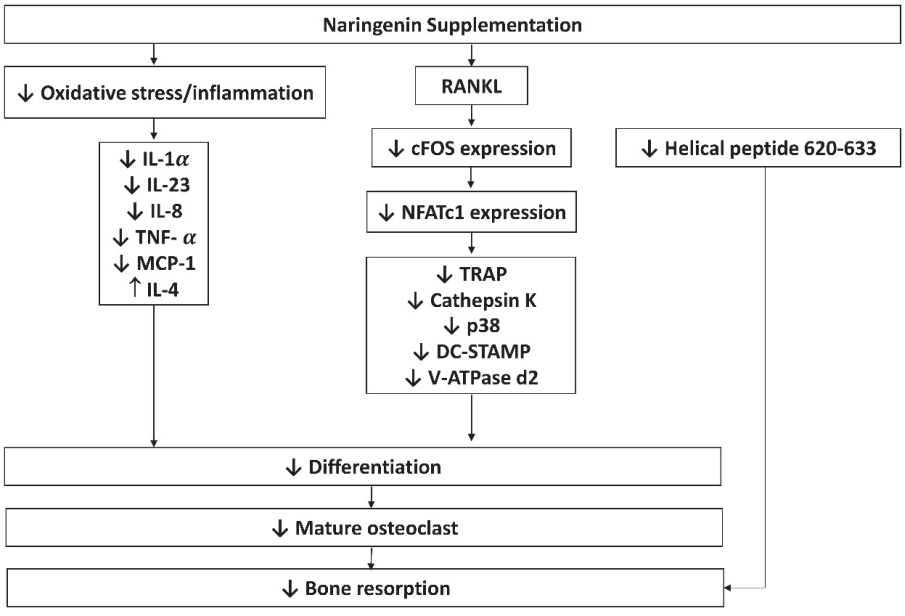


Figure 4. Possible mechanism of actions of naringenin on osteoclasts. Abbreviations: IL-1 α, -23, -8, -4, interleukin-1 alpha, -23, -8, -4; NFATc1, nuclear factor of activated T- cells; OPG, osteoprotegerin; OSX, osterix; RANKL, receptor activator of nuclear factor-kappa B; ligand; TNF-α, tumour necrosis factor-alpha; TRAP, tartrate-resistant acid phosphatase; p38, phosphoinositide 38; DC-STAMP, dendritic cell-specific transmembrane protein; V-ATPase d2, vacuolar-type proton-translocating ATPase; MCP-1, monocyte chemoattractant protein-1.

3.4. Findings from In Vivo Studies

Postmenopausal bone loss due to oestrogen deficiency is caused by a high bone remodelling phenomenon that results in an imbalance between bone formation and resorption [37]. Gera et al., (2022) [29] reported that naringenin supplementation (20 mg/kg for 60 days) in female Wistar rats with OVX-induced osteoporosis increased ALP and decreased acid phosphatase in the serum. They also reported improved cortical and trabecular bone architecture following naringenin supplementation. Kaczmarczyk-Sedlak et al., 2016 [31] reported that naringenin supplementation (50 mg/kg for 4 weeks) in female Wistar rats with OVX-induced osteoporosis increased the width of trabecular in the epiphysis, decreased organic substances in the tibia, periosteal transverse growth of the diaphysis as well lower the ratio of the transverse cross-section area of the marrow cavity/diaphysis. However, the study showed no skeletal biomechanical and mineral (calcium and phosphate) changes probably because of the short duration of supplementation. The supplementation also did not alter the transverse cross-sectional area of cortical bone in the diaphysis and cartilage width compared to the negative group.

Swarnkar et al., (2012) [23] discovered that naringenin (10 mg/kg for 6 weeks) treatment in Balb/cByJ mice mitigated OVX-induced trabecular bone changes by increasing the mineralized nodule, mineral apposition rate and bone formation rate/bone surface (BFR/BS) compared to negative group. This effect was probably attributed to improved osteoblastogenesis indicated by the mRNA gene expression of runt-related transcription factor 2 (Runx-2) and type I collagen. In a preventive model, the same group revealed a lack of changes in trabecular microstructure at the distal femoral epiphysis and tibial proximal metaphysis. In the therapeutic model (5 mg/kg/day of naringenin (i.p.) for 6 weeks), they reported decreased trabecular bone pattern factor but no changes in trabecular microstruc-

ture in the distal femoral epiphysis and tibial proximal metaphysis with supplementation. Wu et al., (2008) [27] also claimed that treating OVX ICR mice with naringenin (10 mg/mL every 2 days for 4 weeks) could enhance BMD, bone mineral content, ALP activity, and BMP-2 expression.

Retinoic acid-induced bone loss in rats has been used to evaluate the skeletal impacts of multiple substances on the skeletal system [38]. According to Oršolić et al., (2014) [32], the administration of naringenin (100 mg/kg for 14 days) in Y59 female rats with retinoic acid-induced bone loss resulted in increased calcium and phosphorus content in the femur, higher BMD in the proximal and distal femur and improved femur length. They attribute the protection to the improvement of the redox status of the rats, but the redox parameters, such as glutathione and malondialdehyde, were measured in the liver and kidney. Oršolić et al., (2022) [34] also reported that the administration of naringenin (100 mg/kg for 14 days) in Y59 female rats with retinoic acid-induced bone loss resulted in decreased serum β-CTx, IL-1β, IL-6, TNFα, and chemokine ligand 5 (CCL5/RANTES). They also reported decreased MDA and increased GSH and CAT in the ovary and kidney. However, they reported a lack of changes in circulating bone formation marker (OCN) level, circulating, bone calcium, and phosphorus levels, densitometry, and cortical geometry in the supplemented group. A short duration of treatment might be responsible for the lack of skeletal effects of the treatment.

Osteolysis is the progressive degeneration of periprosthetic bony tissue, which appears on serial radiographs as progressive radiolucent lines and/or cavitation at the implant-bone or cement-bone interface. Osteolysis can progress to aseptic loosening and implant failure if not treated promptly [39]. Based on the study performed by Wang et al., (2014) [25], naringenin supplementation (10 and 25 mg/kg for 2 weeks) reduced TRAP-positive multinucleated osteoclasts and lowered the number of pores and the percentage porosity in the calvarial region of interest in *C*-57/BL6 mice with Ti-particle induce osteolysis. Moreover, an increase in trabecular bone volume was reported compared to the negative group.

Periodontal hypofunction can occur as a consequence of disassociation with an opposing tooth in certain malocclusions, such as open bites and ectopic teeth [40]. Wood (2005) [33] showed that naringenin supplementation (0.09%, 0.18%, 0.36%, and 0.72% for 42 days) reduced physiological molar crestal alveolar bone (CAB)-cemento-enamel junction (CEJ) distance in buccal maxilla and mandible as well as in lingua maxilla and mandible in Sprague Dawley rats with soft diet-induced periodontal hypofunction compared to the unsupplemented group.

4. Discussion

The currently available evidence shows that naringenin exerts stimulatory effects on osteoblasts through MAPK, PI3K/Akt, and CXCR4/SDF-1 pathways. Osteoblasts are responsible for osteogenesis by synthesising and mineralising organic bone matrix (osteoid) during skeleton construction and bone remodelling [41]. The MAPK cascades regulate Runx2 phosphorylation and transcription, which promote osteoblast differentiation. MAPK pathways and their components, JNK, ERK, and p38, which enforce osteoblastogenesis and establish the non-canonical BMP-2 signal transduction pathways [42–45]. Naringenin stimulates osteogenic gene activation, indicating that it has a stimulatory effect on osteogenic differentiation [21,22]. Activation of the PI3K/Akt signalling pathway also promotes osteoblast proliferation, differentiation, and bone formation activity [46]. Naringenin has been reported to stimulate BMP-2-dependent osteoblastogenesis through the activation of the PI3K/Akt signalling pathway [27]. Activation of the CXCR4/SDF-1 signalling pathway is critical in early osteoblastogenesis and its suppression leads to lower bone formation and mineralisation [47,48].

Osteoclasts are bone resorption cells originating from hematopoietic lineage cells [49]. Bone resorption is vital in bone remodelling, but excessive resorption can result in pathological bone loss. Osteoclast differentiation and activation are governed by various hormones and cytokines. The cytokines RANKL and M-CSF, in particular, are required for osteoclastic differentiation [50]. To promote osteoclast differentiation, preservation and bone resorption,

the M-CSF binds to the colony-stimulating factor 1 receptor, whilst RANKL binds to the RANK receptor [51,52]. TRAF factors such as TRAF 6 are recruited by RANK-RANKL binding [53], leading to the activation of NF-kB, Akt, and MAPKs (ERK/p38/JNK) pathways. Furthermore, the RANKL signalling stimulates *c*-Fos and then NFATc1, a major switch that plays a role in controlling osteoclast terminal differentiation [54,55]. Naringenin was reported to reduce M-CSF and RANKL-induced expression of critical markers of osteoclast differentiation markers such as cathepsin K, *c*-Fos, and NFATc1 [24,25].

The biological properties of naringenin suggest a broad range of clinical applications. Naringenin decreased CAB-CEJ distance in buccal maxilla and mandible as well as in lingua maxilla and mandible, indicating that naringenin supplementation protects against alveolar bone loss in rats with induced periodontal disease [33]. Supplementation of naringenin also improved bone mineral microstructure, mineral, and biomechanical strength [23,27,31,32]. However, the bone loss models that have been used to test the effects of naringenin have been limited to OVX and retinol-induced models. Thus, results from other models, such as testosterone deficiency and glucocorticoid models, are indispensable before it is tested on patients with other causes of osteoporosis. Following joint replacement, the abrasive particles initiated by the prosthesis are primarily responsible for osteolysis [56]. Naringenin supplementation prevented Ti-particle-induced osteolysis, implying that it may be preferable for treating periprosthetic osteolysis [25].

Pharmacokinetics and safety issues of naringenin should be considered before it is used clinically. From the pharmacokinetic aspects, naringenin has very low in vivo bioavailability due to its hydrophobic nature, which limits its practical use. It has a short half-life and is easily converted to its crystalline form, and therefore it is poorly absorbed by the digestive system [57–60]. Previous researchers developed a variety of methods to improve naringenin absorption and low bioavailability, including particle size reduction, complexation with cyclodextrins [61], salt formation, solid dispersions [62], surfactant usage, nanoparticles, nanocarriers [63], and self-emulsifying drug delivery system, as well as prodrug formation [64]. Nanotechnology proved to be an efficient way to improve the bioavailability of naringenin by multiple delivery routes to enhance its effectiveness in the treatment of cancer, inflammation, diabetes, liver, brain, and ocular diseases mostly through numerous in vitro and in vivo methods [65]. Meanwhile, Rodríguez-Fragoso et al., (2011) [66] found out that naringenin inhibits some drug-metabolizing cytochrome P450 enzymes, including CYP3A4 and CYP1A2, potentially leading to drug-drug interactions in the intestine and liver, where phytochemical concentrations are higher. Modification in cytochrome P450 and other enzymatic activity may influence the outcome of drugs that go through extensive first-pass metabolism. An acute toxicity study using Wistar rats reported the lethal dose (LD_{50}) value of naringenin to be 340 mg/kg body weight [67]. Using body surface ratio conversation [68], the human equivalent dose is 64 mg/kg.

The term "naringenin" was searched for on https://clinicaltrials.gov/ (accessed on 31 August 2022) and the search revealed thirteen registered clinical trials on naringenin. The trials investigate the effects of naringenin on healthy subjects (NCT02627547, NCT04867655, NCT05073523, NCT02380144), hepatitis virus/HCV infection/chronic HCV/Hepatitis C (NCT01091077), energy expenditure/safety issues/glucose metabolism (NCT04697355), safety issues/pharmacokinetics (NCT03582553), subjective cognitive decline (NCT04744922), cardiovascular disease risk factors (NCT00539916), intestinal disease (NCT03032861), metabolic syndrome/vascular compliance/predisposition to cardiovascular disease (NCT04731987), pharmacokinetics of new curcumin formulations/safety of new curcumin formulations (NCT01982734) and cardiovascular risk factor/type-2 diabetes mellitus/insulin sensitivity/metabolic syndrome (NCT03527277). Seven of these clinical trials have been completed, four are still recruiting, one with an unknown status, and lastly one trial is still active but not recruiting. However, no attempt has been made to conduct a human clinical trial to evaluate the impact of naringenin on skeletal diseases. Since pure naringenin has only been studied in limited clinical trials, more research on free drug and naringenin-loaded nanosystems in humans is warranted. Further exploration into the interactions of these

nanoformulations with the human body is required before they can be translated into pharmaceuticals and nutraceutical supplements [69].

This review also has several limitations. We only include articles written in English in this review, which potentially excludes studies published in other languages. We did not exclude studies based on their quality because the number of studies is limited. Nevertheless, the current review provides an overview of the skeletal effects of naringenin and prospects of its clinical application as a functional food component to protect bone health.

5. Conclusions

Preclinical findings demonstrate that naringenin protects the skeleton by suppressing osteoclastogenesis and bone resorption while enhancing osteoblastogenesis and bone formation. Human clinical trials to justify naringenin's skeletal effects are lacking. Hence, comprehensive clinical studies should be performed to validate naringenin's skeletal properties and reveal the safety of this flavanone compound.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14224851/s1, Table S1: Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) Checklist. Reference [70] is cited in the supplementary materials.

Author Contributions: Conceptualization, methodology, M.L.N.M., S.O.E. and K.-Y.C.; validation, S.-K.W. and K.-Y.C.; writing-original draft preparation, M.L.N.M.; writing—review and editing, S.O.E., S.-K.W., and K.-Y.C.; visualization, M.L.N.M. and S.O.E.; supervision, K.-Y.C.; funding acquisition, K.-Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Universiti Kebangsaan Malaysia through Research University Grant (GUP-2020-021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: This manuscript does not contain original data.

Acknowledgments: The authors thank Universiti Kebangsaan Malaysia for the support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sozen, T.; Ozisik, L.; Calik Basaran, N. An overview and management of osteoporosis. *Eur. J. Rheumatol.* **2017**, *4*, 46–56. [CrossRef] [PubMed]
2. Cosman, F.; de Beur, S.J.; LeBoff, M.S.; Lewiecki, E.M.; Tanner, B.; Randall, S.; Lindsay, R. Clinician's Guide to Prevention and Treatment of Osteoporosis. *Osteoporos. Int.* **2014**, *25*, 2359–2381. [CrossRef] [PubMed]
3. Deeks, E.D. Denosumab: A Review in Postmenopausal Osteoporosis. *Drugs Aging* **2018**, *35*, 163–173. [CrossRef] [PubMed]
4. Baber, R.J.; Panay, N.; Fenton, A. 2016 IMS Recommendations on womens midlife health and menopause hormone therapy. *Climacteric* **2016**, *19*, 109–150. [CrossRef]
5. Shoback, D.; Rosen, C.J.; Black, D.M.; Cheung, A.M.; Murad, M.H.; Eastell, R. Pharmacological Management of Osteoporosis in Postmenopausal Women: An Endocrine Society Guideline Update. *J. Clin. Endocrinol. Metab.* **2020**, *105*, 587–594. [CrossRef] [PubMed]
6. Black, D.M.; Rosen, C.J. Clinical Practice. Postmenopausal Osteoporosis. *N. Engl. J. Med.* **2016**, *374*, 254–262. [CrossRef] [PubMed]
7. Sleeman, A.; Clements, J.N. Abaloparatide: A new pharmacological option for osteoporosis. *Am. J. Health-Syst. Pharm.* **2019**, *76*, 130–135. [CrossRef]
8. Lacativa, P.G.S.; de Farias, M.L.F. Osteoporose e inflamação. *Arq. Bras. Endocrinol. Metabol.* **2010**, *54*, 123–132. [CrossRef]
9. Ginaldi, L.; Di Benedetto, M.C.; De Martinis, M. Osteoporosis, inflammation and ageing. *Immun. Ageing* **2005**, *2*, 14. [CrossRef]
10. Domazetovic, V.; Marcucci, G.; Iantomasi, T.; Brandi, M.L.; Vincenzini, M.T. Oxidative stress in bone remodeling: Role of antioxidants. *Clin. Cases Miner. Bone Metab.* **2017**, *14*, 209–216. [CrossRef]
11. Tang, B.M.; Eslick, G.D.; Nowson, C.; Smith, C.; Bensoussan, A. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: A meta-analysis. *Lancet* **2007**, *370*, 657–666. [CrossRef]
12. Ekeuku, S.O.; Pang, K.L.; Chin, K.Y. Effects of caffeic acid and its derivatives on bone: A systematic review. *Drug Des. Dev. Ther.* **2021**, *15*, 259–275. [CrossRef] [PubMed]

13. Chen, H.J.; Inbaraj, B.S.; Chen, B.H. Determination of phenolic acids and flavonoids in Taraxacum formosanum kitam by liquid chromatography-tandem mass spectrometry coupled with a post-column derivatization technique. *Int. J. Mol. Sci.* **2012**, *13*, 260–285. [CrossRef] [PubMed]
14. Renugadevi, J.; Prabu, S.M. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. *Toxicology* **2009**, *256*, 128–134. [CrossRef] [PubMed]
15. Shi, Y.; Dai, J.; Liu, H.; Li, R.R.; Sun, P.L.; Du, Q.; Pang, L.L.; Chen, Z.; Yin, K.S. Naringenin inhibits allergen-induced airway inflammation and airway responsiveness and inhibits NF-κB activity in a murine model of asthma. *Can. J. Physiol. Pharmacol.* **2009**, *87*, 729–735. [CrossRef]
16. Tsai, S.J.; Huang, C.S.; Mong, M.C.; Kam, W.Y.; Huang, H.Y.; Yin, M.C. Anti-inflammatory and antifibrotic effects of naringenin in diabetic mice. *J. Agric. Food Chem.* **2012**, *60*, 514–521. [CrossRef]
17. Chung, T.W.; Li, S.; Lin, C.C.; Tsai, S.W. Antinociceptive and anti-inflammatory effects of the citrus flavanone naringenin. *Tzu Chi Med. J.* **2019**, *31*, 81–85. [CrossRef]
18. Annadurai, T.; Muralidharan, A.R.; Joseph, T.; Hsu, M.J.; Thomas, P.A.; Geraldine, P. Antihyperglycemic and antioxidant effects of a flavanone, naringenin, in streptozotocin-nicotinamide-induced experimental diabetic rats. *J. Physiol. Biochem.* **2012**, *68*, 307–318. [CrossRef]
19. Rashmi, R.; Magesh, S.B.; Ramkumar, K.M.; Suryanarayanan, S.; SubbaRao, M.V. Antioxidant potential of naringenin helps to protect liver tissue from streptozotocin-induced damage. *Rep. Biochem. Mol. Biol.* **2017**, *7*, 76–84.
20. La, V.D.; Tanabe, S.; Grenier, D. Naringenin inhibits human osteoclastogenesis and osteoclastic bone resorption. *J. Periodontal Res.* **2009**, *44*, 193–198. [CrossRef]
21. Ming, L.G.; Ge, B.F.; Wang, M.G.; Chen, K.M. Comparison between 8-prenylnarigenin and narigenin concerning their activities on promotion of rat bone marrow stromal cells' osteogenic differentiation in vitro. *Cell Prolif.* **2012**, *45*, 508–515. [CrossRef] [PubMed]
22. Ming, L.-G.; Lv, X.; Ma, X.-N.; Ge, B.-F.; Zhen, P.; Song, P.; Zhou, J.; Ma, H.-P.; Xian, C.J.; Chen, K.-M. The Prenyl Group Contributes to Activities of Phytoestrogen 8-Prenynaringenin in Enhancing Bone Formation and Inhibiting Bone Resorption In Vitro. *Endocrinology* **2013**, *154*, 1202–1214. [CrossRef] [PubMed]
23. Swarnkar, G.; Sharan, K.; Siddiqui, J.A.; Mishra, J.S.; Khan, K.; Khan, M.P.; Gupta, V.; Rawat, P.; Maurya, R.; Dwivedi, A.K.; et al. A naturally occurring naringenin derivative exerts potent bone anabolic effects by mimicking oestrogen action on osteoblasts. *Br. J. Pharmacol.* **2012**, *165*, 1526–1542. [CrossRef] [PubMed]
24. Wang, W.; Li, M.; Luo, M.; Shen, M.; Xu, C.; Xu, G.; Chen, Y.; Xia, L. Naringenin inhibits osteoclastogenesis through modulation of helper T cells-secreted IL-4. *J. Cell. Biochem.* **2018**, *119*, 2084–2093. [CrossRef]
25. Wang, W.; Wu, C.; Tian, B.; Liu, X.; Zhai, Z.; Qu, X.; Jiang, C.; Ouyang, Z.; Mao, Y.; Tang, T.; et al. The inhibition of RANKL-induced osteoclastogenesis through the suppression of p38 signaling pathway by naringenin and attenuation of titanium-particle-induced osteolysis. *Int. J. Mol. Sci.* **2014**, *15*, 21913–21934. [CrossRef]
26. Wang, Y.; Bai, S.; Cheng, Q.; Zeng, Y.; Xu, X.; Guan, G. Naringenin promotes sdf-1/cxcr4 signaling pathway in bmscs osteogenic differentiation. *Folia Histochem. Cytobiol.* **2021**, *59*, 66–73. [CrossRef]
27. Wu, J.-B.; Fong, Y.C.; Tsai, H.Y.; Chen, Y.F.; Tsuzuki, M.; Tang, C.H. Naringin-induced bone morphogenetic protein-2 expression via PI3K, Akt, c-Fos/c-Jun and AP-1 pathway in osteoblasts. *Eur. J. Pharmacol.* **2008**, *588*, 333–341. [CrossRef]
28. Zhang, L.; He, H.; Zhang, M.; Wu, Y.; Xu, X.; Yang, M.; Mei, L. Assessing the effect and related mechanism of naringenin on the proliferation, osteogenic differentiation and endothelial differentiation of human periodontal ligament stem cells. *Biochem. Biophys. Res. Commun.* **2021**, *534*, 337–342. [CrossRef]
29. Gera, S.; Sampathi, S.; Maddukuri, S.; Dodoala, S.; Junnuthula, V.; Dyawanapelly, S. Therapeutic Potential of Naringenin Nanosuspension: In Vitro and In Vivo Anti-Osteoporotic Studies. *Pharmaceutics* **2022**, *14*, 1449. [CrossRef]
30. Wu, Y.-W.; Chen, S.-C.; Lai, W.-F.T.; Chen, Y.-C.; Tsai, Y.-H. Screening of flavonoids for effective osteoclastogenesis suppression. *Anal. Biochem.* **2013**, *433*, 48–55. [CrossRef]
31. Kaczmarczyk-Sedlak, I.; Wojnar, W.; Zych, M.; Ozimina-Kamińska, E.; Bońka, A. Effect of dietary flavonoid naringenin on bones in rats with ovariectomy-induced osteoporosis. *Acta Pol. Pharm.-Drug Res.* **2016**, *73*, 1073–1081.
32. Oršolić, N.; Goluža, E.; Dikić, D.; Lisičić, D.; Sašilo, K.; Rodak, E.; Jeleč, Ž.; Lazarus, M.V.; Orct, T. Role of flavonoids on oxidative stress and mineral contents in the retinoic acid-induced bone loss model of rat. *Eur. J. Nutr.* **2014**, *53*, 1217–1227. [CrossRef] [PubMed]
33. Wood, N. The effects of dietary naringenin supplementation on physiological changes in molar crestal alveolar bone-cemento-enamel junction distance in young rats. *J. Med. Food* **2005**, *8*, 31–35. [CrossRef] [PubMed]
34. Oršolić, N.; Nemrava, J.; Jeleč, Ž.; Kukolj, M.; Odeh, D.; Jakopović, B.; Jazvinšćak Jembrek, M.; Bagatin, T.; Fureš, R.; Bagatin, D. Antioxidative and Anti-Inflammatory Activities of Chrysin and Naringenin in a Drug-Induced Bone Loss Model in Rats. *Int. J. Mol. Sci.* **2022**, *23*, 2872. [CrossRef]
35. Dirckx, N.; Moorer, M.C.; Clemens, T.L.; Riddle, R.C. The role of osteoblasts in energy homeostasis. *Nat. Rev. Endocrinol.* **2019**, *15*, 651–665. [CrossRef] [PubMed]
36. Schett, G. Cells of the synovium in rheumatoid arthritis. Osteoclasts. *Arthritis Res. Ther.* **2007**, *9*, 203. [CrossRef]
37. Eastell, R.; O'Neill, T.W.; Hofbauer, L.C.; Langdahl, B.; Reid, I.R.; Gold, D.T.; Cummings, S.R. Postmenopausal osteoporosis. *Nat. Rev. Dis. Prim.* **2016**, *2*, 16069. [CrossRef]

38. Wei, M.; Yang, Z.; Li, P.; Zhang, Y.; Sse, W.C. Anti-osteoporosis activity of naringin in the retinoic acid-induced osteoporosis model. *Am. J. Chin. Med.* **2007**, *35*, 663–667. [CrossRef]
39. Saleh, K.J.; Thongtrangan, I.; Schwarz, E.M. Osteolysis: Medical and surgical approaches. *Clin. Orthop. Relat. Res.* **2004**, *427*, 138–147. [CrossRef]
40. Sringkarnboriboon, S.; Matsumoto, Y.; Soma, K. Root resorption related to hypofunctional periodontium in experimental tooth movement. *J. Dent. Res.* **2003**, *82*, 486–490. [CrossRef]
41. Tanaka, Y.; Nakayamada, S.; Okada, Y. Osteoblasts and osteoclasts in bone remodeling and inflammation. *Curr. Drug Targets Inflamm. Allergy* **2005**, *4*, 325–328. [CrossRef] [PubMed]
42. Xiao, G.; Gopalakrishnan, R.; Jiang, D.; Reith, E.; Benson, M.D.; Franceschi, R.T. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J. Bone Miner. Res.* **2002**, *17*, 101–110. [CrossRef] [PubMed]
43. Massagué, J.; Blain, S.W.; Lo, R.S. TGFβ signaling in growth control, cancer, and heritable disorders. *Cell* **2000**, *103*, 295–309. [CrossRef]
44. Nohe, A.; Hassel, S.; Ehrlich, M.; Neubauer, F.; Sebald, W.; Henis, Y.I.; Knaus, P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J. Biol. Chem.* **2002**, *277*, 5330–5338. [CrossRef] [PubMed]
45. Guo, X.; Wang, X.F. Signaling cross-talk between TGF-β/BMP and other pathways. *Cell Res.* **2009**, *19*, 71–88. [CrossRef] [PubMed]
46. Xi, J.C.; Zang, H.Y.; Guo, L.X.; Xue, H.B.; Liu, X.D.; Bai, Y.B.; Ma, Y.Z. The PI3K/AKT cell signaling pathway is involved in regulation of osteoporosis. *J. Recept. Signal Transduct.* **2015**, *35*, 640–645. [CrossRef]
47. Xu, J.; Chen, Y.; Liu, Y.; Zhang, J.; Kang, Q.; Ho, K.; Chai, Y.; Li, G. Effect of SDF-1/Cxcr4 Signaling Antagonist AMD3100 on Bone Mineralization in Distraction Osteogenesis. *Calcif. Tissue Int.* **2017**, *100*, 641–652. [CrossRef]
48. Luan, J.; Cui, Y.; Zhang, Y.; Zhou, X.; Zhang, G.; Han, J. Effect of CXCR4 inhibitor AMD3100 on alkaline phosphatase activity and mineralization in osteoblastic MC3T3-E1 cells. *Biosci. Trends* **2012**, *6*, 63–69. [CrossRef]
49. Soysa, N.S.; Alles, N. Osteoclast function and bone-resorbing activity: An overview. *Biochem. Biophys. Res. Commun.* **2016**, *476*, 115–120. [CrossRef]
50. Ono, T.; Nakashima, T. Recent advances in osteoclast biology. *Histochem. Cell Biol.* **2018**, *149*, 325–341. [CrossRef]
51. Suda, T.; Takahashi, N.; Udagawa, N.; Jimi, E.; Gillespie, M.T.; Martin, T.J. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev.* **1999**, *20*, 345–357. [CrossRef] [PubMed]
52. Mun, S.H.; Park, P.S.U.; Park-Min, K.H. The M-CSF receptor in osteoclasts and beyond. *Exp. Mol. Med.* **2020**, *52*, 1239–1254. [CrossRef]
53. Bharti, A.C.; Takada, Y.; Shishodia, S.; Aggarwal, B.B. Evidence That Receptor Activator of Nuclear Factor (NF)-κB Ligand Can Suppress Cell Proliferation and Induce Apoptosis through Activation of a NF-κB-independent and TRAF6-dependent Mechanism. *J. Biol. Chem.* **2004**, *279*, 6065–6076. [CrossRef] [PubMed]
54. Takayanagi, H.; Kim, S.; Koga, T.; Nishina, H.; Isshiki, M.; Yoshida, H.; Saiura, A.; Isobe, M.; Yokochi, T.; Inoue, J.; et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell* **2002**, *3*, 889–901. [CrossRef]
55. Ishida, N.; Hayashi, K.; Hoshijima, M.; Ogawa, T.; Koga, S.; Miyatake, Y.; Kumegawa, M.; Kimura, T.; Takeya, T. Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. *J. Biol. Chem.* **2002**, *277*, 41147–41156. [CrossRef] [PubMed]
56. Yao, J.; Ma, S.; Feng, W.; Wei, Y.; Lu, H.; Zhong, G.; Wu, Z.; Wang, H.; Su, W.; Li, J. Tanshinone IIA protects against polyethylene particle-induced osteolysis response in a mouse calvarial model. *Int. J. Clin. Exp. Pathol.* **2018**, *11*, 4461–4471.
57. Kumar, R.P.; Abraham, A. PVP- coated naringenin nanoparticles for biomedical applications—In vivo toxicological evaluations. *Chem. Biol. Interact.* **2016**, *257*, 110–118. [CrossRef]
58. Shpigelman, A.; Shoham, Y.; Israeli-Lev, G.; Livney, Y.D. β-Lactoglobulin-naringenin complexes: Nano-vehicles for the delivery of a hydrophobic nutraceutical. *Food Hydrocoll.* **2014**, *40*, 214–224. [CrossRef]
59. Yang, L.J.; Ma, S.X.; Zhou, S.Y.; Chen, W.; Yuan, M.W.; Yin, Y.Q.; Yang, X.D. Preparation and characterization of inclusion complexes of naringenin with β-cyclodextrin or its derivative. *Carbohydr. Polym.* **2013**, *98*, 861–869. [CrossRef]
60. Martinez, S.E.; Lillico, R.; Lakowski, T.M.; Martinez, S.A.; Davies, N.M. Pharmacokinetic analysis of an oral multicomponent joint dietary supplement (Phycox®) in dogs. *Pharmaceutics* **2017**, *9*, 30. [CrossRef]
61. Shulman, M.; Cohen, M.; Soto-Gutierrez, A.; Yagi, H.; Wang, H.; Goldwasser, J.; Lee-Parsons, C.W.; Benny-Ratsaby, O.; Yarmush, M.L.; Nahmias, Y. Enhancement of naringenin bioavailability by complexation with hydroxypropoyl-β-cyclodextrin. *PLoS ONE* **2011**, *6*, e18033. [CrossRef] [PubMed]
62. Kanaze, F.I.; Kokkalou, E.; Niopas, I.; Georgarakis, M.; Stergiou, A.; Bikiaris, D. Dissolution enhancement of flavonoids by solid dispersion in PVP and PEG matrixes: A comparative study. *J. Appl. Polym. Sci.* **2006**, *102*, 460–471. [CrossRef]
63. Bali, V.; Ali, M.; Ali, J. Nanocarrier for the enhanced bioavailability of a cardiovascular agent: In vitro, pharmacodynamic, pharmacokinetic and stability assessment. *Int. J. Pharm.* **2011**, *403*, 46–56. [CrossRef] [PubMed]
64. Khan, A.W.; Kotta, S.; Ansari, S.H.; Sharma, R.K.; Ali, J. Potentials and challenges in self-nanoemulsifying drug delivery systems. *Expert Opin. Drug Deliv.* **2012**, *9*, 1305–1317. [CrossRef] [PubMed]

65. Teng, H.; Zheng, Y.; Cao, H.; Huang, Q.; Xiao, J.; Chen, L. Enhancement of bioavailability and bioactivity of diet-derived flavonoids by application of nanotechnology: A review. *Crit. Rev. Food Sci. Nutr.* **2021**, 1–16. [CrossRef]
66. Rodríguez-Fragoso, L.; Martínez-Arismendi, J.L.; Orozco-Bustos, D.; Reyes-Esparza, J.; Torres, E.; Burchiel, S.W. Potential Risks Resulting from Fruit/Vegetable-Drug Interactions: Effects on Drug-Metabolizing Enzymes and Drug Transporters. *J. Food Sci.* **2011**, *76*, R112–R124. [CrossRef]
67. Selvam, M.; Kaliyaperumal, M. Determination of LD 50 of Naringenin for its effects on diabetic nephropathy in rats-A pilot study. *J. Chem. Pharm. Res.* **2015**, *7*, 550–553.
68. Shin, J.; Seol, I.; Son, C. Interpretation of Animal Dose and Human Equivalent Dose for Drug Development. *J. Korean Orient. Med.* **2010**, *31*, 1–7.
69. Bhia, M.; Motallebi, M.; Abadi, B.; Zarepour, A.; Pereira-Silva, M.; Saremnejad, F.; Santos, A.C.; Zarrabi, A.; Melero, A.; Jafari, S.M.; et al. Naringenin nano-delivery systems and their therapeutic applications. *Pharmaceutics* **2021**, *13*, 291. [CrossRef]
70. Tricco, A.C.; Lillie, E.; Zarin, W.; O'Brien, K.K.; Colquhoun, H.; Levac, D.; Moher, D.; Peters, M.D.J.; Horsley, T.; Weeks, L.; et al. PRISMA Extension for Scoping Reviews (PRISMA-ScR): Checklist and Explanation. *Ann. Intern. Med.* **2018**, *169*, 467–473. [CrossRef]

nutrients

MDPI

Article

Uric Acid Levels Are Associated with Bone Mineral Density in Mexican Populations: A Longitudinal Study

Karina Robles-Rivera [1,†], Anna D. Argoty-Pantoja [1,†], Alberto Hidalgo-Bravo [2], Amado D. Quezada-Sánchez [3], Guadalupe León-Reyes [4], Yvonne N. Flores [5,6,7,8], Jorge Salmerón [1], Rafael Velázquez-Cruz [4] and Berenice Rivera-Paredez [1,*]

1 Research Center in Policies, Population and Health, School of Medicine, National Autonomous University of Mexico (UNAM), Mexico City 04510, Mexico
2 Department of Genetics, National Institute of Rehabilitation (INR), Mexico City 14389, Mexico
3 Center for Evaluation and Surveys Research, National Institute of Public Health (INSP), Cuernavaca 62100, Mexico
4 Genomics of Bone Metabolism Laboratory, National Institute of Genomic Medicine (INMEGEN), Mexico City 14610, Mexico
5 Epidemiological and Health Services Research Unit, Morelos Delegation, Mexican Institute of Social Security, Cuernavaca 62000, Mexico
6 Department of Health Policy and Management and UCLA-Kaiser Permanente Center for Health Equity, Fielding School of Public Health, University of California, Los Angeles, CA 90095, USA
7 UCLA Center for Cancer Prevention and Control Research, Fielding School of Public Health, Los Angeles, CA 90095, USA
8 Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095, USA
* Correspondence: bereriveraparedez7@gmail.com; Tel.: +52-55-56-22-66-66 (ext. 82355)
† These authors contributed equally to this work.

Citation: Robles-Rivera, K.; Argoty-Pantoja, A.D.; Hidalgo-Bravo, A.; Quezada-Sánchez, A.D.; León-Reyes, G.; Flores, Y.N.; Salmerón, J.; Velázquez-Cruz, R.; Rivera-Paredez, B. Uric Acid Levels Are Associated with Bone Mineral Density in Mexican Populations: A Longitudinal Study. *Nutrients* **2022**, *14*, 4245. https://doi.org/10.3390/nu14204245

Academic Editor: Qian Zhang

Received: 31 August 2022
Accepted: 9 October 2022
Published: 12 October 2022

Abstract: Background: Inconsistent epidemiological evidence between uric acid (UA) and bone mineral density (BMD) has been observed. Therefore, we evaluated the association between UA and BMD in Mexican adults. Methods: This analysis was conducted on 1423 participants from the Health Workers Cohort Study. We explored cross-sectional associations using linear regression and longitudinal associations using fixed-effects linear regression by sex and age groups (<45 and ≥45 years). Results: In females <45 years old, the cross-sectional analysis showed that UA levels were positively associated with total hip BMD. However, in the longitudinal analysis, we observed a negative association with the femoral neck and lumbar spine BMD. In contrast, in males <45 years old, we found an increase in total hip and femoral neck BMD in the groups with high levels of UA in the longitudinal association. On the other hand, in females ≥45 years old, we observed a longitudinal association between UA and loss of BMD at different sites. We did not observe an association between UA levels and BMD in males ≥45 years old. Conclusions: Our results suggest higher serum UA levels are associated with low BMD at different skeletal sites in Mexican females. Further studies are needed to delineate the underlying mechanisms behind this observation.

Keywords: uric acid levels; bone mineral density; Mexican population

1. Introduction

Uric acid (UA) is an organic compound generated as the final product of purine metabolism [1]. UA has been considered an essential antioxidant [2]; it is a potent scavenger of free radicals and may contribute as an endogenous systemic antioxidant against bone deterioration [3,4]. Nevertheless, abnormally high levels could increase the risk of different diseases [5]. To date, the role of UA in bone mineral density (BMD) remains unclear. Different observational studies have demonstrated a positive association between UA and BMD (subjects with higher UA levels tend to have higher BMD). These findings have been observed at different skeletal sites such as the lumbar spine, hip, and femoral neck in both

men and women, and most of the evidence has been obtained through cross-sectional studies [3,6–8]. However, recent studies have found a negative association between UA and BMD; a cross-sectional study showed that high levels of UA are a risk factor for vertebral fractures in postmenopausal women [9]. Longitudinal analysis showed that UA was negatively associated with BMD, and both studies were performed on subjects with type 2 diabetes (T2D) [9,10]. Nevertheless, other longitudinal studies did not find an association between UA and BMD changes in postmenopausal women [11] or between UA and incident fractures [12,13]. Furthermore, a mendelian randomization analysis in 241 elderly men and 1108 postmenopausal women did not show a causal association of UA with BMD [14]. Additionally, experiments using hyperuricemic and normouricemic rats reported no relationship between UA with BMD [15]. Therefore, we aim to evaluate the cross-sectional and longitudinal association between UA levels and BMD in Mexican adults.

2. Materials and Methods

2.1. Study Population

The data used in this study were derived from the Health Worker Cohort Study (HWCS), collected at baseline from 2004–2006 and through follow-up waves from 2010–2012 [16]. Workers and their relatives were invited by the Mexican Institute of Social Security (IMSS) of Cuernavaca Morelos. In this analysis, we included 1737 participants aged >20 with BMD measurement. Participants with kidney damage (n = 23), missing data on the food-frequency questionnaire (FFQ; those who answered <75% of the questionnaire or who had missing data in an entire section of the questionnaire; n = 99), or with implausible energy consumption estimated through a generalized extreme studentized deviate test (n = 49; <500 kcal/d or >6500 kcal/d) were excluded. We also excluded individuals with missing information on UA (n = 3), creatinine (n = 14), or smoking (n = 126). The final sample for this analysis consisted of 1423 participants (Supplementary Figure S1). The Research, Ethics, and Biosecurity Committee from the IMSS evaluated and accepted the study protocol (12CEI 09 006 14). Written informed consent was obtained from all the participants.

2.2. Bone Mineral Density Measurement

BMD measurements were obtained with a dual-energy X-ray absorptiometry (DEXA) Lunar DPX NT instrument (Lunar Radiation Corp., Madison, WI, USA) by trained examiners. The same machine was used for both study waves (2004–2006 and 2010–2012). Measurement sites were the femoral neck, total hip, and lumbar spine (L1–L4). Trained technicians performed daily quality control checks using the manufacturer phantom; the daily variation coefficient was within usual operational standards, and the in vivo variation coefficient was lower than 1.0–1.5% [16].

2.3. Uric Acid Level Assessment

At baseline and follow-up, fasting UA levels were determined through the enzymatic colorimetric method using a SYNCHRON CX system from Beckman Coulter [16]. We evaluated UA changes as a continuous as well as a categorical variable (categories defined by quartiles and hyperuricemia by serum concentrations UA cut-off points of $\geq$7.0 mg/dL in men and $\geq$5.7 mg/dL in women) [17].

2.4. Covariate Assessment

Demographic data such as age, sex, medication use (hormone replacement therapy [HRT]), consumption of calcium supplements, smoking, physical activity, and dietary intake were obtained from self-administered questionnaires in both waves [16].

Baseline age was categorized as <45 and $\geq$45 years old because, based on previous studies, endocrine changes affecting BMD in women occur around the age of 45 years [18,19]. Furthermore, we explored the age categories <50 and $\geq$50 years according to the WHO criteria for osteoporosis [20,21]. Smoking status was classified as never smokers, former smokers, and current smokers. Information on dietary intake was assessed

using a 116-item semi-quantitative FFQ based on a previously validated FFQ [16] and using food composition tables compiled by the National Institute of Public Health [16]. Dietary inflammatory index (DII) scores were calculated using a method previously reported by Shivappa et al., with 30 of the 45 food parameters, such as total energy, carbohydrate, protein, total fat, cholesterol, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), alcohol, fiber, caffeine, iron, magnesium, niacin, riboflavin, selenium, thiamine, beta carotene, zinc, folic acid, n-3 fatty acids, n-6 fatty acids, onion, trans fat, and vitamins A, C, D, E, B6, and B12 [22]. The leisure time physical activity (PA) was estimated with data from a previously validated PA questionnaire [23]. World Health Organization's definition was used to establish two categories: inactive (<150 min/week of moderate to vigorous activity) or active ($\geq$150 min/week of moderate to vigorous activity) [24]. T2D was defined as self-reported physician-diagnosed diabetes, use of hypoglycemic medication, or fasting glucose established cut-off points of >126.0 mg/dL [25].

Standing height and weight were measured with standardized procedures. We calculated participants' body mass index (BMI) (kg/m^2) at each wave, and the WHO's cut-off points were used for the classification of BMI status [26,27]. Serum creatinine levels were determined using a compensated kinetic Jaffe assay that offers results traceable to the isotope dilution mass spectrometry reference method. Glomerular filtration rate (GFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI), and values were expressed as mL/min/1.73 m^2.

2.5. Statistical Analyses

For descriptive purposes, continuous variables were summarized with means and standard deviations (SD) or median and 25th percentile (P25)–75th percentile (P75), and categorical variables using percentages by sex and age groups. For Table 1, the differences by sex at baseline were analyzed with Wilcoxon for continuous variables and the z-statistic for testing the equality of proportions for categorical variables. Differences between UA categories at baseline were analyzed with Dunn's test for continuous variables and the z-statistic for testing the equality of proportions for categorical variables.

Differences between study stages were analyzed with the Wilcoxon rank-sum test for paired samples, the paired t-test for continuous variables, and McNemar's test for categorical variables.

Cross-sectional associations between UA levels (continuous and categorical) and BMD were analyzed by linear regression models stratified by sex and age groups. The models were adjusted by age, BMI category (normal, overweight, and obesity, use of HRT, T2D, calcium supplement consumption, CKD-EPI equation, DII, energy-adjusted calcium intake, smoking, and leisure time PA. Longitudinal associations with UA levels continuously and categorically were analyzed by fixed-effects (FE) linear regression models stratified by sex and age groups. FE models analyze within-person change while eliminating time-invariant confounding (between-individual differences) [28]. The models were adjusted by covariates changing over time, such as BMI category (normal, overweight, and obese), use of HRT, T2D, CKD-EPI equation, DII, calcium supplements consumption, energy-adjusted calcium intake, smoking, and leisure time PA.

The level of statistical significance was set at $\alpha < 0.05$. All statistical analyses were performed using Stata 14.2.

Table 1. Baseline characteristics of the Health Workers Cohort Study (HWCS) by sex.

	Total n = 1423	**Females n = 1051**	**Males n = 372**
Age [a], years	46(37–55)	46(37–56)	45(36–54) *
BMI [a], kg/m^2	26.1(23.6–29.0)	25.9(23.5–29.0)	26.5(24.2–28.9)
Overweight, %	43.1	40.8	49.5 **
Obesity, %	19.3	19.6	18.6
Body fat proportion [a]	40.7(33.8–45.8)	43.2(38.7–47.2)	30.6(27.0–35.0) ***
Diabetes, %	8.6	8.7	8.3
Uric acid [a], mg/dL	4.8(3.9–5.9)	4.4(3.7–5.3)	6.0(5.2–6.8) ***
Hyperuricemia, %	18.2	16.9	21.8 *
Creatinine [a], mg/dL	0.9(0.8–1)	0.8(0.7–1.0)	1.0(0.9–1.2) ***
CKD-EPI equation [a]	98.1(91.1–105.5)	98.1(90.8–105.9)	97.8(91.6–104.7)
Dietary inflammatory index [a]	0.09(−1.41, 1.85)	−0.05(−1.48, 1.78)	0.37(−1.09, 2.25) **
Energy [a], kcal/day	1980(1528–2536)	1928(1512–2451)	2150(1612–2785) ***
Smoking status, %			
Past, %	28.3	23.8	41.1 ***
Current, %	16.0	13.7	22.3 ***
Phosphorous intake [a], mg/day	1326(1019–1742)	1298(999–1689)	1419(1085–1835) **
Calcium intake [a], mg/day	985(817–1203)	993(825–1208)	950(794–1196)
Calcium supplements, %	14.3	17.9	4.3 ***
Hormone replacement therapy, %	-	6.1	-
Hip BMD [a], g/cm^2	1.019(0.923–1.112)	0.996(0.904–1.082)	1.084(0.997–1.189) ***
Hip T-score [a]	−0.10(−0.82, 0.61)	−0.09(−0.85, 0.61)	−0.11(−0.72, 0.64)
Low hip BMD, %	20.2	21.0	17.7
Femoral neck BMD [a], g/cm^2	0.978(0.884–1.077)	0.960(0.867, 1.050)	1.029(0.942, 1.145) ***
Femoral neck T-score [a]	−0.58(−1.35, 0.22)	−0.69(−1.45, 0.09)	−0.34(−0.95, 0.58) ***
Lumbar spine BMD [a], g/cm^2	1.113(1.009–1.223)	1.102(0.999–1.213)	1.148(1.041–1.258) ***
Lumbar spine T-score [a]	−0.78(−1.63, 0.09)	−0.78(−1.63, 0.09)	−0.77(−1.64, 0.09)
High leisure time physical activity, %	38.5	35.3	47.4 ***

[a] Median (P25-P75). Hyperuricemia was defined as 7.0 mg/dL among males and 5.7 mg/dL among females. The *p*-values of the statistical tests were calculated using the Wilcoxon test for continuous variables and the tests on the equality of proportions for categorical variables. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Cross-Sectional Associations between UA and BMD Levels by Sex and Age Groups

We explored the characteristics by sex. Statistically significant differences were observed between females and males. Exposures such as amount overweight, UA, hyperuricemia, creatinine, DII, energy, smoking status, and phosphorous intake were higher in males than females. Additionally, females had higher body fat proportions, calcium supplements, and CKD-EPI than males. Furthermore, we observed that females had less hip BMD, femoral neck BMD, and lumbar spine BMD than males. We did not observe statistically significant differences in calcium intake by sex (Table 1). In addition, we explored the characteristics at baseline by UA categories. In females and males <45 years, statistically significant differences were observed in BMI, amount overweight, body fat proportion, hyperuricemia, DII, and calcium intake between the lowest and the highest quartile of UA. In addition, differences in obesity and low hip BMD were observed in females. In the ≥45 years old group, BMI, amount overweight, obesity, body fat proportion, and hyperuricemia showed significant differences between UA categories in females and males. Furthermore, differences in females were observed at the hip and femoral neck BMD, while in males they were found in CKD-EPI and MDRD equations and current smoking status (Supplementary Table S1).

Results from the linear regression showed a positive association between UA with hip and femoral neck BMD in females <45 years old. The adjusted model showed that an increase in 1 mg/dL of UA was associated with an increase in total hip BMD of 0.011 g/cm^2 (95% CI 0.0009, 0.021). Furthermore, at hip BMD, this association was observed with hyperuricemia, and at the femoral neck, the association remained in the high and very high

categories of UA (Figure 1 and Supplementary Table S2). In females ≥45 years old, we observed UA levels and hyperuricemia to be positively associated with hip BMD (β = 0.010, 95% CI 0.002, 0.018; and β = 0.033, 95% CI 0.011, 0.055; respectively). The association between UA and lumbar spine BMD was not significant in both age groups of females. We did not observe a cross-sectional association between UA and BMD in males in both age groups (Supplementary Tables S2 and S4).

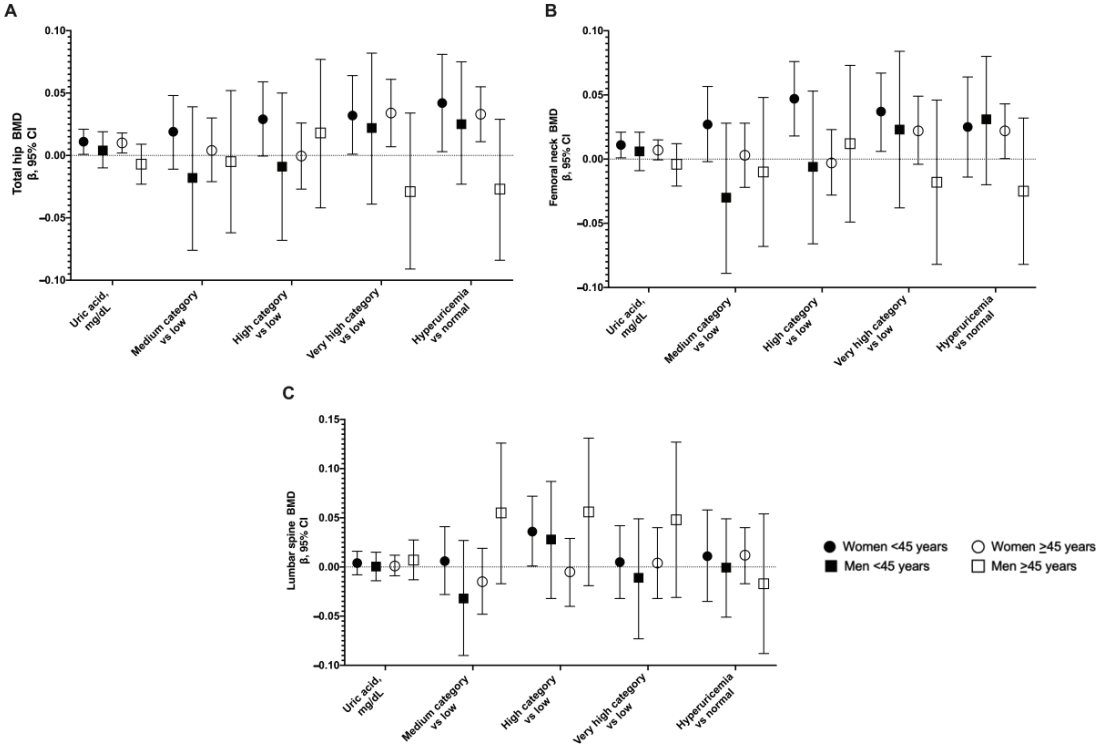


Figure 1. Cross-sectional association between UA and total hip BMD (**A**), femoral neck BMD (**B**), and lumbar spine BMD (**C**). Errors bars represent 95% confidence intervals.

3.2. Longitudinal Associations between UA and BMD Levels by Sex and Age Groups

The final analysis included 1423 participants with a mean time between baseline and second wave of 6.8 years (SD 1.4). Females made up 73.9% of the participants, of which 56.7% were ≥45 years old. At baseline, the median age of the group <45 years old was 36 years (P25-P75: 31–41) in females and males, while for the ≥45 years old group, the median age was 55 years (P25-P75: 49–61) for females and 54 years (P25-P75: 55–67) for males.

Measurements along the two-time points showed a significant increase of UA in females in both age groups and a decrease in total hip and lumbar spine BMD. Femoral neck BMD increased in <45 years and decreased in ≥45 years. In both age groups, hyperuricemia, BMI, body fat proportion, DII, and smoking status increased, while creatinine, energy, and calcium intake decreased. Furthermore, in females ≥45 years old, the HRT and the high leisure time physical activity decreased.

On the other hand, in both age groups of males, a significant increase in body fat proportion and a decrease in creatinine, energy calcium intake, hip BMD, and femoral neck BMD were observed. In addition, in <45 years, BMI, CKD-EPI equation, and smoking status increased. In ≥45 years, UA levels, hyperuricemia, lumbar spine BMD, and DII score increased (Table 2).

Table 2. Baseline and follow-up characteristics of the Health Workers Cohort Study (HWCS) by sex and age groups (n = 1423).

	<45 Years				≥45 Years			
	Females (n = 455)		Males (n = 181)		Females (n = 596)		Males (n = 191)	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-up	Baseline	Follow-Up
Age [a], years	36 (31–41)	43 (38–48) ***	36 (31–40)	43 (38–47) ***	55 (49–61)	61 (56–68) ***	54 (48–60)	60 (55–67) ***
BMI [a], kg/m^2	24.7 (22.5–27.8)	25.7 (23.3–29.2) ***	26.2 (24.2–28.8)	27.9 (24.7–29.5) ***	26.8 (24.5–29.9)	26.9 (24.6–30.0) *	26.7 (24.5–29.0)	26.9 (24.0–29.1)
Overweight, %	33.0	34.4	51.4	49.7	46.7	47.0	49.5	47.5
Obesity, %	13.6	19.9*	18.2	21.0	24.1	24.9	18.6	186
Body fat proportion [a]	40.8 (40.2–41.4)	44.0 (43.4–44.6) ***	31.1 (29.6–32.5)	32.3 (31.3–33.2) *	43.9 (43.4–44.4)	45.0 (44.5–45.5) ***	30.9 (30.0–31.8)	31.9 (31.0–32.8) **
Uric acid [a], mg/dL	4.1 (4.0–4.2)	4.4 (4.3–4.5) ***	6.4 (6.2–6.6)	6.3 (6.1–6.5)	4.9 (4.8–5.0)	5.2 (5.1–5.3) ***	5.9 (5.7–6.1)	6.1 (5.9–6.3) *
Hyperuricemia, %	8.5 (6.0–11.1)	12.7 (9.6–15.8) *	26.0 (19.5–324)	24.9 (18.5–31.2)	23.8 (20.4–27.2)	30.0 (26.4–33.6) **	17.5 (12.1–22.9)	25.3 (19.0–31.4) *
Creatinine [a], mg/dL	0.84 (0.83–0.86)	0.72 (0.71–0.73) ***	1.03 (1.01–1.06)	0.90 (0.87–0.92) ***	0.84 (0.83–0.86)	0.72 (0.71–0.74) ***	1.04 (1.01–1.06)	0.93 (0.90–0.96) ***
CKD-EPI equation [a]	106.6 (105.8–107.4)	106.6 (105.8–107.4)	104.7 (103.5–105.9)	106.2 (104.8–107.6) *	92.4 (91.7–93.1)	92.6 (91.8–93.3)	92.0 (90.2–93.9)	91.4 (90.0–92.8)
Dietary inflammatory index [a]	0.11 (−1.43, 1.84)	0.70 (−1.09, 2.36) **	0.63 (−1.03, 2.40)	0.90 (−0.68, 2.50)	−0.12 (−1.57, 1.66)	0.50 (−0.90, 2.14) ***	0.25 (−1.11, 2.02)	0.81 (−0.75, 2.38) *
Energy [a], kcal/day	2148 (2068–2227)	1880 (1806–1953) ***	2310 (2177–2442)	2122 (1985–2261) **	2008 (1944–2073)	1750 (1694–1807) ***	2224 (2103–2346)	1908 (1803–2013) ***
Smoking status, %								
Past, %	21.2	26.9 *	34.8	47.5 *	25.9	32.5 *	46.4	55.7
Current, %	14.9	12.7	25.4	18.8	13.0	7.9 **	19.1	12.4
Phosphorous intake [a], mg/day	1308 (1015–1731)	1070 (766–1451) ***	1504 (1126–1838)	1190 (944–1621) ***	1294 (994–1681)	1045 (786–1434) ***	1378 (1037–1815)	1089 (794–1491) ***
Calcium intake [a], mg/day	1062 (1009–1113)	867 (820–914) ***	1001 (927–1075)	894 (814–975) *	1059 (1013–1105)	867 (828–906) ***	1043 (961–1126)	842 (774–909) ***
Calcium supplements, %	7.0	6.8	1.1	0.0	26.2	26.1	7.7	0.0 ***
Hormone replacement therapy, %	3.3	3.7	-	-	8.4	5.4 *	-	-
Diuretics, %	0.9	1.5	0	0.6	1.2	2.9	0.5	3.1
Hip BMD [a], g/cm^2	1.040 (1.029–1.051)	1.027 (1.015–1.038) ***	1.126 (1.104–1.147)	1.111 (1.089–1.132) **	0.962 (−0.051, −0.044)	0.914 (0.904, 0.925) ***	1.061 (1.039–1.082)	1.037 (1.015–1.058) ***
Hip T-score [a]	0.20 (−0.37, 0.90)	0.06 (−0.57, 0.81) ***	0.16 (−0.49, 0.86)	0.09 (−0.65, 0.66) ***	−0.40 (−1.11, 0.41)	−0.75 (−1.44, −0.09) ***	−0.32 (−0.95, 0.34)	−0.47 (−1.11, 0.27) ***
Low hip BMD, %	9.8	12.9	11.6	14.4	29.8	41.3 ***	31.4	23.7

Table 2. *Cont.*

	<45 Years				≥45 Years			
	Females (n = 455)		Males (n = 181)		Females (n = 596)		Males (n = 191)	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-up	Baseline	Follow-Up
Femoral neck BMD [a], g/cm^2	1.008 (0.996–1.019)	1.019 (1.008–1.030) ***	1.096 (1.073, 1.118)	1.073 (1.051–1.095) ***	0.917 (0.907–0.928)	0.868 (0.858–0.877) ***	0.994 (0.973–1.026)	0.962 (0.941–0.983) ***
Femoral neck T-score [a]	−0.19 (−0.86, 0.55)	−0.30 (−0.84, 0.35)	0.11 (−0.67, 0.93)	−0.03 (−0.75, 0.60)	−1.03 (−1.74, −0.24)	−1.25 (−1.84, −0.61)	−0.63 (−1.29, 0.06)	−0.86 (−1.46, −0.15)
Lumbar spine BMD [a], g/cm^2	1.172 (1.159–1.184)	1.159 (1.147–1.172) **	1.162 (1.142–1.183)	1.162 (1.140–1.184)	1.045 (1.032–1.058)	1.006 (0.994–1.018) ***	1.152 (1.127–1.178)	1.167 (1.142–1.193) **
Lumbar spine T-score [a]	−0.26 (−0.88, 0.45)	−0.41 (−1.08, 0.44) ***	−0, 70 (−1.45, 0.16)	−0.76 (−1.45, 0.12)	−1.33 (−2.15, −0.40)	−1.70 (−2.42, −0.78) ***	−0.88 (−1.80, 0.09)	−0.77 (−1.71, 0.21) *
High leisure time physical activity, %	30.4	32.0	47.8	39.2	38.9	32.0 *	46.9	40.7

[a] Median (P25-P75). The *p*-values of the statistical tests were calculated using the Wilcoxon rank-sum test (for paired samples) for continuous variables and McNemar's test for categorical variables. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In females ≥45 years old, the fixed-effects linear regression showed that UA was negatively associated with hip, femoral neck, and lumbar spine BMD change (Figure 2 and Supplementary Table S3). The adjusted model showed that an increase in 1 mg/dL of UA was associated with a decrease in total hip BMD of 0.007 g/cm^2 (95% CI −0.011, −0.002), in femoral neck −0.0009 g/cm^2 (95% CI −0.014, −0.004), and in lumbar spine −0.010 g/cm^2 (95% CI −0.017, −0.004). We observed that these findings were maintained when evaluating change from a low to a high category of UA and from a low to a very high category of UA for the different sites. In females <45 years old, significant associations of UA levels were observed for femoral neck and lumbar spine BMD (Figure 2 and Supplementary Table S3). When we stratified <50 and ≥50 years, the associations remained in the same direction but were not statistically significant at >50 years (Supplementary Table S5).

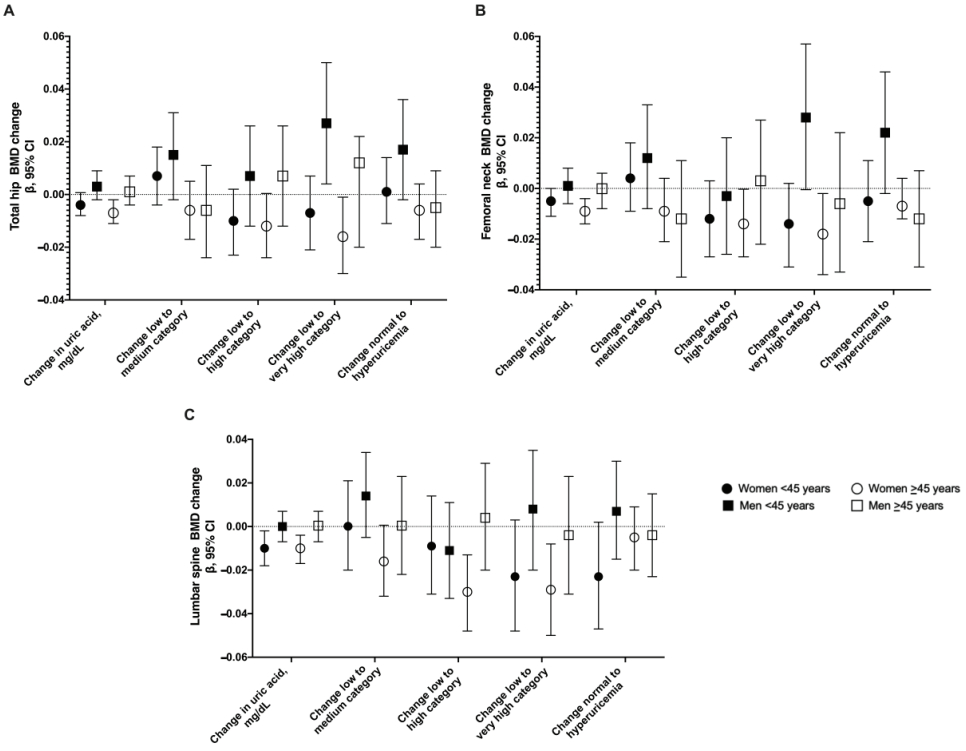


Figure 2. Longitudinal association between changes in UA and changes in total hip BMD (**A**), femoral neck BMD (**B**), and lumbar spine BMD (**C**). Errors bars represent 95% confidence intervals.

In males, we did not observe a significant association between UA and BMD in both age groups as a continuous variable. Nevertheless, in the evaluation by change of categories in <45 years old, we found a positive association; change from a low to a very high category of UA increased hip BMD by 0.027 g/cm^2 (95% CI 0.004, 0.050). In addition, we also observed a change from normal to hyperuricemia increased femoral neck BMD in 0.022 g/cm^2 (95% CI 0.002, 0.046) (Figure 2 and Supplementary Table S3). When we stratified <50 and ≥50 years, the associations were similar (Supplementary Table S5).

4. Discussion

Our results suggest a positive cross-sectional association between UA with hip and femoral neck BMD in females <45 years old. Nevertheless, a longitudinal negative association was observed between increased levels of UA and decreased hip, femoral neck, and

lumbar spine BMD in Mexican women ≥45 years old. Meanwhile, in women <45 years old, this association was only present at the femoral neck and lumbar spine BMD.

It is essential to interpret the results of the cross-sectional analysis with caution due to potential residual confounding with unmeasured variables (shared genetic variants or environmental causes). In our cross-sectional analysis, we observed in women <45 years old a positive association between UA levels and total hip and femoral neck BMD. Estrogens could partially explain this observation; estrogens are one key regulator of bone metabolism, having direct effects on osteocytes (decreasing bone remodeling), osteoblasts (maintaining bone formation), and osteoclasts (decreasing bone resorption). Therefore, a decrease in BMD has been observed during menopause due to estrogen deficiency [29]. In previous studies, women with low estradiol levels (<11 pg/mL) had a more than twofold increased relative risk of fracture (Risk Ratio [RR] 2.2, 95% CI 1.2, 4.0) [30]. A longitudinal study of 1902 pre-or early perimenopausal women assessed bone loss at each transition stage due to a decline of ovarian function. Little change was observed during the pre-or early menopause stages in the lumbar spine and total hip BMD, but the bone loss was accelerated in late perimenopause (0.018 g/cm^2 and 0.010 per year, respectively, $p < 0.001$) when adjusted for age, menopausal stage, weight, smoking, ethnicity, intake of vitamin D, calcium, and alcohol [31]. Estrogens have also been studied because of their effect on UA levels, due to a higher renal clearance of urate [32,33]. To our knowledge, there is no evidence evaluating the association between estrogen and UA levels with BMD. Additional studies are needed to determine the role of estrogen and uric acid levels on BMD in the Mexican population.

Several studies have shown a positive association between UA levels and BMD, especially in postmenopausal women. A large cross-sectional study of 7502 postmenopausal women found, after adjusting for multiple confounders (age, weight, height, GFR, calcium and phosphorus concentrations, and lifestyle factors), that serum UA levels were positively associated with BMD at all sites (per 1 mg/dL UA increase: lumbar spine β: 0.009 g/cm^2, femoral neck β: 0.004 g/cm^2, total hip β: 0.006 g/cm^2, and trochanter β: 0.005 g/cm^2, $p < 0.001$) [34]. Similarly, it was observed in a cross-sectional analysis with 356 twin women ≥45 years old that UA levels were positively associated with BMD in all skeletal sites (lumbar spine 0.190 g/cm^2, femoral neck 0.169 g/cm^2, total hip 0.167 g/cm^2, total forearm 0.136 g/cm^2, and whole body 0.170 g/cm^2, $p < 0.01$), after adjusting for multiple confounders (age, GFR, calcium, CTX-I levels, FM/LM/Ht^2, HRT, and lifestyle factors) [35].

In agreement with other studies, we observed higher UA levels in men than in women and across age categories in women. [36,37].The National Health and Nutrition Examination Survey (NHANES) 2015–2016 reported that men had higher UA levels (6.04 mg/dL) than women (4.79 mg/dL), and the hyperuricemia prevalence increased according to age categories; the highest was at >80 years (27.8%), followed by 26.1% (60–79 years) and 18.7% (40–59 years) [37]. Compared to older men, one explanation for the decreased UA levels in older women is the use of HRT due to its effect on insulin sensitivity, reducing UA concentrations by increasing its renal excretion [38].

On the other hand, some studies have shown that uric acid causes mitochondrial oxidative stress, stimulating fat accumulation independent of excessive caloric intake [39]. A meta-analysis found that for obesity (BMI > 30 kg/m^2), the age-adjusted relative ratio of gout was 2.24 (95% CI 1.76–2.86) [40]. In our research, we found in men and women a higher prevalence of obesity in those participants with UA levels in the very high category compared to those in lower categories. However, the association between the two variables must be taken with caution because serum uric acid influenced obesity, most likely together with other related factors not analyzed in this study, such as diet.

For our longitudinal analysis, it is important to consider that the fixed-effects method controls for unchanging variables, such as genetic factors. While there is currently controversial information regarding the association between UA and BMD, few studies have demonstrated a negative association. A cross-sectional study, including 356 postmenopausal women and 512 men older than 50 diagnosed with T2D, found that higher UA levels were

significantly associated with vertebral fractures only in women (odds ratio [OR] = 1.38, 95% CI = 1.01–1.88, *p*-value = 0.041) [41]. In older adults from a Cardiovascular Health Study, men with higher urate levels had a 60% increased risk of hip fracture (Hazard Ratio [HR] 1.6; 95% CI 1.1, 2.5). In addition, it was observed that urate levels >10 mg/dL had a higher risk of hip fracture (HR 3.0, 95% CI 1.5, 6.1), followed by urate >9 mg/dL (HR 1.9, 95% CI 1.2, 3.0) and >8 mg/dL (HR 1.4; 95% CI 1.1, 1.8). In contrast, there was no association between UA levels and hip fractures in women [13]. One explanation for this negative association is the generation of oxidative stress by hyperuricemia, which inhibits bone cell differentiation and promotes osteoclast function [38,42,43]. Furthermore, during an acute gout attack, proinflammatory cytokines (interleukin-1, interleukin-6, interleukin-8, and Tumor Necrosis Factor-α) are released and promote osteoclast differentiation, increasing bone resorption [36].

There is little information regarding the association between UA and BMD in men, especially younger adults. In men, we did not observe an association between UA and BMD in the cross-sectional and longitudinal analysis while considering BMD as a continuous variable. Nevertheless, we found a positive association when category change was observed, from a low to a very high UA with increased hip BMD and from normal to hyperuricemia with femoral neck BMD. The evidence reinforces that the female sex hormones may be regulating UA handling machinery [33]. Estrogen could explain the observed sex differences in UA levels associated with BMD. Therefore, understanding the role of estrogen in regulating serum uric acid levels is critical to unraveling the complex mechanisms of UA and BMD.

In a cross-sectional study based on the NHANES from 1999 to 2006 with 6704 men >18 years, no statistically significant association between UA and lumbar spine BMD was observed after adjusting for confounding factors [44]. Similar results were observed in a study with 943 males and 4256 postmenopausal females; the association between UA levels and femoral neck, total hip, and L1-L4 BMD was observed in women, but not in men after adjusting for confounders [45]. A possible explanation could be the paradoxical antioxidant effects of UA acting as an electron donor and chelating metal ions to convert them into less reactive forms incapable of catalyzing free radical reactions, preventing osteoclast bone resorption [2]. However, studies in younger populations of men are needed to confirm these findings.

The potential causes for discrepancy between prior studies might be due to different population characteristics (genetic background, age, obesity, and body fat proportion) or differences in potential confounders adjusted, primarily related to lifestyle factors. In our case, we adjusted by DII, but in other studies, adjustments were not made for vegetables, alcohol, and calcium consumption.

The present study has some strengths and limitations. The main strength is the longitudinal design that includes women and men of a wide range of ages, allowing age stratification (<45 and ≥45 years). Standardized procedures with validated instruments and trained personnel were used to assess the clinical variables. The main limitations were the low prevalence of osteoporosis and the small number of women who used HTR; therefore, it was impossible to evaluate these associations and interactions. Unfortunately, we did not have information on menopausal status for all women; therefore, we used age categories as a proxy according to previous studies [18,19]. We have no information regarding the consumption of medications such as diphosphonate, glucocorticoids, and allopurinol that affect BMD or UA. However, the consumption of HRT and diuretics is low, so we consider that these could not affect the results.

5. Conclusions

In conclusion, our results suggest that higher serum UA levels are associated with low BMD at different skeletal sites in Mexican females. This effect on bone metabolism is independent of the confounding variables analyzed (BMI category, use of HRT, calcium supplement consumption, energy-adjusted calcium intake, smoking, and PA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14204245/s1, Figure S1: Flowchart of study population; Table S1: Baseline characteristics of the Health Workers Cohort Study (HWCS) by uric acid categories (n = 1423); Table S2: Cross-sectional association between uric acid and BMD at baseline by sex and age groups; Table S3: BMD change according to changes in uric acid between baseline and follow-up by sex and age groups; Table S4: Cross-sectional association between uric acid and BMD at baseline by sex and age groups (<50 and ≥50 years old); Table S5: BMD change according to changes in uric acid between baseline and follow-up by sex and age groups (<50 and ≥50 years old).

Author Contributions: R.V.-C. and B.R.-P.; Data curation, B.R.-P.; Formal analysis, B.R.-P.; Funding acquisition, Y.N.F., J.S. and R.V.-C.; Investigation, B.R.-P.; Methodology, R.V.-C.; Writing—original draft, K.R.-R., A.D.A.-P. and B.R.-P.; Writing—review & editing, K.R.-R., A.D.A.-P., A.H.-B., A.D.Q.-S., G.L.-R., Y.N.F., J.S., R.V.-C. and B.R.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Mexican Council of Science and Technology (CONACyT): (Grant numbers: 7876, 87783, 262233, 26267M, SALUD-2010-01-139796, SALUD-2011-01-161930, and CB-2013-01-221628). YNF was supported by NIH/NCI K07CA197179. RVC was supported by grants from the CONACyT: Grant INFR-2016-01-270405 and partially supported by the Instituto Nacional de Medicina Genómica project 346-05/2018/I.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of The Mexican Social Security Institute (12CEI 09 006 14).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets analyzed in this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors would like to acknowledge the staff at Unidad de Investigación Epidemiológica y en Servicios de Salud, IMSS, Cuernavaca, Morelos.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Skoczyńska, M.; Chowaniec, M.; Szymczak, A.; Langner-Hetmańczuk, A.; Maciążek-Chyra, B.; Wiland, P. Pathophysiology of hyperuricemia and its clinical significance—A narrative review. *Reumatologia* **2020**, *58*, 312–323. [CrossRef] [PubMed]
2. Glantzounis, G.K.; Tsimoyiannis, E.C.; Kappas, A.M.; Galaris, D.A. Uric acid and oxidative stress. *Curr. Pharm. Des.* **2005**, *11*, 4145–4151. [CrossRef] [PubMed]
3. Ibrahim, W.N.; Younes, N.; Shi, Z.; Abu-Madi, M.A. Serum Uric Acid Level Is Positively Associated with Higher Bone Mineral Density at Multiple Skeletal Sites Among Healthy Qataris. *Front. Endocrinol.* **2021**, *12*, 653685. [CrossRef] [PubMed]
4. Sánchez-Rodríguez, M.A.; Ruiz-Ramos, M.; Correa-Muñoz, E.; Mendoza-Núñez, V.M. Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterized by antioxidant enzymes. *BMC Musculoskelet. Disord.* **2007**, *8*, 124. [CrossRef] [PubMed]
5. Zhao, L.; Cao, L.; Zhao, T.-Y.; Yang, X.; Zhu, X.-X.; Zou, H.-J.; Wan, W.-G.; Xue, Y. Cardiovascular events in hyperuricemia population and a cardiovascular benefit-risk assessment of urate-lowering therapies: A systematic review and meta-analysis. *Chin. Med. J.* **2020**, *133*, 982–993. [CrossRef] [PubMed]
6. Hwang, J.; Hwang, J.H.; Ryu, S.; Ahn, J.K. Higher serum uric acid is associated with higher lumbar spine bone mineral density in male health-screening examinees: A cross-sectional study. *J. Bone Miner. Metab.* **2019**, *37*, 142–151. [CrossRef]
7. Veronese, N.; Carraro, S.; Bano, G.; Trevisan, C.; Solmi, M.; Luchini, C.; Manzato, E.; Caccialanza, R.; Sergi, G.; Nicetto, D.; et al. Hyperuricemia protects against low bone mineral density, osteoporosis and fractures: A systematic review and meta-analysis. *Eur. J. Clin. Investig.* **2016**, *46*, 920–930. [CrossRef]
8. Yao, X.; Chen, L.; Xu, H.; Zhu, Z. The Association between Serum Uric Acid and Bone Mineral Density in Older Adults. *Int. J. Endocrinol.* **2020**, *7*, 3082318. [CrossRef] [PubMed]
9. Tanaka, K.-I.; Kanazawa, I.; Notsu, M.; Sugimoto, T. Higher Serum Uric Acid is a Risk Factor of Reduced Muscle Mass in Men with Type 2 Diabetes Mellitus. *Exp. Clin. Endocrinol. Diabetes* **2021**, *129*, 50–55. [CrossRef]
10. Yang, K.; Miao, H.; Zhao, R.; Wu, X.; Liu, B.; Zheng, S.; Huang, D.; Ping, Z. Association between serum uric acid and bone mineral density in patients with type 2 diabetes: A 6-year longitudinal study in China. *Medicine* **2021**, *100*, e25733. [CrossRef] [PubMed]
11. Kang, S.; Kwon, D.; Lee, J.; Chung, Y.-J.; Kim, M.-R.; Namkung, J.; Jeung, I.C. Association between Serum Uric Acid Levels and Bone Mineral Density in Postmenopausal Women: A Cross-Sectional and Longitudinal Study. *Healthcare* **2021**, *9*, 1681. [CrossRef] [PubMed]

12. Veronese, N.; Bolzetta, F.; De Rui, M.; Maggi, S.; Noale, M.; Zambon, S.; Corti, M.C.; Toffanello, E.D.; Baggio, G.; Perissinotto, E.; et al. Serum uric acid and incident osteoporotic fractures in old people: The PRO.V.A study. *Bone* **2015**, *79*, 183–189. [CrossRef] [PubMed]
13. Mehta, T.; Bůžková, P.; Sarnak, M.J.; Chonchol, M.; Cauley, J.A.; Wallace, E.; Fink, H.A.; Robbins, J.; Jalal, D. Serum urate levels and the risk of hip fractures: Data from the Cardiovascular Health Study. *Metabolism* **2015**, *64*, 438–446. [CrossRef]
14. Xiong, A.; Yao, Q.; He, J.; Fu, W.; Yu, J.; Zhang, Z. No causal effect of serum urate on bone-related outcomes among a population of postmenopausal women and elderly men of Chinese Han ethnicity—A Mendelian randomization study. *Osteoporos. Int.* **2016**, *27*, 1031–1039. [CrossRef] [PubMed]
15. Zhang, D.; Bobulescu, I.A.; Maalouf, N.M.; Adams-Huet, B.; Poindexter, J.; Park, S.; Wei, F.; Chen, C.; Moe, O.W.; Sakhaee, K. Relationship between serum uric Acid and bone mineral density in the general population and in rats with experimental hyperuricemia. *J. Bone Miner. Res.* **2015**, *30*, 992–999. [CrossRef]
16. Denova-Gutiérrez, E.; Flores, Y.N.; Gallegos-Carrillo, K.; Ramírez-Palacios, P.; Rivera-Paredez, B.; Muñoz-Aguirre, P.; Velázquez-Cruz, R.; Torres-Ibarra, L.; Meneses-León, J.; Méndez-Hernández, P.; et al. Health workers cohort study: Methods and study design. *Salud Publica Mex.* **2016**, *58*, 708–716. [CrossRef] [PubMed]
17. Choi, J.W.J.; Ford, E.S.; Gao, X.; Choi, H.K. Sugar-sweetened soft drinks, diet soft drinks, and serum uric acid level: The Third National Health and Nutrition Examination Survey. *Arthritis Rheum.* **2008**, *59*, 109–116. [CrossRef] [PubMed]
18. Davis, S.R.; Lambrinoudaki, I.; Lumsden, M.; Mishra, G.D.; Pal, L.; Rees, M.; Santoro, N.; Simoncini, T. Menopause. *Nat. Rev. Dis. Prim.* **2015**, *1*, 15004. [CrossRef] [PubMed]
19. Emaus, N.; Berntsen, G.K.R.; Joakimsen, R.; Fonnebø, V. Longitudinal changes in forearm bone mineral density in women and men aged 45–84 years: The Tromso Study, a population-based study. *Am. J. Epidemiol.* **2006**, *163*, 441–449. [CrossRef]
20. Zamani, M.; Zamani, V.; Heidari, B.; Parsian, H.; Esmaeilnejad-Ganji, S.M. Prevalence of osteoporosis with the World Health Organization diagnostic criteria in the Eastern Mediterranean Region: A systematic review and meta-analysis. *Arch. Osteoporos.* **2018**, *13*, 129. [CrossRef] [PubMed]
21. Schousboe, J.T.; Ensrud, K.E. Diagnostic criteria for osteoporosis should not be expanded. *Lancet Diabetes Endocrinol.* **2015**, *3*, 236–238. [CrossRef]
22. Shivappa, N.; Steck, S.E.; Hurley, T.G.; Hussey, J.R.; Hébert, J.R. Designing and developing a literature-derived, population-based dietary inflammatory index. *Public Health Nutr.* **2014**, *17*, 1689–1696. [CrossRef] [PubMed]
23. Martínez-González, M.A.; López-Fontana, C.; Varo, J.J.; Sánchez-Villegas, A.; Martinez, J.A. Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. *Public Health Nutr.* **2005**, *8*, 920–927. [CrossRef]
24. WHO. *Global Recommendations on Physical Activity for Health*; World Health Organization: Geneva, Switzerland, 2010. [CrossRef]
25. American Diabetes Association Professional Practice Committee. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2022. *Diabetes Care* **2021**, *45*, S17–S38. [CrossRef]
26. Institute of Medicine (US) Committee on Nutrition Services for Medicare Beneficiaries. *The Role of Nutrition in Maintaining Health in the Nation's Elderly: Evaluating Coverage of Nutrition Services for the Medicare Population Washington (DC)*; National Academies Press: Washington, DC, USA, 2000; ISBN 0309068460.
27. WHO (World Health Organization). *WHO Obesity and Overweight Fact Sheet No 311*; World Health Organization: Geneva, Switzerland, 2013.
28. Allison, P. *Fixed Effects Regression Models*; SAGE Publications: Thousand Oaks, CA, USA, 2009; ISBN 9780761924975.
29. Cauley, J.A. Estrogen and bone health in men and women. *Steroids* **2015**, *99*, 11–15. [CrossRef]
30. Garnero, P.; Sornay-Rendu, E.; Claustrat, B.; Delmas, P.D. Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: The OFELY study. *J. Bone Miner. Res.* **2000**, *15*, 1526–1536. [CrossRef]
31. Finkelstein, J.S.; Brockwell, S.E.; Mehta, V.; Greendale, G.A.; Sowers, M.R.; Ettinger, B.; Lo, J.C.; Johnston, J.M.; Cauley, J.A.; Danielson, M.E.; et al. Bone mineral density changes during the menopause transition in a multiethnic cohort of women. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 861–868. [CrossRef]
32. Antón, F.M.; García Puig, J.; Ramos, T.; González, P.; Ordás, J. Sex differences in uric acid metabolism in adults: Evidence for a lack of influence of estradiol-17 beta (E2) on the renal handling of urate. *Metabolism* **1986**, *35*, 343–348. [CrossRef]
33. Halperin Kuhns, V.L.; Woodward, O.M. Sex Differences in Urate Handling. *Int. J. Mol. Sci.* **2020**, *21*, 4269. [CrossRef]
34. Ahn, S.H.; Lee, S.H.; Kim, B.-J.; Lim, K.-H.; Bae, S.J.; Kim, E.H.; Kim, H.-K.; Choe, J.W.; Koh, J.-M.; Kim, G.S. Higher serum uric acid is associated with higher bone mass, lower bone turnover, and lower prevalence of vertebral fracture in healthy postmenopausal women. *Osteoporos. Int.* **2013**, *24*, 2961–2970. [CrossRef]
35. Makovey, J.; Macara, M.; Chen, J.S.; Hayward, C.S.; March, L.; Seibel, M.J.; Sambrook, P.N. Serum uric acid plays a protective role for bone loss in peri- and postmenopausal women: A longitudinal study. *Bone* **2013**, *52*, 400–406. [CrossRef] [PubMed]
36. Lin, K.-M.; Lu, C.-L.; Hung, K.-C.; Wu, P.-C.; Pan, C.-F.; Wu, C.-J.; Syu, R.-S.; Chen, J.-S.; Hsiao, P.-J.; Lu, K.-C. The Paradoxical Role of Uric Acid in Osteoporosis. *Nutrients* **2019**, *11*, 2111. [CrossRef] [PubMed]
37. Chen-Xu, M.; Yokose, C.; Rai, S.K.; Pillinger, M.H.; Choi, H.K. Contemporary Prevalence of Gout and Hyperuricemia in the United States and Decadal Trends: The National Health and Nutrition Examination Survey, 2007–2016. *Arthritis Rheumatol.* **2019**, *71*, 991–999. [CrossRef] [PubMed]
38. Ovalle, F.; Bell, D.S. Hormone replacement therapy and serum uric acid. *Lancet* **1999**, *354*, 1643, author reply 1644. [CrossRef]

39. Johnson, R.J.; Nakagawa, T.; Sanchez-Lozada, L.G.; Shafiu, M.; Sundaram, S.; Le, M.; Ishimoto, T.; Sautin, Y.Y.; Lanaspa, M.A. Sugar, uric acid, and the etiology of diabetes and obesity. *Diabetes* **2013**, *62*, 3307–3315. [CrossRef]
40. Evans, P.L.; Prior, J.A.; Belcher, J.; Mallen, C.D.; Hay, C.A.; Roddy, E. Obesity, hypertension and diuretic use as risk factors for incident gout: A systematic review and meta-analysis of cohort studies. *Arthritis Res. Ther.* **2018**, *20*, 136. [CrossRef]
41. Tanaka, K.-I.; Kanazawa, I.; Notsu, M.; Sugimoto, T. Higher Serum Uric Acid is a Risk Factor of Vertebral Fractures in Postmenopausal Women with Type 2 Diabetes Mellitus. *Exp. Clin. Endocrinol. Diabetes* **2020**, *128*, 66–71. [CrossRef]
42. Lee, Y.J.; Hong, J.Y.; Kim, S.C.; Joo, J.K.; Na, Y.J.; Lee, K.S. The association between oxidative stress and bone mineral density according to menopausal status of Korean women. *Obstet. Gynecol. Sci.* **2015**, *58*, 46–52. [CrossRef]
43. Bai, X.; Lu, D.; Bai, J.; Zheng, H.; Ke, Z.; Li, X.; Luo, S. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. *Biochem. Biophys. Res. Commun.* **2004**, *314*, 197–207. [CrossRef]
44. Li, X.; Li, L.; Yang, L.; Yang, J.; Lu, H. No association between serum uric acid and lumbar spine bone mineral density in US adult males: A cross sectional study. *Sci. Rep.* **2021**, *11*, 15588. [CrossRef]
45. Yan, D.-D.; Wang, J.; Hou, X.-H.; Bao, Y.-Q.; Zhang, Z.-L.; Hu, C.; Jia, W.-P. Association of serum uric acid levels with osteoporosis and bone turnover markers in a Chinese population. *Acta Pharmacol. Sin.* **2018**, *39*, 626–632. [CrossRef] [PubMed]

nutrients

MDPI

Review

Lipid Metabolism in Cartilage Development, Degeneration, and Regeneration

Zhanpeng Su [1], Zhixian Zong [2,3], Jinxia Deng [1], Jianping Huang [4], Guihua Liu [5], Bo Wei [1], Liao Cui [6], Gang Li [2,3], Huan Zhong [1,*] and Sien Lin [1,2,3,*]

[1] Orthopaedic Center, Affiliated Hospital of Guangdong Medical University, Guangdong Medical University, Zhanjiang 524013, China
[2] Musculoskeletal Research Laboratory, Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, China
[3] Stem Cells and Regenerative Medicine Laboratory, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, China
[4] Department of Prosthodontics, Yonsei University College of Dentistry, Seoul 03722, Korea
[5] Institute of Orthopaedics, Huizhou Municipal Central Hospital, Huizhou 516001, China
[6] Department of Pharmacology, Marine Biomedical Research Institute, Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical Unversity, Zhanjiang 524023, China
* Correspondence: nq6584@163.com (H.Z.); sienlin@cuhk.edu.hk (S.L.); Tel.: +852-3763-6153 (S.L.)

Abstract: Lipids affect cartilage growth, injury, and regeneration in diverse ways. Diet and metabolism have become increasingly important as the prevalence of obesity has risen. Proper lipid supplementation in the diet contributes to the preservation of cartilage function, whereas excessive lipid buildup is detrimental to cartilage. Lipid metabolic pathways can generate proinflammatory substances that are crucial to the development and management of osteoarthritis (OA). Lipid metabolism is a complicated metabolic process involving several regulatory systems, and lipid metabolites influence different features of cartilage. In this review, we examine the current knowledge about cartilage growth, degeneration, and regeneration processes, as well as the most recent research on the significance of lipids and their metabolism in cartilage, including the extracellular matrix and chondrocytes. An in-depth examination of the involvement of lipid metabolism in cartilage metabolism will provide insight into cartilage metabolism and lead to the development of new treatment techniques for metabolic cartilage damage.

Keywords: lipid metabolism; cartilage; osteoarthritis; fatty acid; cholesterol; phospholipid

Citation: Su, Z.; Zong, Z.; Deng, J.; Huang, J.; Liu, G.; Wei, B.; Cui, L.; Li, G.; Zhong, H.; Lin, S. Lipid Metabolism in Cartilage Development, Degeneration, and Regeneration. *Nutrients* **2022**, *14*, 3984. https://doi.org/10.3390/nu14193984

Academic Editor: Sara Baldassano

Received: 23 August 2022
Accepted: 20 September 2022
Published: 25 September 2022

1. Introduction

Cartilage is a connective tissue dominated by the extracellular matrix (ECM) that lacks vascular, lymphatic, and nerve supply. Human cartilage is classified into three types: hyaline, fibrous, and elastic cartilage [1]. Among these, hyaline cartilage is the most common one in the body. With a glassy appearance and sitting on the surface of the joint, hyaline cartilage reduces the friction caused by joint movement. Two major components are present in the ECM of cartilage: collagens and proteoglycans. Proteoglycans are formed by glycosaminoglycans covalently linked to core proteins through side chains [2]. Hyaline cartilage contains a large amount of proteoglycans with a gel-like structure and water-rich glycosaminoglycan chains that provide compressive elasticity to the cartilage, which is the reason why cartilage can absorb and distribute the pressure applied to the joint [3]. Although they take up only 5–10% of the total volume of the cartilage, chondrocytes and their progenitor play an important role in maintaining the homeostasis of cartilage [4,5]. Chondrocytes synthesize the ECM and are embedded themselves in the matrix [6]. Chondrocytes and the ECM are functionally interrelated. Chondrocytes play a crucial role in cartilage formation, degradation and regeneration, and the ECM provides the microenvironment

in which chondrocytes exist healthily and maintain the normal structure of cartilage [7]. Besides chondrocytes, progenitor cells are also identified in the cartilage which may be responsible for cartilage repair due to their self-renewal, multilineage differentiation, and migratory abilities [4].

Lipids including triacylglycerols, phospholipids, and cholesteryl esters play important roles in many physiological processes in the body [8]. They are known to act as energy storage substances and elemental components of the cell membranes, as well as precursors of inflammatory cytokines. Meanwhile, they also maintain metabolic homeostasis in association with intestinal flora, enzyme activators, glycosyl carriers, and regulating the function of immune cells [9–11]. The dysfunction of lipid metabolism, however, may lead to a number of disorders such as obesity, type II diabetes, and atherosclerosis [12]. Cartilage has many saturated fatty acids such as linoleic acid, oleic acid, and palmitic acid, which are involved in maintaining its structure and function. When lipid content or metabolism are disrupted, cartilage dysfunction results [13]. This article aims to review the function of lipid metabolism in the formation, degeneration, and regeneration of cartilage, which may shed light on potential therapeutics for cartilage illnesses.

2. Cartilage Development, Degeneration, and Regeneration

2.1. Chondrogenesis and Endochondral Ossification in Cartilage Development

Except for the cranium, the bones in the mesial and appendicular skeleton are generated from a hyaline cartilage template. The coagulation of mesenchymal cells, which occurs in the craniofacial region (e.g., middle ear bone and temporal bone) or in the mesoderm elsewhere in the body as the beginning of endochondral ossification, is essential for cartilage differentiation [14]. One of the earliest morphogenetic processes during embryonic development is the synthesis of cartilage, which serves as the starting point for osteogenesis inside cartilage, and then progresses to gradually forming bones and skeletal tissue. Mesenchymal cells condense, differentiate into chondrocytes, and then, cartilage tissue transforms into bone (Figure 1). This process is subject to stringent regulation by multiple stakeholders. The first stage is for undifferentiated mesenchymal cells to migrate to the osteogenesis site, where they begin to cluster together. The clumping of cells is induced by changed mitotic activity and the inability of cells to leave the center, which is caused by cell aggregation toward the center. This cell activity causes an increase in the density of mesenchymal stem cell (MSC) accumulation and a constant aggregation, but not an increase in cell proliferation.

This process could be mediated by the ECM promoting cell migration via intercellular adhesion molecules and gap junctions associated with increased intercellular contact, allowing for intercellular communication and small-molecule transfer. The process of mesenchymal cell condensation is critical in cartilage formation. This process is controlled by cell–cell and cell–matrix interactions, as well as the interrelationships of secreted substances with their corresponding receptors. [15]. Mesenchymal cells secrete an ECM rich in hyaluronic acid and type I collagen prior to coagulation, preventing intimate cell–cell interactions. Hyaluronan promotes cell motility. However, a rise in hyaluronidase activity abolishes cell–cell interactions induced by hyaluronan. The activation of one or more signal transduction pathways that results in the differentiation of mesenchymal cells into chondrocytes may entail cell–cell interactions. Cell–matrix interactions, in addition to cell–cell interactions, appear to be important in MSC coagulation. The condensation of mesenchymal cells generates a scaffold for the formation of skeletal elements within cartilage, determining the shape, size, location, and number of skeletal elements.

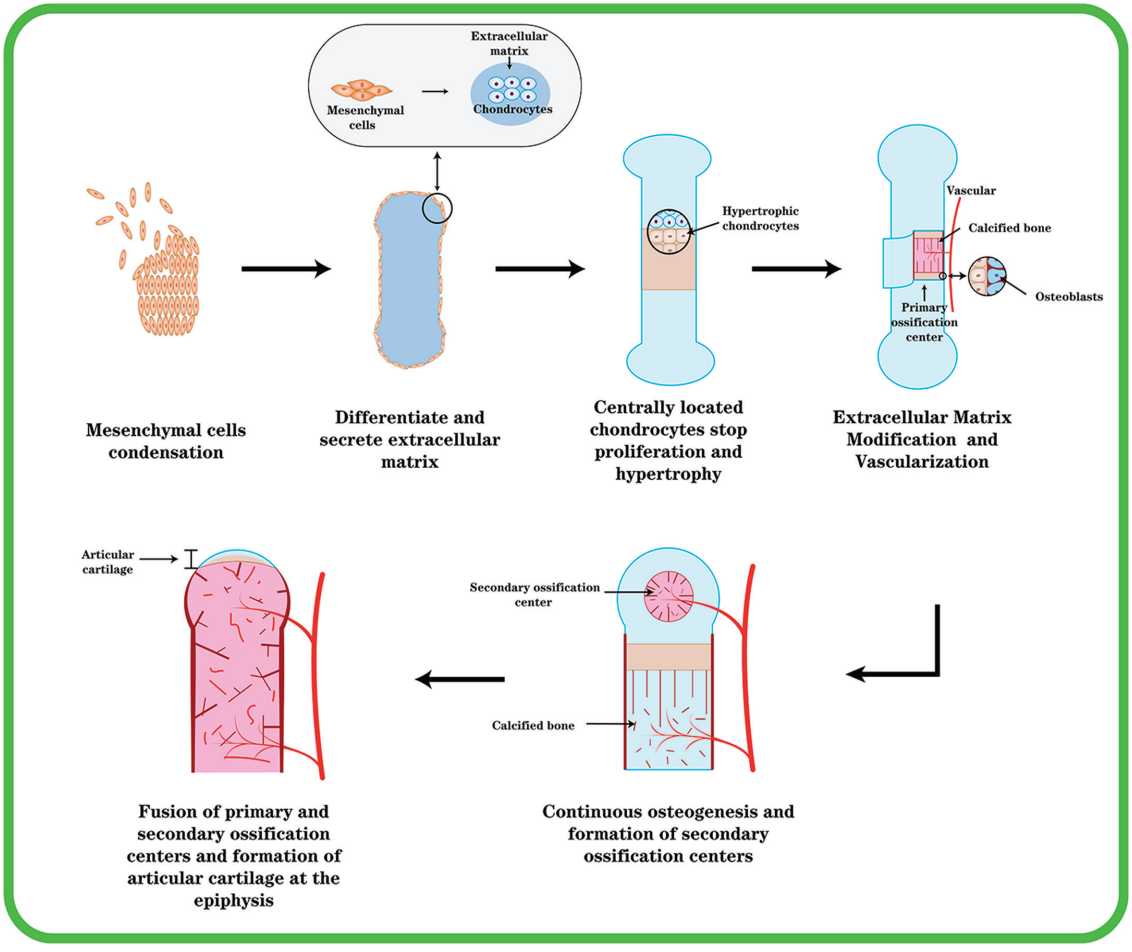


Figure 1. The formation of endochondral ossification and articular cartilage. Initially, mesenchymal cells undergo agglutination, mesenchymal cells in the center differentiate into chondrocytes and secrete type II collagen, chondrocytes continue to proliferate and secrete ECM, and cells around the agglutination express type I collagen and become cartilage membrane. The cartilage center's cells then transform into hypertrophic chondrocytes, and blood vessels invade and create main ossification centers. Cartilage is progressively replaced by bone, and the cartilage membrane transforms into periosteum. At the ends of the elements, secondary ossification centers are created, and the epiphyseal cartilage plate is generated between the secondary and primary ossification centers. At last, the epiphyseal plate ossifies, leaving permanent articular cartilage at the element's end.

Through the gradual differentiation of mesenchymal cells into chondrocytes and prechondral condensation, the ECM's composition changes. Chondrocytes begin to produce cartilage-specific type II collagen, chondroitin sulfate-rich proteoglycans, aggrecan, and fibronectin, whereas type I collagen expression is suppressed [16]. Chondrocytes in the center of condensation undergo rapid proliferation and linear growth of skeletal elements, after which centrally located chondrocytes stop proliferating and enter the maturation process; after further differentiation and hypertrophy, chondrocytes express type X collagen, whereas type II collagen expression decreases, and cartilage development appears in a different zone [15]. Toward the end of the element are zones with a relatively flattened

morphology where cells accumulate with each other and chondrocytes have a high proliferative capacity. Chondrocytes in the zone where proliferation is relatively quiescent (resting zone) are rounded. In the center of the element are chondrocytes that stop proliferating and become hypertrophic (the "hypertrophic zone") [17]. The cells surrounding the condensate retain the fibroblast morphology and continue to express type I collagen, giving rise to the chondrocyte membrane. Chondrocytes undergo hypertrophy and cell death during the process of endochondral ossification, and hypertrophic chondrocytes play an important role in the process. There are two types of cartilage: temporary and permanent. The healthy cartilage in a joint is referred to as permanent or resting cartilage. Permanent cartilage has a low proliferative capacity and does not undergo terminal differentiation under normal conditions, whereas temporary cartilage will eventually form bone. A portion of chondrocytes undergo hypertrophic changes and transform into hypertrophic chondrocytes during their proliferation [18]. Cellular hypertrophy refers to the increase in cell size and volume. Both collagenase activity and the cellular matrix are altered during the process of chondrocyte hypertrophy. During hypertrophy, the ECM changes as a result of the chondrocyte volume change, resulting in tissue failure and an increase in collagen type X [19]. Hypertrophic chondrocytes express elevated levels of type X collagen, runt-associated transcription factor 2 (Runx2), and matrix metalloproteinase 13 (MMP-13). In contrast, cartilage markers such as aggregated glycan, type II collagen, and Sox9 were reduced in hypertrophic chondrocytes. Type X collagen is a well-known marker of chondrocyte hypertrophy. Although the function of type X collagen is unknown, its presence at sites of chondrocyte hypertrophy and calcification suggests that it may play a role in the early stages of bone formation within cartilage [20]. Most of these hypertrophic cells at the core of the element perish over time, whereas the ECM stays intact. In this region, hypertrophic cartilage produces MMP-13 to modify the extracellular matrix (ECM), thereby promoting vascular invasion and subsequent vascularization in a process that transforms cartilage tissue lacking vascular supply into blood-rich bone tissue and necessitates the initiation of angiogenic programs that require potent angiogenic factors such as vascular endothelial growth factor (VEGF) [21]. Osteoblasts penetrate cartilage via blood arteries and begin replacing it with mineralized bone. Endochondral osteogenesis [22] outlines the production of cartilage, hypertrophy, and mineralization, followed by bone formation.

As stated, the process of endochondral osteogenesis takes place in growth plate cartilage, which differs from articular cartilage in terms of ECM content, cellular organization, and mechanical properties. Articular cartilage contains fewer chondrocytes and is embedded as separate cells in a distinct ECM with more collagen crosslinks than growth plate cartilage [21]. Mature articular cartilage has a banded organization that divides into a calcified layer, a deep layer, and a calcified layer from the articular cartilage surface toward the bone. The three groups of the non-hypertrophic Sox (Sox9/5/6) program are maintained in this cartilage, and the chondrocyte differentiation process is terminated to form permanent cartilage at the ends of the long bones [22].

2.2. Cartilage Homeostasis

The ECMs and chondrocytes have a mutually beneficial relationship: chondrocytes produce ECMs to form a highly organized molecular framework, and chondrocytes require the surrounding matrix to support their functions. Under normal physiological conditions, chondrocytes regulate the collapse and synthesis of the ECM in a dynamic balance. Chondrocytes are encapsulated in the ECM, and the stability of the ECM provides a stable microenvironment for chondrocytes. The mechanism of chondrocyte differentiation and phenotypic stability is regulated by a complex network of signaling molecules. When cartilage is injured, the chondrocyte phenotype changes, or even will become apoptotic, which is followed by ECM remodeling [23].

A wide range of signaling molecules influence cartilage matrix synthesis and maintenance, which are regulated via multiple signaling pathways. TGF-family members include BMPs, and are considered the major molecules in cartilage anabolism, along with insulin

growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and BMP-7. TGF-β binds to the type I receptors of activin-like kinases (ALK) ALK1 and ALK5, activating the Smad1/5/8 pathway and phosphorylating Smad2 and 3, respectively, causing a cascade of biological effects [24]. Smad2 and Smad3 inhibit chondrocyte hypertrophy as well as the entry of chondrocytes into the terminal differentiation state [25]. The activation of ALK5 and Smad2/3, on the other hand, induces chondrocyte synthesis of type II collagen and aggregated glycans, maintains chondrocyte stability, and plays an important role in chondrogenic differentiation regulation [26]. Reduced expression levels of ALK5 in senescent chondrocytes or OA cause disruption of the differentiation state of chondrocytes and promote the conversion of chondrocytes to a hypertrophic phenotype. In contrast, increased ALK1 expression levels activate the Smad1/5/8 signaling pathway, whereas Runx2 seems to have some synergistic effects with ALK1, promoting chondrocyte hypertrophy with increased synthesis of type X collagen and MMP-13, leading to cartilage matrix degradation [27].

BMP regulates cartilage development by regulating the Smad1/5/8 and Smad4 signaling pathways, and it is a critical regulator in all stages of cartilage development [28]. Various members of BMPs have different effects on chondrocytes, either in terms of cartilage matrix degradation and chondrocyte hypertrophy or in terms of chondrocyte proliferation and matrix synthesis. BMP can increase the synthesis of type II collagen and aggregated glycans in animal models of cartilage defects, whereas inhibiting BMP activity causes cartilage damage and reduced synthesis of proteoglycans [29]. In chondrocytes, BMP-7 is thought to induce extracellular matrix synthesis while inhibiting IL-1, IL-6, and fibronectin fragment-induced catabolism, promoting cartilage matrix synthesis while inhibiting catabolism [30]. BMP-2 and BMP-7 have different effects on chondrocytes, with BMP-2 promoting hypertrophy and BMP-7 inhibiting hypertrophy and maintaining cartilage stability [31].

2.3. Chondrocytes and Progenitor Cells in Cartilage Regeneration

Self-repair following cartilage injury is a tough procedure. When articular cartilage is injured, the mechanical loading of the cartilage changes, and oxidative stress mediates the harmful effects of shear stress on chondrocytes, resulting in chondrocyte senescence or death [32]. Normal articular cartilage is in a stable state of differentiation, secreting the ECM to preserve the structural integrity of the cartilage matrix and the function of the cartilage. Chondrocytes are induced to diversify into distinct phenotypes when OA arises as a result of changes in the chondrocyte microenvironment and cell death. Chondrocytes may undergo dedifferentiation alterations in OA, regaining the ability to proliferate, replenish chondrocytes that die in inflammation, or replace ECM-degrading components [33]. Both dedifferentiated chondrocytes and chondroprogenitor cells have the ability to proliferate. Indeed, numerous dedifferentiation marker genes, including collagen type II (IIA and IIB) and VI, tendin, and others, are increased in OA, implying a return of the chondroprogenitor phenotype [34]. Furthermore, during proliferation, osteoarthritic chondrocytes become senescent and hypertrophic, secreting proteins such as type X collagen and MMP-13 that are implicated in the matrix breakdown and calcification in OA [18].

Cartilage progenitor cells (CPCs), synovial-derived MSCs (SD-MSCs), synovial fluid-derived MSCs (SF-MSCs), bone marrow-derived MSCs (BM-MSCs), infrapatellar fat pad-derived MSCs (IF-MSCs), and MSCs from other origins all play a role in cartilage repair [35]. Bone marrow MSCs have been studied widely, and transplantation of bone marrow MSCs may reduce pain, enhance joint function, and halt the course of cartilage deterioration in OA patients [36]. Chondroprogenitor cells are found in both the superficial and deep zones of cartilage; however, the superficial zone seems to be more abundant, and the properties of chondroprogenitor cells vary by area. Chondroprogenitor cells in the deeper layers of cartilage tissue have a larger ability for chondrogenesis, are less phenotypically differentiated, have more flexibility, and may differentiate into a range of cell types. They can restore cartilage that has been damaged or degenerated. When cartilage is activated by injury or inflammation, the cells proliferate faster and migrate to the site of injury for repair. Superficial chondroprogenitor cells may originate from deeper layers, showing

higher lubricant expression than the deeper counterpart, and proliferating slowly [37]. Quiescent MSCs are also discovered in the synovial membrane of the mouse knee, where they proliferate and differentiate to heal cartilage injury [38]. The majority of cells expressing growth and differentiation factor 5 (Gdf5) are located in the synovial lining, including fibroblast-like synoviocytes (FLS) that express lubricant (or proteoglycan 4 (Prg4)) and the Yes-associated protein (Yap) to maintain synovial lining proliferation and promote cartilage regeneration [39]. Besides, MSCs from the infrapatellar fat pad and synovial fluid may also play important roles in cartilage repair as well as OA development [40–43].

Cartilage damage generates an unfavorable microenvironment for chondrocytes and the ECM. As recruitment cues for bone marrow MSCs, cell lysates, ECM catabolic fragments, and high-mobility group box chromosomal protein 1 (HMGB1) can all function [18]. In damaged joints, growth factors, chemokines (chemokine (C-C motif) ligands (CCLs), chemokine (C-X-C motif) ligands (CXCLs), and chemokine (C-X3-C motif) ligands (CX3CLs)), and several other cytokines can function as recruitment signals to regulate endogenous bone marrow mesenchymal stem cells. Synovial fluid can create several chemokines when joint lesions develop, with CCL25, CXCL10, and XCL1 strongly attracting human subchondral mesenchymal progenitor cells, whereas the cell migration of other cells is missing or suppressed [44]. In response to recruitment cues, bone marrow mesenchymal stem cells may undertake directed motility and migration [45]. Bone marrow MSCs move into injured cartilage to produce a bone marrow MSC–chondrocyte co-culture system. Bone marrow MSCs can assist chondrocytes in regeneration, and chondrocytes can support bone marrow MSCs in differentiation and matrix synthesis, therefore interacting to heal cartilage [46]. In addition to regenerating the ECM, recruited bone marrow MSCs can ameliorate the microenvironment in damaged cartilage and support chondrocyte survival. MSCs can suppress the gene expression of COX2 (PTGS2), PGE2, TNF-α, IL-1β, IL-6, and CXCL8 (IL-8), as well as inflammatory proteins IL-6 and IL-8. CCL2, CCL3, CCL5, and CCL1 levels are also reduced [47]. In the presence of MSCs, the expression levels of chondrocyte fibrosis and hypertrophy genes were downregulated, whereas the expression levels of pro-chondrogenic-related genes, such as ACNA and COL2A1, were increased [47]. Mesenchymal stem cells can also have a supporting impact on chondrocytes by secreting paracrine cytokines such as PDGF and TGF-β, thus stimulating the proliferation of chondrocytes and playing an essential part in the repair of articular cartilage [48].

2.4. ECMs and Chondrocytes in Cartilage Degeneration

Cartilage is essential in joints, and its malfunction can lead to a variety of disorders, the most frequent of which is OA. OA is a widespread chronic degenerative disease characterized by cartilage degeneration, osteophyte formation, and joint sclerosis, with pain as the primary symptom. OA can affect any joint, including the joints of the hands and feet, hips, and knees [49]. OA is a considerable expense on society and families, as well as a serious global public health concern. The annual cost of OA treatment ranges from 1% to 5% of GDP, and with the rising trend of obesity and aging, the incidence and cost of OA will continue to increase [50].

Mechanical stress injury is a significant cause of OA. Injury induces the upregulation of Runx2 and IHH, as well as the expression of proteases. Aggrecan degradation is an early event in cartilage degradation, and lysosomal histone proteases found in cartilage can degrade glycoprotein at an acidic pH [51]. The aggrecan domain is cleaved at the interglobular structural domain (IGD) between the N-terminal G1 and G2 globular structural domains of aggrecan by ADAMTSs, which are zinc-dependent metalloproteases of the metalloprotein family. All of the ADAMTS enzymes degrade aggrecan, but ADAMTS-5 is the most active "aggrecanase" in vitro, followed by ADAMTS-4, and thus, both are thought to be the main enzymes responsible for the pathological cleavage of aggrecan on the Glu373~Ala374 bond in IGD [52]. Drugs that inhibit ADATMS-4/5 are currently being researched as a promising OA therapy that targets cartilage degradation and has the potential to reduce joint pain and restore normal function [53]. The specific order of degradation of cartilage tissue's various

components is unknown. Aggrecan is thought to protect against collagen degradation, which is a reversible process in OA. However, collagen degradation is irreversible, and cartilage cannot be repaired once collagen is degraded [54,55]. MMP-1, MMP-3, MMP-8, and MMP-13 are all involved in collagen degradation, and MMP-13 is considered to be the main collagenase with increased expression in OA cartilage [56,57]. In addition, other MMPs (e.g., MMP-28), a disintegrin and metalloproteinases (e.g., ADAM-12, ADAM-15), and ADAMTSs (e.g., ADAMTS-16, ADAMTS-17) are also increased in OA [58]. Cathepsin K, a papain-like cysteine protease, is the only enzyme other than collagenolytic MMP that hydrolyzes natural triple-helix type I and type II collagen. Cathepsin K expression in chondrocytes is increased in OA, and the enzyme is thought to be involved in collagen degradation in the cartilage matrix and subchondral bone [59].

Besides ECMs, chondrocytes also play an important role in cartilage health maintenance and disease development. The phenotype of chondrocytes is determined by the microenvironment in which they are located. Mechanical stress and pathological stimuli such as TNF-α, IL-1β, and NO cause chondrocytes to change into various phenotypes, and these phenotypes respond to inflammatory stimuli in various ways [60]. Chondrocytes of different phenotypes play different roles in OA, either repairing cartilage or degrading the joint, and among these phenotypes, the catabolic phenotype dominates, promoting cartilage matrix degradation [61]. The catabolic phenotype is founded on the secretion of various hydrolytic proteases. The anabolic phenotype is founded on ECM regeneration and repair (including type II collagen and proteoglycans). The hypertrophic phenotype is founded on type X collagen secretion followed by apoptosis. The dedifferentiated phenotype is based on type I collagen secretion. Additionally, the chondrogenic phenotype is dominated by synthetic fetal type II collagen (IIA) and type III collagen [62].

3. Lipid Metabolism in Cartilage

Metabolic disorders such as obesity, diabetes mellitus, and cardiovascular diseases have been identified as risk factors of OA, and they are regarded as predictive of faster deterioration of the OA joint. Emerging evidence reveals the role of common pathways of metabolic diseases and OA, such as lipid metabolism, may have a direct systemic effect on joints [63].

Lipids are large molecules with a multitude of complex structures that play an important role in maintaining cellular functions [64]. There are four main lipid classes in the human body, including triglycerides (TGs), fatty acids (FAs), cholesterol, and phospholipids. Fats are important nutrients in the daily diet because they provide energy but also serve a variety of other functions, such as aiding in the absorption of fat-soluble vitamins and playing a role in the production and regulation of eicosanoids [65]. Fats are digested by lipase into fatty acids, glycerol, and monoacylglycerols, which are subsequently absorbed by the small intestine via bile salts [66]. Fatty acids can be absorbed by cells and become an important component of cell membranes. Lipids and their metabolic components can play an important role in signal transduction as second messengers between cells, and lipids are important in cartilage (Figure 2) [67].

In cartilage, the transport of lipids is a complex process. The complex structure of the cartilage matrix endows articular cartilage the ability to resist compressive loads and also determines how molecules diffuse into the matrix [68]. Recent research suggests two modes of lipid transport: synovial fluid diffusion and subchondral bone exchange. Synovial fluid is thought to be the primary source of molecules in articular cartilage metabolism. It has been discovered that synovial fluid, not subchondral bone, provides sufficient nutrients to maintain the structure and function of mature articular cartilage. The diffusion of solute molecules in the synovial fluid is significantly impacted by compression and circulatory loads in the joint [69]. The cartilage matrix is negatively charged, and only small, neutral molecules can diffuse freely through the joint fluid, whereas the diffusion of large and small negatively charged molecules is restricted [68]. To increase solubility, lipids are typically present in the blood as lipoproteins or bound to carrier plasma proteins. Once

bound to large molecules, the transport of lipids into cartilage will be restricted. Since size is the determining factor for molecular diffusion, for the diffusion of non-charged small molecules, there is no discernible difference between immature and mature cartilage [70].

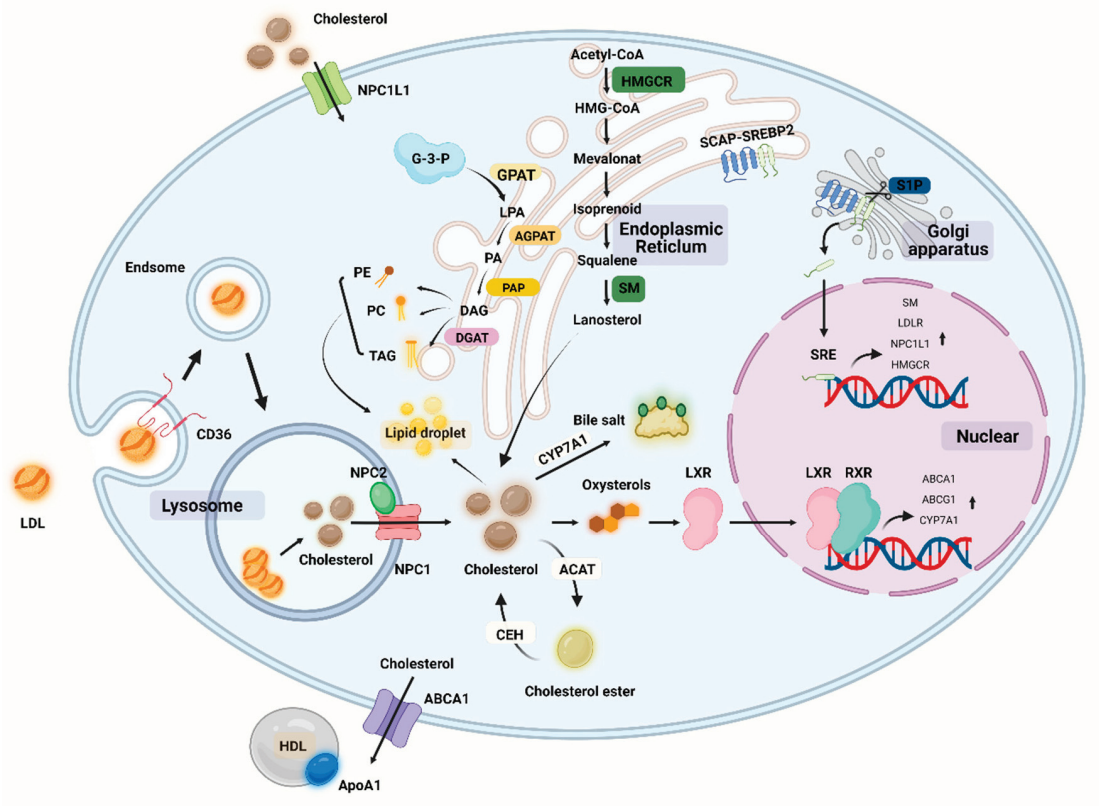


Figure 2. Overview of lipid metabolism in cells including chondrocytes. NPC1L1: Niemann–Pick C1-like 1, SM: squalene monooxygenase, HMGCR: hydroxymethylglutarate monoacyl coenzyme A reductase, HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A, CEH: cholesteryl ester hydrolase, ACAT: acyl coenzyme cholesterol acyltransferase, CYP7A1: cholesterol 7α-hydroxylase, LXR: liver x receptor, RXR: retinoic acid X receptor, SCAP: SREBP cleavage activation protein, SREBP: sterol-regulatory element binding protein, TAG: triglyceride, PE: phosphatidylethanolamine, PC: phosphatidyl choline (lecithin), GPAT: glycerol-3-phosphate acyltransferase, AGPAT: lysophospholipid acyltransferase antibody, DGAT: diacylglycerol acyltransferase, G-3-P: glyceraldehyde 3-phosphate, LPA: lysophosphatidic acid, PA: phosphatidic acid DAG: glycerol diester, PAP: phosphatidic acid phosphatase. (Figure was created with Biorender.com and accessed 20 September 2022).

In contrast to synovial fluid, vascular penetration from the subchondral bone allows molecules to be transported in mature joints' calcified cartilage. The bone–chondral junction in articular cartilage is viewed as a complex three-dimensional structure known as the tide-mark, which enables the transport of molecules as the uncalcified cartilage extends across the calcified cartilage and makes contact with the vessels of bone and bone marrow [71]. However, in mature joints, there is calcified cartilage at the bone–chondral interface, and this natural barrier greatly limits the entry of lipids and other nutrients from calcified cartilage to non-calcified cartilage [72]. Recently, it was reported that regardless of body mass index (BMI), abnormal lipid accumulation in OA chondrocytes may be responsible for the development and progression of OA [73].

3.1. Fatty Acid

Previous evidence shows that total free fatty acids (FFAs) and the accumulation of substantial lipids in articular cartilage have been reported in OA patients prior to the appearance of histopathological OA features [74]. A higher level of saturated fatty acids (SFAs) is also closely related to the progression of cartilage degradation, and SFA deposition in cartilage may alter its metabolism and lead to cartilage damage [73]. Even an SFA-rich diet is associated with increased OA-like pathophysiological changes [75].

Fatty acids can be taken up from food, but they can also be synthesized by acetyl coenzyme A through a series of reactions in glycolysis and the citric acid cycle for lipid synthesis. However, acetyl coenzyme A itself is a byproduct of fatty acid oxidation. Acetyl coenzyme A, malonyl coenzyme A, and NADPH are required for the synthesis of fatty acids by fatty acid synthase (FAS) [76]. Malonyl coenzyme A is an important inhibitor of the carnitine/palmitoyl shuttle system and a C2 donor in the de novo synthesis of fatty acids. Two different enzymes, acetyl coenzyme A carboxylase 1 (ACC1) and acetyl coenzyme A carboxylase 2 (ACC2), have evolved for fatty acid synthesis and oxidation [77]. Based on their carbon chain length, fatty acids can be classified into short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and long-chain fatty acids (LCFAs). Based on their degree of saturation, fatty acids can be categorized as SFAs, monounsaturated fatty acids (MUFAs), or polyunsaturated fatty acids (PUFAs), and each fatty acid has different biological effects (Figure 3) [78].

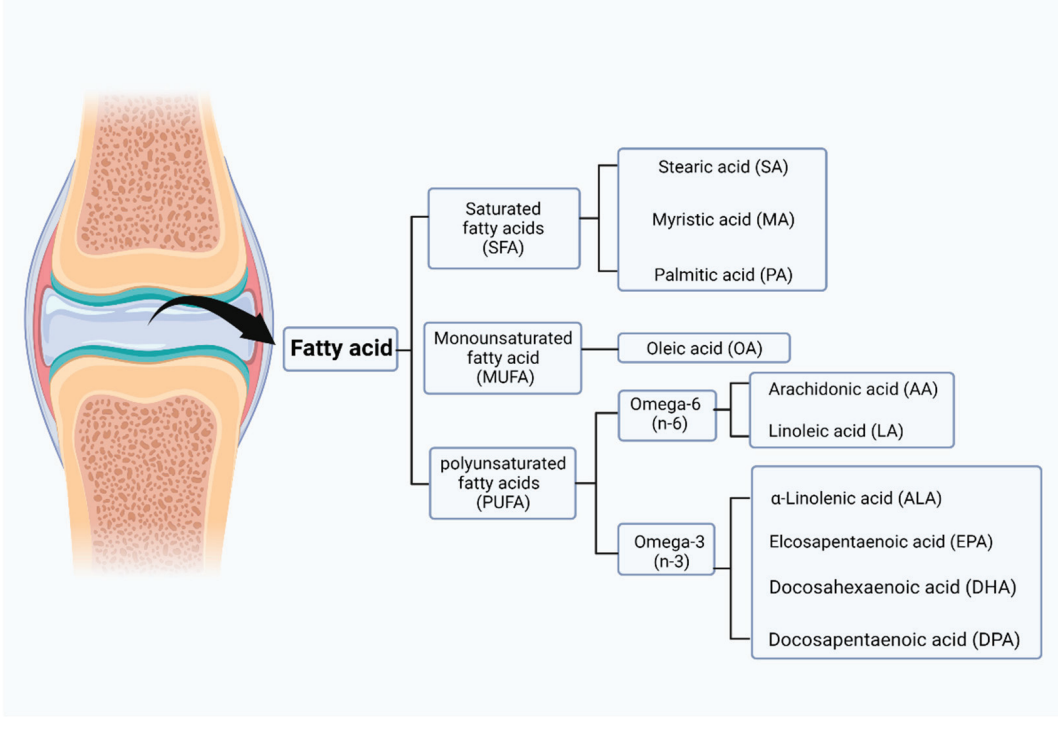


Figure 3. Classification of fatty acids in joint. Based on the degree of saturation, fatty acids can be categorized as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), or polyunsaturated fatty acid (PUFAs). Those fatty acids can be further categorized into lower grades. (Figure was created with Biorender.com and accessed 20 September 2022).

Stearate, a proinflammatory fatty acid, can partially activate hypoxia-inducible factor 1 (HIF-1) in chondrocytes to induce inflammation [79]. Palmitate acts as another proinflammatory fatty acid and catabolic factor in OA, which induces cystathione activation and cell death, stimulates normal chondrocytes via IL-1, and upregulates IL-6 and cyclooxygenase 2 (COX2) expression in chondrocytes through the Toll-like receptor 4 (TLR-4) signaling pathway [80]. Through the NADPH oxidase 4 (NOX-4) signaling pathway, high palmitic and oleic acid concentrations in articular cartilage mediate ROS production and chondrocyte apoptosis [81].

Differences in the desaturation sites of unsaturated fatty acids have resulted in the emergence of two distinct families of essential fatty acids: omega-3 and omega-6, which play different roles in the body. Alpha-linolenic acid (ALA) and linoleic acid (LA) are the dietary precursors of omega-3 unsaturated fatty acids (n-3 PUFAs) and omega-6 unsaturated fatty acids (n-6 PUFAs). In alpha-linolenic acid (ALA), 18 carbons and 3 double bonds are present, whereas 18 carbons and 2 double bonds are present in linoleic acid (LA) (18:2). Alpha-linolenic acid (ALA) and linoleic acid (LA) are both essential fatty acids because linoleic acid is desaturated by D15 desaturase to produce alpha-linolenic acid (ALA), but humans lack D15 desaturase. The conversion of n-3 unsaturated fatty acids to eicosapentaenoic acid (EPA FA, 20:5, n-3) and docosahexaenoic acid is possible (DHA FA, 22:6, n-3). In inflammation, omega-6 unsaturated fatty acids such as linolenic acid (LA FA, 18:2, n-6) are converted to gamma-LA (GLA FA, 18:3, n-6) and arachidonic acid (AA FA, 20:4, n-6) and play their own unique roles [82]. These fatty acids are precursors to a wide variety of bioactive lipids. In contrast to the proinflammatory effects of SFAs and n-6 PUFAs, omega-3 PUFAs can be oxidized to specialized proresolving molecules (SPMs), such as resolvins, protectins, and maresins, as well as other oxylipins with potent anti-inflammatory and pro-catabolic functions. SPMs function as signaling molecules that promote the restoration of vascular integrity, tissue regeneration, and repair, and decrease inflammation by inhibiting inflammatory lipid mediators and cytokines [83]. n-6 PUFAs, like SFAs, have proinflammatory effects that are mediated by lipid metabolites produced downstream of the precursor. Arachidonic acid is found in n-6 PUFAs and can be converted by cyclooxygenase (COX) and lipoxygenase (LOX) to eicosanoid compounds, which are biologically active lipids such as prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs). Due to the requirement for the same enzyme class for the conversion of a-linolenic acid to EPA and arachidonic acid to arachidonic acid, the synthesis of EPA and arachidonic acid will compete to regulate anti-inflammatory and proinflammatory homeostasis [84]. Normal cartilage typically contains low levels of n-6 polyunsaturated fatty acids (PUFAs) and high levels of n-9 fatty acids. Because the n-9 fatty acids may not function as a substrate for cyclooxygenase (COX), the cartilage is protected from prostaglandin (PG)-induced inflammatory responses. During aging, n-9 PUFAs in cartilage decrease while n-6 PUFAs increase [85]. Arachidonic acid is a widely distributed n-6 unsaturated fatty acid with 20 carbon and 4 double bonds (20:4) that is present in cell membranes and is an essential component of the lipid bilayer. However, in the presenting of inflammatory factors, phospholipase digestion of lipid molecules is stimulated, allowing arachidonic acid to be liberated in the cell membrane and then converted to PGH2 by COX, and then to leukotrienes by lipid oxidase. In diseased articular cartilage, the prostaglandins and leukotrienes produced by n-6 PUFAs were found to have strong proinflammatory and catabolic effects [86]. Furthermore, levels of prostaglandin E2 (PGE2) and 15-HETE (an oxytocin derived from arachidonic acid) in the synovial fluid of patients with OA were found to correlate with the degree of lesions [87].

Prostaglandin analogues are synthesized from PGH2 by activating PGD synthase or PGE synthase (PGEs). There are at least four types of PGE2 receptors (EP) in humans (EP1, EP2, EP3, and EP4), and different PGE2 receptors (EP) on the chondrocyte surface bind to PGE2 with different effects. When EP2 and EP4 are stimulated simultaneously in rats, for instance, they promote chondrocyte differentiation [88]. In the diseased joints of OA patients, synovial fibroblasts and chondrocytes produce proinflammatory cytokines

(e.g., IL-1β and TNF-α), which result in the upregulation of the expression of MMPs and cause degenerative changes in articular cartilage. Additionally, that research reveals that PEG2 can inhibit the expression of MMP-1 and MMP-13 via EP4 and inhibition of the MKK4-JNK MAP kinase-c-JUN pathway [89]. IL-1β stimulates the production of PGE2 and upregulates the expression of EP1 and EP4 in rabbit chondrocytes, thereby increasing the sensitivity of chondrocytes to prostaglandins; however, the biological effects induced by the subsequent binding of the receptor to PGE2 are dependent on the cell's state [90]. Microsomal prostaglandin E synthase 1 (mPGES-1), the terminal enzyme of PGE2 synthesis, is upregulated in many inflammatory conditions, including OA, and is regulated by MAP kinase phosphatase 1 (MKP-1), an enzyme that limits the activity of the proinflammatory MAP kinases p38 and JNK; thus, MKP-1 may be a new target for the treatment of OA [91]. The response of chondrocytes to different levels of PGE2 varies according to COX/mPGES and EP expression levels, mechanical stress stimulation in the diseased joint, and the action of growth factors and cytokines.

3.2. Cholesterol Metabolism

Total cholesterol is derived from the biosynthetic pathway of cellular cholesterol and dietary intake. Most tissues in the body, including chondrocytes, can synthesize cholesterol, and the liver is the primary site of synthesis, which occurs in the endoplasmic reticulum and cytoplasm of cells [92]. The synthetic enzymes required for cholesterol synthesis are found in the cytoplasm and cytosolic reticulum and are produced through a series of enzymatic reactions in which ATP provides energy and NADPH provides hydrogen. Cholesterol synthesis begins with acetyl coenzyme A, from which 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is synthesized, followed by mevalonate (MVA), isopentenyl pyrophosphate (IPP), and finally, squalene is converted to cholesterol [93]. Cholesterol is important in the human body not only as a structural component of cell membranes, but also as a precursor to biosynthetic compounds such as bile acids, vitamin D, and some steroid hormones. Cholesterol is converted in the adrenal cortex to adrenocortical hormones, in the gonads to sex hormones such as androgens, estrogens, and progestins, in the skin to 7-dehydrocholesterol, by ultraviolet radiation to vitamin D3, and in the liver to bile acids, which aid in lipid digestion and absorption [94,95]. Along with other intermediates of the cholesterol biosynthesis pathway, cholesterol regulates signaling molecules including Ras, Hedgehog, and Rho [96–98].

Given that cholesterol is a major component of cell membranes, any alteration may impair the function and fluidity of cell membranes, resulting in abnormal cellular behavior and cholesterol accumulation [93]. Cholesterol is distributed in chondrocytes as scattered dots, primarily in the nucleus. Cholesterol is important in promoting chondrocyte differentiation and maturation. It was discovered in animal experiments. Cholesterol may regulate cartilage formation and promote endochondral osteogenesis by altering the activity of IHH, which is inhibited when cholesterol synthesis is inhibited [99]. However, inflammatory processes can be triggered by the accumulation of persistent low-density lipoproteins (LDLs) in the cartilage extracellular matrix. The LDL is the primary carrier of cholesterol from the liver to the cells, and once taken up by the cells via the LDL receptor (LDLR), lysosomes hydrolyze LDL cholesterol to release cholesterol [100]. Oxidized LDLs (ox-LDLs) binding to the chondrocyte LOX-1 stimulates ROS production in chondrocytes and activates NF-κB, thereby promoting the expression of catabolic genes such as MMP-1, MMP-9, ADAMTS-5, and ADAMTS-13 [101–103]. In addition, it has been discovered that ox-LDLs binding to LOX-1 under conditions of oxidative stress promotes the expression of the chondrocyte runt-related transcriptional gene 2, which induces chondrocyte hypertrophy and the production of type X collagen [104]. C-reactive protein (CRP) is a sensitive indicator of inflammation, and OA is associated with increased levels of CRP and interleukin-6 (IL-6) [105]. Recent studies clearly demonstrate the link between inflammation and imbalance in cholesterol homeostasis, with osteoarthritic cartilage exhibiting a considerable downregulation of cholesterol efflux gene (e.g., ApoA1 and ABCA1) ex-

pression as compared to normal cartilage. When cholesterol accumulates in cells, the liver X receptor (LXR) transcription factor is activated and binds to the promoter sequence of the ATP-binding cassette transporter protein A1 (ABCA1) gene, and ABCA1 plays a key role in cholesterol efflux by acting as a lipid pump to efflux intracellular cholesterol and phospholipids to apolipoprotein A1 (ApoA1), a major protein component of HDLs [106]. ABCA1 appears to have a function in anti-inflammation; ABCA1 controls the production of inflammatory proteins and chemokines in macrophage cells, and an increase in intracellular free cholesterol content in ABCA1 gene-deficient macrophages is associated with an increase in the macrophage inflammatory response [107]. Cholesterol metabolism-related proteins have a rigorous regulation mechanism in chondrocytes, with ApoA1 and ABCA1 expression increasing during differentiation and decreasing during hypertrophic differentiation. Serum amyloid A (SAA) has been reported to have proinflammatory effects, and SAA levels in OA plasma and synovial fluid rise with an increasing Kellgren–Lawrence grade in OA patients [108]. ApoA1 binds and transports cholesterol during chondrocyte differentiation, but it is possible that ApoA1 is replaced by SAA in response to inflammatory factor stimulation and is present in inflamed hypertrophic chondrocytes, as the mRNA for SAA is found to be maximally expressed when chondrocytes undergo hypertrophy [109]. ApoA1 stimulates the production of IL-6, MMP-1, and MMP-3 in primary chondrocytes and fibroblast-like synoviocytes, and increased SAA, HDL, and LDL concentrations lead to the inflammatory features of the cholesterol regulation-related protein ApoA1 in chondrocytes [110]. All protein regulation inside chondrocytes aims to strictly manage cholesterol levels in order to prevent hypercholesterolemia from depositing cholesterol in the joint. High cholesterol levels may play a role in OA etiology. We are familiar with hypercholesterolemia, which causes atherosclerosis of the blood arteries, damaging them and lowering the blood supply. When there is an accumulation of cholesterol in the joints, there is a danger of vascular injury, which impairs the blood flow to the subchondral bone. When cartilage loses oxygen and nutrients, this may result in histopathology and contribute to the development of OA [111]. Furthermore, hypercholesterolemia may also cause lipid oxidation and deposition in tissues, resulting in cartilage damage. In the presence of excessive cholesterol intake, the development of medial joint OA is significantly correlated with cholesterol exposure. In mice fed a cholesterol-rich diet and devoid of the low-density lipoprotein receptor (LDLr-/-), the size of the osteophyte at the edge of the tibial plateau increased [112]. The increased expression of cholesterol hydroxylases (CH25H and CYP7B1) and oxysterol metabolite synthesis, increased cholesterol absorption and cholesterol content, and increased expression of retinoic acid-related orphan receptor alpha (ROR) encouraged chondrocyte hypertrophy and catabolic-related proteins (e.g., MMP-13, ADAMTS-4, and ADAMTS-5). The severity of OA was reduced in mice whose cholesterol levels were lowered [113]. The process of cholesterol-related synthesis and regulation can be seen in Figure 2.

Cholesterol can also function as a signaling agent in chondrocytes, and signaling during cholesterol overload can increase LXR, upregulate genes for cholesterol efflux-related proteins (such as ABCA1 and ApoA1) to prevent cholesterol accumulation in cells, and limit chondrocyte hypertrophy [114]. SREBP transcription factors are crucial processes for regulating cholesterol homeostasis, and there are three SREBP proteins, SREBP-1a, SREBP-1c, and SREBP-2, each of which has a particular role. SREBP-2 is involved in activating cholesterol-related genes, boosting cholesterol absorption and synthesis, interacting with Smad3, and engaging in the OA process [115]. Through the TGF-β1/Akt/SREBP-2 pathway and the cholesterol efflux-related genes ABCA1 and ApoA1, miR-33a controls cholesterol production in OA chondrocytes. The expression of miR-33a and its host gene SREBP-2 was dramatically increased in OA chondrocytes, and cholesterol production via the TGF-1/Akt/SREBP-2 pathway stimulated a significant increase in chondrocyte MMP-13 expression levels; hence, the OA phenotype is enhanced and contributes to the inflammatory process [116]. Cholesterol can potentially facilitate as a second messenger in the signaling process. Hedgehog signaling is critical in the development of bone and cartilage.

The G-protein-coupled receptor-like protein Smoothened (SMO) and downstream effectors transduce Hedgehog signaling pathway activation, and cholesterol is an essential component of the HH signaling pathway. Both cholesterol and sterol can activate HH signaling and cause SMO, but sterol deficiency is sufficient to inhibit HH signaling and cholesterol is required for HH activation. Subchondral bone has been demonstrated to stimulate HH signaling in animal OA models and to decrease the course of the lesion in vivo by SMO blocking [117]. Upregulation of the enzymes human 3-hydroxy-3-methylglutaryl coenzyme A synthase 1 (HMGCS1) and cytochrome P450 oxidase (CYP51A1), which synthesize cholesterol in chondrocytes, increases cholesterol biosynthesis during chondrogenesis, and cholesterol synthesis can promote the formation of growth plate cartilage by protecting against apoptosis and IHH expression [99,118].

3.3. Phospholipid Metabolism

Except for mature red blood cells, phospholipids can be synthesized in all human tissues, with the liver and kidneys serving as the primary sites. Phospholipids can be classified as either glycerophospholipids or sphingolipids, which are composed of glycerol and sphingomyelin, respectively. Sphingolipids are primarily synthesized in brain tissue. Coenzymes such as pyridoxal phosphate, NADPH+, and H+ are required in the reaction process, and the basic raw materials are palmitoyl coenzyme A and serine. Sphingolipids are formed after the formation of sphingomyelin and ceramides. Sphingomyelinase, an enzyme that hydrolyzes the phosphate bond to produce phosphorylated choline and ceramides, affects the degradation and metabolism of sphingolipids. Glycerophospholipids are the most common type of phospholipid. After being synthesized in the endoplasmic reticulum of the cytoplasm and processed by the Golgi apparatus, glycerophospholipids can be used to form cell membranes or secreted as lipoproteins outside the cell. Cardiolipin (CL) is a characteristic phospholipid of the inner mitochondrial membrane, where its synthesis also occurs. In addition to constituting biological membranes, glycerophospholipids are also bioactive components of the bile and membrane surface. Glycerophospholipids are also essential for protein recognition and cell membrane signaling [119,120].

LPCAT and PLA are critical for the metabolism of glycerophospholipids since they enhance the conversion of PC to lysolecithin (LysoPC). Elevated amounts of LPCAT4 and the essential regulating enzyme phospholipase A2 (PLA2) and its isoenzyme PLA2G5 have been discovered in OA cartilage, leading to increased levels of LysoPC. LysoPC is not only linked to an increased incidence of OA, but it may also be utilized to clinically predict arthroplasty after 10 years. In clinical investigations, the ratio of LysoPC to PC in synovial fluid has been identified as a novel marker for OA. LysoPC is a potent membrane lysis agent, and elevated levels are linked to chondrocyte apoptosis and cartilage degradation in OA, as well as to promoting cartilage inflammation by producing inflammatory lipid factors throughout the transition process [121]. In addition to its function in chondrocyte differentiation, hypertrophy, and mineralization, LPCAT4 is also involved in chondrocyte hypertrophy and mineralization. In mice, LPCAT4 mRNA expression was elevated in the hypertrophic zone, and LPCAT4 mRNA expression was also elevated in ATDC5 cells at late stages of development. When LPCAT4 was inhibited, the mRNA levels of cartilage differentiation markers, including Col10, alkaline phosphatase (ALP), aggregation protein (aggrecan), and Col10, were decreased [122]. OA synovial fluid is exposed to several proinflammatory stimuli that encourage the elevation of phospholipase A2 expression, and an increase in phospholipid composition may influence the inflammatory state of the joint [122]. Ceramide is one of the substrates for sphingolipid synthesis; it may be digested by ceramidase to generate sphingosine, which can then be phosphorylated by the sphingosine kinase to generate sphingosine-1-phosphate (S1P) [123]. Explants of rabbit articular cartilage treated with high concentrations of C2-ceramide elevated the matrix metalloproteinase-stimulated MMP-1, -3, and -13 expression and triggered chondrocyte death [67]. S1P stimulates chondrocyte proliferation by inducing phospholipase c-mediated

intracellular calcium release while also activating the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated kinase [124].

4. Interaction of Lipid Metabolism with Other Metabolisms

Vitamin D (VitD) is a micronutrient that may be taken through diet, but is mostly created in the human body through the utilization of UV-provided energy. When exposed to UV light, the process of cholesterol production creates 7-deoxycholesterol (vitamin D prodigiosa), which transforms into provitamin D3. Provitamin D3 is converted to 25(OH)D3 in the liver by 25-hydroxylases (CYP2R1 and CYP27A1). 25(OH)D-1-hydroxylase (CYP27B1) transforms 25(OH)D3 to 1,25(OH)2D3. VitD circulates in the body and reaches tissues with vitamin D receptors (VDRs), including bone and chondrocytes. Most biological effects of VitD are mediated by VDRs, which form heterodimers with retinoid X receptors and regulate target genes containing VDR response elements (VDREs), increasing calcium absorption and bone formation. VitD affects calcium and phosphorus homeostasis and promotes calcium absorption and appropriate bone mineralization [125]. It is intimately connected to lipid metabolism, and VDR activity governs the process. As a key enzyme in the synthetic cholesterol pathway, DHCR7 is controlled by the sterol regulatory element binding protein (SREBP), together with the other enzymes involved in cholesterol production. The proteasomal degradation of DHCR7 decreases cholesterol synthesis, hence, changing the flow to vitamin D3. DHCR7 regulates the synthesis of vitamin D and cholesterol [126]. SREBP regulates lipid homeostasis by binding to and anchoring in the endoplasmic reticulum with the SREBP cleavage activator protein (SCAP), which is needed for SREBP activation (Figure 2). It has been discovered that 25-hydroxyvitamin D (25OHD) suppresses SREBP activation independently of VDRs, affecting SREBP activation via the initiating protein hydrolysis processes and ubiquitin-mediated SCAP degradation. 25OHD physically binds to SCAP at first, allowing serine protease to hydrolyze the SREBP-binding domain in SCAP. Then, the SREBP-binding domain is hydrolyzed, enabling 25OHD-dependent ubiquitination. SREBP is destabilized as a result of SCAP deterioration. As a result, downstream adipogenesis-related genes are downregulated in response to SREBP [127]. The activation of the VDRs by vitamin D increases the expression of MOGAT1 and LPGAT1 in the MG pathway and AGPAT2 in the glycerol-3-phosphate pathway. The activation of VDRs also causes an increase in phosphatidate cytidylyltransferase 1 (CDS1) expression, which facilitates the conversion of PA to CDP-DAG, PI, and PG [128]. VitD has a vital regulatory function in bone and cartilage, and VitD deficiency can result in normal structural alterations in patellar tendons and collateral ligaments due to fatty infiltration and inflammation. Low VitD levels are linked to muscle weakness and poor function, and muscle injury leads to diminished joint cushioning capacity, resulting in cartilage deterioration, which is considered to be connected with early OA. VitD supplementation lowers fatty infiltration and inflammation while also benefitting muscular function. Leptin induces cartilage deterioration by damaging chondrocytes. Lipocalin, in contrast, has anti-inflammatory properties and inhibits MMPs while suppressing inflammatory molecules such as IL-6 and TNF-α. VitD raises lipocalin levels while decreasing leptin levels, lowering inflammation and protecting against OA [129]. In addition, VitD may alleviate the progression of OA by regulating the ideal condition of the skeletal response to the disease's pathological process. In a rat model of VitD deficiency, cartilage matrix degradation and proteoglycan loss occurred, which is comparable to early OA, demonstrating the detrimental effects of low VitD levels on cartilage [130]. The expression of MMP-9 and MMP-13 induced by TNF-α could be suppressed in vitro by vitamin D. VitD may have a cell-state-dependent effect on chondrocytes and could effectively regulate chondrocytes in the OA state [131]. VitD inhibits the beginning of mTORC1 complex-induced autophagy in chondrocytes by downregulating mTOR levels and then blocking the AMPK-mTOR signaling pathway. Increased autophagic flow in OA synovial membranes following VitD therapy protects against cartilage destruction. In OA animals, an additional VitD therapy reduced pain, cartilage damage, and inflammation, as well as caused a substantial drop

in synovial MMP-13, IL-1, and MCP-1 levels [132]. By influencing the conversion of the macrophage polarization phenotype, VitD modulates the inflammatory response in OA. M2 appears mostly in the late phase of inflammation and has anti-inflammatory effects, and VitD promotes the preponderance of M2 phenotype macrophages in inflammation. VitD supplementation increases the expression of VDR and IL-37 in macrophages, reduces inflammation, and controls immunological function [133].

Lipid metabolism interacts closely with glucose metabolism and protein metabolism, not only in the traditional metabolic organs, but also in cartilage, in a relatively slower grade (Figure 4). Glucose is an essential source of energy, is involved in the composition of the extrachondral matrix, is engaged in signaling and metabolic control, and plays a crucial role in cartilage development and maintenance [134]. The transport of glucose, which requires the involvement of glucose transporters, is the first key step in glucose metabolism. Glucose transporter 1 (GLUT1), which regulates glucose transport, permits chondrocytes to continue developing normally. GLUT1 deficiency impairs cartilage development, whereas GLUT1 overexpression causes excessive glucose uptake and the production of advanced glycation end products (AGEs), resulting in cartilage degradation. After glucose is transported to the chondrocytes, it can either contribute to the synthesis of other substances or undergo glycolysis [135,136]. Under normal oxygen levels, one molecule of glucose enters glycolysis for metabolism, resulting in the production of two molecules of pyruvate, which then enter the mitochondria for oxidative phosphorylation. Pyruvate enters the tricarboxylic acid cycle via oxidative phosphorylation to produce 36 molecules of ATP, which serves as the cell's energy source. In contrast, glucose is phosphorylated horizontally through anaerobic glycolytic substrates at low oxygen levels to generate a small amount of ATP, and eventually, lactate [137,138]. Because the oxygen supply decreases with depth in cartilage, articular chondrocytes have fewer mitochondria than other tissues, and ATP is mostly obtained by the Embden–Meyerhof glycolytic route, with only 10% of ATP derived from aerobic oxidation; however, mitochondrial activity is important in cartilage homeostasis and metabolism [139,140].

The tricarboxylic acid cycle is an important metabolic pathway that connects glucose, fats, and amino acids. Glucose is metabolized to produce acetyl coenzyme A, which can then be used to synthesize lipids, whereas glycerol in lipids is metabolized to produce glycerol phosphate, which can then be converted to glucose, and amino acids can be converted to glucose after deamination to produce alpha-keto acids, which can then be used as raw materials for phospholipids [141]. In OA cartilage, glucose and pyruvate consumption rose dramatically, as did the production of lactate and some TCA intermediates such as citrate, cis-aconitate, and malate, as well as glutamine and succinate levels. The tricarboxylic acid cycle acts as a connection between the three primary nutrients, and variations between distinct metabolic pathways may have consequences for cartilage homeostasis [142].

Different metabolic pathways are affected in pathological states, but the metabolic pathways are not isolated from one another; rather, they are intricately interconnected and serve distinct functions. Cholesterol accumulation in cartilage causes degenerative changes in cartilage. On the one hand, increased PLA2 activity and, thus, more PC conversion to AA activate eicosanoid and prostaglandin pathways [143]. On the other hand, n-6 PUFAs and SFAs act as proinflammatory substances, eventually leading to an inflammatory cartilage microenvironment. The ß-oxidation route catabolizes and provides energy from fatty acids, and carnitine plays an important regulatory function in mitochondrial fatty acid absorption. In OA, acylcarnitine levels are lowered and correspond with OA severity, reducing fatty acid catabolism and ATP generation [144].

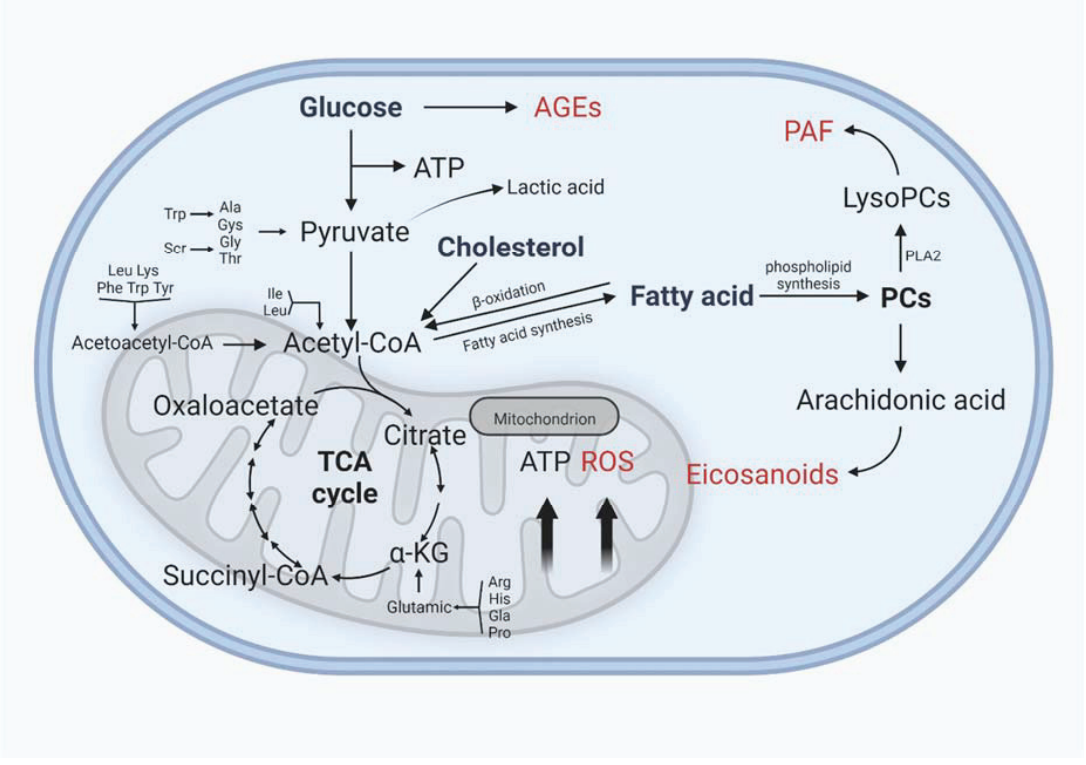


Figure 4. Interactions between glucose and protein metabolism and lipid metabolism in chondrocytes. AGEs: advanced glycation end products, ROS: reactive oxygen species, PAF: platelet-activating factor. AGEs, ROS, PAF, and eicosanoids are implicated in the inflammatory process of osteoarthritis, and their levels rise as OA progresses. (Figure was created with Biorender.com and accessed 20 September 2022.)

Chondrocytes in OA exist in a proinflammatory milieu where hypoxia induces the elevation of pyruvate kinase M2 (PKM2) expression, reduces the energy supply through oxidative phosphorylation, and increases ATP generation by glycolysis. Chondrocyte proliferation and differentiation are hindered as a result of increased glycolysis [145]. Animal models of OA were developed using the mechanism through which monosodium iodoacetate (MIA) alters glycolysis by inhibiting glyceraldehyde-3-phosphate dehydrogenase [146]. Lactate dehydrogenase converts pyruvate to lactate during accelerated anaerobic glycolysis, creating a tiny quantity of ATP and causing lactate buildup and the creation of an acidic microenvironment. It has been demonstrated that creating an acidic milieu (the usual pH of cartilage is 6.6) inhibits chondrocyte extracellular matrix formation [147]. The greater ATP demand of chondrocytes in OA is related to increase NO generation. Arginine, a physiological nitrogenous substrate that targets iNOs, and NO generation in chondrocytes are linked to the pathophysiology of OA. Specific activation of arginase II in chondrocytes via the NF-κB pathway leads to the increased production of MMP-3 and MMP-13 in chondrocytes, resulting in cartilage degradation [148].

5. Lipid Metabolism and OA Treatment

Total plasma n-6 PUFAs and DHA are adversely related with cartilage degradation, whereas n-6 PUFAs are proinflammatory in the development of OA. Dietary lipid consumption influences systemic lipid levels, and the proper composition of dietary lipids

may play an important role in preventing articular cartilage health. In an OA mouse model, adding Antarctic krill oil to the diet enhanced the intake of EPA-PL and DHA-PL and had a low n-6/n-3 PUFA ratio to block articular cartilage deterioration and slow the course of OA [149]. According to one study, dietary treatment with tiny levels of n-3 PUFAs helped repair damaging OA and decreased the consequences of obesity. Dietary SFAs and n-6 PUFAs, on the other hand, increase the development of OA, and food and metabolism appear to be more relevant than body weight in the relationship between obesity and post-traumatic OA [75]. Hypercholesterolemia causes mitochondrial dysfunction in chondrocytes, resulting in increased ROS and apoptosis, and antioxidants and statins have been shown in animal experiments to help preserve cartilage [149]. ABCA1 is essential for cholesterol metabolism. In OA, the expression of serum and glucocorticoid-inducible kinase 1 (SGK1) is increased, and ABCA1 exerts action mediated by the camp response element-binding protein (CREB1). As SGK1 silencing stimulates chondrocyte growth and reduces inflammation, it may be a unique method for treating OA [150]. In OA cartilage, the increased expression of miR-155 leads to the downregulation of cholesterol efflux-related gene expression such as LXR, resulting in cholesterol buildup and the development of inflammation, whereas the serine protease inhibitor vaspin (a protein released by white adipose tissue) has been found to inhibit miR-155 expression and enhance cholesterol efflux, therefore slowing the progression of OA [151]. The use of slow-release products containing PLA2 inhibitors can inhibit PLA2, hence lowering inflammatory reactions induced by membrane phospholipid hydrolysis that generates inflammatory mediators such as AA and lysophospholipids [152]. S1P has been demonstrated to inhibit IL-1-induced iNOS, ADAMTS-4, and MMP-13 expression [153]. MSC MVs require S1P for enrichment and penetrate cartilage in a cd44-dependent way to drive chondrocyte proliferation, matrix deposition, and cartilage defect repair, and S1P blockage inhibits the effect of MSC MVs on cartilage repair [154]. The investigation of S1P agonist-like medicines has the potential to lead to a significant advancement in the treatment of OA. The effects of lipid metabolism on cartilage are summarized in Table 1.

Table 1. Summary of the effects of lipid metabolism on cartilage.

Author, Year	Study Population	Methods	Main Outcomes
Shufang Wu, 2003 [99]	Sprague Dawley rats	Treat with inhibitor of the cholesterol synthesis	Cholesterol production suppression inhibits chondrocyte proliferation, hypertrophy, and differentiation. Cholesterol controls growth plate chondrogenesis and longitudinal bone formation, potentially through enhancing IHH's action in the growth plate.
H. Kishimoto, 2010 [104]	Bovine articular chondrocytes	Treat with ox-LDL	ox-LDL stimulates the hypertrophy of OA chondrocytes, in part, through binding to LOX-1. The binding of ox-LDL to LOX-1 promotes intracellular ROS generation and oxidative stress in chondrocytes.
Aspasia Tsezou, 2010 [106]	Human chondrocytes with OA	Treat with LXR agonist	In osteoarthritis patients, the expression of ABCA1 and ApoA1 transcriptional regulators LXRa and LXRb was dramatically reduced. LXR agonists enhance cholesterol efflux in chondrocytes with osteoarthritis.

Table 1. *Cont.*

Author, Year	Study Population	Methods	Main Outcomes
C. Gentili, 2005 [109]	Chick chondrocytes	Treat with LXR/RXR agonists	Chondrocytes manufacture ApoA1 during development, and ligands that activate LXR or RXR may lead to a significant increase in ApoA1 expression and cholesterol efflux in thicker chondrocytes. Other LXR target genes are implicated in cholesterol transport and homeostasis, including ABCA1 and SREBP1. The expression of chondrocyte SAA is inhibited by ligands that activate LXR or RXR and induce ApoA1.
Dominique de Seny, 2015 [110]	Human chondrocytes with OA	Incubation with ApoA1, rhSAA, HDL, LDL	By boosting TLR4 activation, free ApoA1 particles in OA joints may directly or indirectly contribute to the local inflammatory process. ApoA1's inflammatory characteristics on chondrocytes and fibroblast-like synoviocytes will be influenced by HDL and LDL concentrations that are tightly regulated.
Wouter de Munter, 2013 [112]	LDL receptor-deficient and wild-type control mice with OA	Cholesterol-rich diet	In OA, elevated LDL cholesterol levels stimulate the development of ectopic bone. The underlying process may include TGF -β activation and, to a lesser degree, BMP activation after ox-LDL absorption by synovial intimal macrophages.
W an-Su Choi, 2019 [113]	Mice with OA	High-cholesterol diet vs. regular diet	In osteoarthritic chondrocytes, increased cholesterol absorption, cholesterol hydroxylase levels, and hydroxysterol metabolite synthesis directly activate ROR, contributing to the pathogenesis of OA. Through LOX1-mediated augmentation of CH25H and CYP7B1 absorption and metabolism, increased cholesterol levels in chondrocytes induce experimental OA in mice. The CH25H-CYP7B1-ROR axis of cholesterol metabolism in chondrocytes is a crucial catabolic regulator in the etiology of osteoarthritis.

Table 1. *Cont.*

Author, Year	Study Population	Methods	Main Outcomes
Margaret Man-Ger Sun, 2020 [114]	Mice primary chondrocytes	Treat with specific LXRagonist	Upon activation by its specific agonist, the LXR-Srebp1-Scd1 axis functions as a cholesterol sensor, lowering the buildup of free cholesterol in cells to mitigate cholesterol's cytotoxic effects. LXR is activated by its particular agonist, and LXR's protective impact on OA may be mediated, in part, by increased cholesterol excretion and suppression of chondrocyte hypertrophy.
Fotini Kostopoulou, 2015 [116]	Human cartilage with OA	Treat with TGF-β1	miR-33a may influence the TGF-1/PI3K/Akt signaling pathway via the regulation of Smad7 expression. MiR-33 inhibition in OA chondrocytes raises the levels of ABCA1 and HDL, resulting in a reduction in MMP-13 expression.
Shirou Tabe, 2017 [122]	ATDC5 cells	Silence LPCA4 expression	Other than Col2, Sox9, and Runx2, knockdown of LPCAT4 decreased the mRNA expression of chondrogenic differentiation-related molecules. The expression of LPCAT4 rises during chondrogenic differentiation, and LPCAT4 is implicated in chondrocyte hypertrophy.
Mi-Kyoung Kim, 2006 [124]	Rat primary chondrocytes	Treat with S1P and PhS1P	Sphingosine kinase-1 is the key to the production of S1P from sphingosine. S1P and PhS1P lead to cellular proliferation of rat primary chondrocytes through a ptx-sensitive G-protein-mediated pathway, plc-mediated [Ca^{2+}] increase and activation of MAPKs.
Hongming Miao, 2015 [79]	Male C57BL/6 mice	HFD	In a way reliant on LDH-a, circulating stearic acid elevates lactate levels in plasma and chondrocytes. Stearic acid increases the production of VEGF and cytokines that cause inflammation through a TLR4 pathway and a new lactate/HIF1 pathway.
Oscar lvarez-Garcia, 2014 [80]	Human chondrocytes and fibroblast-like synoviocytes	Human chondrocytes from normal and OA were treated with Palmitate or oleate in combination with IL-1	Palmitate, but not oleate, stimulates IL-1 in normal chondrocytes, causing caspase activation and cell death, and upregulates IL-6 and COX2 in chondrocytes and fibroblast-like synoviocytes through Toll-like receptor-4 signaling.

Table 1. *Cont.*

Author, Year	Study Population	Methods	Main Outcomes
Daniel edina-Luna, 2017 [81]	Human chondrocytes	Treatment with FFA blend consisting of palmitic and oleic acids vs. control	A high-fat milieu causes cartilage degradation by raising chondrocyte IL-1, ROS, and RNS, promoting autocrine production of IL-6 and IL-8.
Chia-Lung Wu, 2014 [75]	Mice with OA	HFD, rich in SFAs, n-3 PUFAs, n-6 PUFAs	n-3 PUFA supplementation may reduce the effects of obesity on OA and enhance healing. SFAs and omega-6 PUFAs are both harmful in OA following joint damage. SFAs may cause synovial macrophages to release IL-1 and TNF, both of which are implicated in cartilage degradation.
M. Miyamoto, 2003 [88]	Primary chondrocytes from rat rib cartilage	Treat with PGE2	Simultaneous stimulation of EP2 and EP4 induces chondrocyte differentiation
H. Davis Adkisson, 1991 [85]	Cartilage from multiple species	Argentation high-performance liquid chromatographic(AHPLC) separationof fatty acid	Low levels of n-6 PUFAs and high levels of abnormal n-9 fatty acids found in normal cartilage
Kohei Nishitani, 2010 [89]	Human chondrocytes	Treat with PGE2	PGE2 inhibits IL-1β-induced MMP-1 and MMP-13 synthesis through EP4 activation and suppression of MKK4, JNK MAP kinase, and c-JUN phosphorylation.
Sunderajhan Sekar, 2017 [73]	Rat	High-Carbohydrate Supplements with SFAs vs. cornstarch diet	High-fat diet can induce metabolic syndrome in rats. Palmitic acid and stearic acid combined with IL-1 mediate cartilage damage by increasing MMP-13, ADAMTS-4, and ADAMTS-5 gene expression and increase the severity of OA
Sujeong Park, 2022 [74]	ACOT12 knockout mice	Treatment with specific agonists and inhibitors of PPARα	ACOT12 is involved in maintaining cartilage homeostasis. Increased accumulation of acetyl-CoA due to PPARα deficiency leads to stimulation of cartilage-degrading enzymes and chondrocyte apoptosis through regulation of ACOT12.

6. Conclusions

OA is a complicated disease with numerous risk factors, including trauma, age, obesity, genetics, and metabolism. The majority of the previous research on OA has focused on traumatic OA, but with the global increase in obesity rates, the role of obesity and metabolism in OA has also received increased attention. Obesity not only increases pressure load to the joints, but the hypercholesterolemia and inflammatory response caused by some adipokines associated with obesity can also cause cartilage damage [155]. Numerous alterations in lipid metabolism, including cartilage, subchondral bone, and periosteum, are involved in the pathogenesis of OA, and these alterations interact with inflammatory mediators to influence the development of the lesion. Previous research on the effect of lipids on cartilage has paved the way for the development of novel articular cartilage lesion treatments. Altering a specific lipid metabolism pathway with drugs or diet may be a

potential treatment target for articular cartilage disease. Because the specific mechanisms underlying the pathogenesis of OA remain unknown, and there are only a few options for cartilage treatment, a metabolic approach could be an alternative. The exploration of lipid metabolism and clear metabolic pathways must be expanded, and further investigation of the sites of action of lipid metabolism on the function of cartilage and its homeostasis under normal physiological conditions is still required.

Author Contributions: Z.S., Z.Z., J.D., J.H. and G.L. (Guihua Liu) carried out the literature review and drafted the manuscript. Z.S., Z.Z. and S.L. revised the manuscript. S.L. and H.Z. finalized and approved the manuscript for submission. S.L., G.L. (Gang Li), B.W. and L.C. provided funding support. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the National Natural Science Foundation of China (81874000 and 82172430) and the Natural Science Foundation of Guangdong Province Science and Technology Department (2020A1515010003 and 2019A1515110724). This project is partially supported by the University Grants Committee, Research Grants Council of the Hong Kong Special Administrative Region, China (14120118, 14108720, 14121721, C7030-18G, T13-402/17-N, and AoE/M-402/20); Hong Kong Innovation Technology Commission Funds (PRP/050/19FX); Hong Kong Medical Research Funds (16170951, 17180831, 08190416, and 09203436); Medical Research Fund of Guangdong Province (A2020151); "Peaking Plan" for the construction of the high-level hospital at the Affiliated Hospital of Guangdong Medical University (20501DFY20190168); and Discipline construction project of Guangdong Medical University (4SG21002G).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wachsmuth, L.; Söder, S.; Fan, Z.; Finger, F.; Aigner, T. Immunolocalization of matrix proteins in different human cartilage subtypes. *Histol. Histopathol.* **2006**, *21*, 477–485. [CrossRef] [PubMed]
2. Naba, A.; Clauser, K.R.; Ding, H.; Whittaker, C.A.; Carr, S.A.; Hynes, R.O. The extracellular matrix: Tools and insights for the "omics" era. *Matrix. Biol.* **2016**, *49*, 10–24. [CrossRef] [PubMed]
3. Knudson, W.; Ishizuka, S.; Terabe, K.; Askew, E.B.; Knudson, C.B. The pericellular hyaluronan of articular chondrocytes. *Matrix Biol.* **2018**, *78–79*, 32–46. [CrossRef] [PubMed]
4. Jiang, Y.; Tuan, R.S. Origin and function of cartilage stem/progenitor cells in osteoarthritis. *Nat. Rev. Rheumatol.* **2014**, *11*, 206–212. [CrossRef]
5. Hunziker, E.B.; Quinn, T.M.; Häuselmann, H.-J. Quantitative structural organization of normal adult human articular cartilage. *Osteoarthr. Cartil.* **2002**, *10*, 564–572. [CrossRef]
6. Archer, C.W.; Francis-West, P. The chondrocyte. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 401–404. [CrossRef]
7. Lin, Z.; Willers, C.; Xu, J.; Zheng, M.-H. The chondrocyte: Biology and clinical application. *Tissue Eng.* **2006**, *12*, 1971–1984. [CrossRef]
8. Fahy, E.; Subramaniam, S.; Brown, H.A.; Glass, C.K.; Merrill, A.H.; Murphy, R.C.; Raetz, C.R.H.; Russell, D.W.; Seyama, Y.; Shaw, W.; et al. A comprehensive classification system for lipids. *J. Lipid Res.* **2005**, *46*, 839–861. [CrossRef]
9. Zhang, C.; Wang, K.; Yang, L.; Liu, R.; Chu, Y.; Qin, X.; Yang, P.; Yu, H. Lipid metabolism in inflammation-related diseases. *Analyst* **2018**, *143*, 4526–4536. [CrossRef]
10. Schoeler, M.; Caesar, R. Dietary lipids, gut microbiota and lipid metabolism. *Rev. Endocr. Metab. Disord.* **2019**, *20*, 461–472. [CrossRef]
11. Yan, J.; Horng, T. Lipid Metabolism in Regulation of Macrophage Functions. *Trends Cell Biol.* **2020**, *30*, 979–989. [CrossRef] [PubMed]
12. Erion, D.M.; Park, H.-J.; Lee, H.-Y. The role of lipids in the pathogenesis and treatment of type 2 diabetes and associated co-morbidities. *BMB Rep.* **2016**, *49*, 139–148. [CrossRef] [PubMed]
13. Villalvilla, A.; Gómez, R.; Largo, R.; Herrero-Beaumont, G. Lipid Transport and Metabolism in Healthy and Osteoarthritic Cartilage. *Int. J. Mol. Sci.* **2013**, *14*, 20793–20808. [CrossRef] [PubMed]
14. Long, F.; Ornitz, D.M. Development of the Endochondral Skeleton. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a008334. [CrossRef]
15. DeLise, A.; Fischer, L.; Tuan, R. Cellular interactions and signaling in cartilage development. *Osteoarthr. Cartil.* **2000**, *8*, 309–334. [CrossRef]
16. Karsenty, G.; Kronenberg, H.M.; Settembre, C. Genetic Control of Bone Formation. *Annu. Rev. Cell Dev. Biol.* **2009**, *25*, 629–648. [CrossRef]
17. Mackie, E.J.; Ahmed, Y.A.; Tatarczuch, L.; Chen, K.-S.; Mirams, M. Endochondral ossification: How cartilage is converted into bone in the developing. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 46–62. [CrossRef]

18. Rim, Y.A.; Nam, Y.; Ju, J.H. The Role of Chondrocyte Hypertrophy and Senescence in Osteoarthritis Initiation and Progression. *Int. J. Mol. Sci.* **2020**, *21*, 2358. [CrossRef]
19. Van Donkelaar, C.C.; Wilson, W. Mechanics of chondrocyte hypertrophy. *Biomech. Model. Mechanobiol.* **2011**, *11*, 655–664. [CrossRef]
20. Van der Kraan, P.M.; van den Berg, W.B. Chondrocyte hypertrophy and osteoarthritis: Role in initiation and progression of cartilage degeneration? *Osteoarthr. Cartil.* **2012**, *20*, 223–232. [CrossRef]
21. Iwamoto, M.; Ohta, Y.; Larmour, C.; Enomoto-Iwamoto, M. Toward regeneration of articular cartilage. *Birth Defects Res. Part C Embryo Today Rev.* **2013**, *99*, 192–202. [CrossRef] [PubMed]
22. Heras, F.L.; Gahunia, H.K.; Pritzker, K.P. Articular Cartilage Development: A Molecular Perspective. *Orthop. Clin. N. Am.* **2012**, *43*, 155–171. [CrossRef] [PubMed]
23. Peng, Z.; Sun, H.; Bunpetch, V.; Koh, Y.; Wen, Y.; Wu, D.; Ouyang, H. The regulation of cartilage extracellular matrix homeostasis in joint cartilage degeneration and regeneration. *Biomaterials* **2020**, *268*, 120555. [CrossRef] [PubMed]
24. Funaba, M.; Zimmerman, C.M.; Mathews, L.S. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. *J. Biol. Chem.* **2002**, *277*, 41361–41368. [CrossRef] [PubMed]
25. Ying, J.; Wang, P.; Zhang, S.; Xu, T.; Zhang, L.; Dong, R.; Xu, S.; Tong, P.; Wu, C.; Jin, H. Transforming growth factor-beta1 promotes articular cartilage repair through canonical Smad and Hippo pathways in bone mesenchymal stem cells. *Life Sci.* **2018**, *192*, 84–90. [CrossRef]
26. ten Dijke, P.; Hill, C.S. New insights into TGF-beta-Smad signalling. *Trends Biochem. Sci.* **2004**, *29*, 265–273. [CrossRef]
27. Davidson, E.N.B.; Remst, D.F.G.; Vitters, E.L.; van Beuningen, H.M.; Blom, A.B.; Goumans, M.J.; van den Berg, W.B.; van der Kraan, P.M. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in humans and mice. *J. Immunol.* **2009**, *182*, 7937–7945. [CrossRef]
28. van der Kraan, P.; Davidson, E.B.; Berg, W.V.D. Bone Morphogenetic Proteins and articular cartilage: To serve and protect or a wolf in sheep clothing's? *Osteoarthr. Cartil.* **2010**, *18*, 735–741. [CrossRef]
29. Davidson, E.N.B.; Vitters, E.L.; Van Lent, P.L.; Van De Loo, F.A.; Berg, W.B.V.D.; Van Der Kraan, P.M. Elevated extracellular matrix production and degradation upon bone morphogenetic protein-2 (BMP-2) stimulation point toward a role for BMP-2 in cartilage repair and remodeling. *Arthritis Res. Ther.* **2007**, *9*, R102. [CrossRef]
30. Elshaier, A.M.; Hakimiyan, A.A.; Rappoport, L.; Rueger, D.C.; Chubinskaya, S. Effect of interleukin-1beta on osteogenic protein 1-induced signaling in adult human articular chondrocytes. *Arthritis Rheum.* **2009**, *60*, 143–154. [CrossRef]
31. Caron, M.; Emans, P.; Cremers, A.; Surtel, D.; Coolsen, M.; van Rhijn, L.; Welting, T. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthr. Cartil.* **2013**, *21*, 604–613. [CrossRef]
32. Martin, J.A.; Buckwalter, J.A. Post-traumatic osteoarthritis: The role of stress induced chondrocyte damage. *Biorheology* **2006**, *43*, 517–521.
33. Charlier, E.; Deroyer, C.; Ciregia, F.; Malaise, O.; Neuville, S.; Plener, Z.; Malaise, M.; de Seny, D. Chondrocyte dedifferentiation and osteoarthritis (OA). *Biochem. Pharmacol.* **2019**, *165*, 49–65. [CrossRef]
34. Aigner, T.; Söder, S.; Gebhard, P.M.; McAlinden, A.; Haag, J. Mechanisms of disease: Role of chondrocytes in the pathogenesis of osteoarthritis—Structure, chaos and senescence. *Nat. Clin. Pract. Rheumatol.* **2007**, *3*, 391–399. [CrossRef] [PubMed]
35. Narcisi, R.; Cleary, M.A.; Sivasubramaniyan, K.; Brama, P.A.J.; van Osch, G.J.V.M. MSC Populations for Cartilage Regeneration. *Cartilage* **2017**, 35–57. [CrossRef]
36. Le, H.; Xu, W.; Zhuang, X.; Chang, F.; Wang, Y.; Ding, J. Mesenchymal stem cells for cartilage regeneration. *J. Tissue Eng.* **2020**, *11*. [CrossRef]
37. Yu, Y.; Zheng, H.; Buckwalter, J.; Martin, J. Single cell sorting identifies progenitor cell population from full thickness bovine articular cartilage. *Osteoarthr. Cartil.* **2014**, *22*, 1318–1326. [CrossRef]
38. Kurth, T.B.; Dell'Accio, F.; Crouch, V.; Augello, A.; Sharpe, P.T.; De Bari, C. Functional mesenchymal stem cell niches in adult mouse knee joint synovium in vivo. *Arthritis Care Res.* **2011**, *63*, 1289–1300. [CrossRef]
39. Roelofs, A.J.; Zupan, J.; Riemen, A.H.K.; Kania, K.; Ansboro, S.; White, N.; Clark, S.M.; De Bari, C. Joint morphogenetic cells in the adult mammalian synovium. *Nat. Commun.* **2017**, *8*, 15040. [CrossRef]
40. Koh, Y.-G.; Choi, Y.-J. Infrapatellar fat pad-derived mesenchymal stem cell therapy for knee osteoarthritis. *Knee* **2012**, *19*, 902–907. [CrossRef]
41. Mantripragada, V.P.; Piuzzi, N.S.; Bova, W.A.; Boehm, C.; Obuchowski, N.A.; Lefebvre, V.; Midura, R.J.; Muschler, G.F. Donor-matched comparison of chondrogenic progenitors resident in human infrapatellar fat pad, synovium, and periosteum - implications for cartilage repair. *Connect. Tissue Res.* **2019**, *60*, 597–610. [CrossRef] [PubMed]
42. McGonagle, D.; Baboolal, T.; Jones, E. Native joint-resident mesenchymal stem cells for cartilage repair in osteoarthritis. *Nat. Rev. Rheumatol.* **2017**, *13*, 719–730. [CrossRef] [PubMed]
43. Sekiya, I.; Ojima, M.; Suzuki, S.; Yamaga, M.; Horie, M.; Koga, H.; Tsuji, K.; Miyaguchi, K.; Ogishima, S.; Tanaka, H.; et al. Human mesenchymal stem cells in synovial fluid increase in the knee with degenerated cartilage and osteoarthritis. *J. Orthop. Res.* **2011**, *30*, 943–949. [CrossRef] [PubMed]

44. Endres, M.; Andreas, K.; Kalwitz, G.; Freymann, U.; Neumann, K.; Ringe, J.; Sittinger, M.; Häupl, T.; Kaps, C. Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells. *Osteoarthr. Cartil.* **2010**, *18*, 1458–1466. [CrossRef]
45. Kawai, H.; Sakamoto, F.; Taguchi, M.; Kitamura, M.; Sotomura, M.; Tsukamoto, G. 2-Oxo-1,3-dioxoles as specific substrates for measurement of arylesterase activity. *Chem. Pharm. Bull* **1991**, *39*, 1422–1425. [CrossRef]
46. Marchan, J.; Wittig, O.; Diaz-Solano, D.; Gomez, M.; Cardier, J.E. Enhanced chondrogenesis from chondrocytes co-cultured on mesenchymal stromal cells: Implication for cartilage repair. *Injury* **2021**, *53*, 399–407. [CrossRef]
47. Zhang, Q.; Chen, Y.; Wang, Q.; Fang, C.; Sun, Y.; Yuan, T.; Wang, Y.; Bao, R.; Zhao, N. Effect of bone marrow-derived stem cells on chondrocytes from patients with osteoarthritis. *Mol. Med. Rep.* **2015**, *13*, 1795–1800. [CrossRef]
48. Xu, L.; Wu, Y.; Xiong, Z.; Zhou, Y.; Ye, Z.; Tan, W.-S. Mesenchymal Stem Cells Reshape and Provoke Proliferation of Articular Chondrocytes by Paracrine Secretion. *Sci. Rep.* **2016**, *6*, 32705. [CrossRef]
49. Glyn-Jones, S.; Palmer, A.J.R.; Agricola, R.; Price, A.J.; Vincent, T.L.; Weinans, H.; Carr, A.J. Osteoarthritis. *Lancet* **2015**, *386*, 376–387. [CrossRef]
50. O'Neill, T.W.; McCabe, P.S.; McBeth, J. Update on the epidemiology, risk factors and disease outcomes of osteoarthritis. *Best Pr. Res. Clin. Rheumatol.* **2018**, *32*, 312–326. [CrossRef]
51. Huber-Lang, M.; Denk, S.; Fulda, S.; Erler, E.; Kalbitz, M.; Weckbach, S.; Schneider, E.M.; Weiss, M.; Kanse, S.M.; Perl, M. Cathepsin D is released after severe tissue trauma in vivo and is capable of generating C5a in vitro. *Mol. Immunol.* **2012**, *50*, 60–65. [CrossRef] [PubMed]
52. Burrage, P.S.; Mix, K.S.; Brinckerhoff, C.E. Matrix Metalloproteinases: Role In Arthritis. *Front. Biosci.* **2006**, *11*, 529–543. [CrossRef] [PubMed]
53. Zhao, P.; Liu, D.; Song, C.; Li, D.; Zhang, X.; Horecny, I.; Zhang, F.; Yan, Y.; Zhuang, L.; Li, J.; et al. Discovery of Isoindoline Amide Derivatives as Potent and Orally Bioavailable ADAMTS-4/5 Inhibitors for the Treatment of Osteoarthritis. *ACS Pharmacol. Transl. Sci.* **2022**, *5*, 458–467. [CrossRef] [PubMed]
54. Pratta, M.A.; Yao, W.; Decicco, C.; Tortorella, M.D.; Liu, R.-Q.; Copeland, R.A.; Magolda, R.; Newton, R.C.; Trzaskos, J.M.; Arner, E.C. Aggrecan Protects Cartilage Collagen from Proteolytic Cleavage. *J. Biol. Chem.* **2003**, *278*, 45539–45545. [CrossRef]
55. Karsdal, M.A.; Madsen, S.H.; Christiansen, C.; Henriksen, K.; Fosang, A.J.; Sondergaard, B.C. Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. *Arthritis Res. Ther.* **2008**, *10*, R63. [CrossRef]
56. Struglics, A.; Larsson, S.; Pratta, M.; Kumar, S.; Lark, M.; Lohmander, L. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloproteinase-generated aggrecan fragments. *Osteoarthr. Cartil.* **2006**, *14*, 101–113. [CrossRef]
57. Hu, Q.; Ecker, M. Overview of MMP-13 as a Promising Target for the Treatment of Osteoarthritis. *Int. J. Mol. Sci.* **2021**, *22*, 1742. [CrossRef]
58. Kevorkian, L.; Young, D.A.; Darrah, C.; Donell, S.T.; Shepstone, L.; Porter, S.; Brockbank, S.M.V.; Edwards, D.R.; Parker, A.E.; Clark, I.M. Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Care Res.* **2004**, *50*, 131–141. [CrossRef]
59. Kozawa, E.; Cheng, X.W.; Urakawa, H.; Arai, E.; Yamada, Y.; Kitamura, S.; Sato, K.; Kuzuya, M.; Ishiguro, N.; Nishida, Y. Increased expression and activation of cathepsin K in human osteoarthritic cartilage and synovial tissues. *J. Orthop. Res.* **2015**, *34*, 127–134. [CrossRef]
60. Aigner, T.; Kurz, B.; Fukui, N.; Sandell, L. Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr. Opin. Rheumatol.* **2002**, *14*, 578–584. [CrossRef]
61. Goldring, S.R.; Goldring, M.B. Changes in the osteochondral unit during osteoarthritis: Structure, function and cartilage–bone crosstalk. *Nat. Rev. Rheumatol.* **2016**, *12*, 632–644. [CrossRef] [PubMed]
62. Valcourt, U.; Gouttenoire, J.; Aubert-Foucher, E.; Herbage, D.; Mallein-Gerin, F. Alternative splicing of type II procollagen pre-mRNA in chondrocytes is oppositely regulated by BMP-2 and TGF-β1. *FEBS Lett.* **2003**, *545*, 115–119. [CrossRef]
63. Zheng, L.; Zhang, Z.; Sheng, P.; Mobasheri, A. The role of metabolism in chondrocyte dysfunction and the progression of osteoarthritis. *Ageing Res. Rev.* **2020**, *66*, 101249. [CrossRef]
64. Santos, A.L.; Preta, G. Lipids in the cell: Organisation regulates function. *Experientia* **2018**, *75*, 1909–1927. [CrossRef] [PubMed]
65. Calder, P.C. Eicosanoids. *Essays Biochem.* **2020**, *64*, 423–441. [PubMed]
66. Maldonado-Valderrama, J.; Wilde, P.; Macierzanka, A.; Mackie, A. The role of bile salts in digestion. *Adv. Colloid Interface Sci.* **2011**, *165*, 36–46. [CrossRef] [PubMed]
67. Sabatini, M.; Rolland, G.; Léonce, S.; Thomas, M.; Lesur, C.; Pérez, V.; de Nanteuil, G.; Bonnet, J. Effects of Ceramide on Apoptosis, Proteoglycan Degradation, and Matrix Metalloproteinase Expression in Rabbit Articular Cartilage. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 438–444. [CrossRef]
68. Sabatini, M.; Rolland, G.; Léonce, S.; Thomas, M.; Lesur, C.; Pérez, V.; de Nanteuil, G.; Bonnet, J. Isolated effects of external bath osmolality, solute concentration, and electrical charge on solute transport across articular cartilage. *Med. Eng. Phys.* **2016**, *38*, 1399–1407.
69. Wang, Y.; Wei, L.; Zeng, L.; He, D.; Wei, X. Nutrition and degeneration of articular cartilage. *Knee Surg. Sports Traumatol. Arthrosc.* **2012**, *21*, 1751–1762. [CrossRef]
70. Arkill, K.P.; Winlove, C.P. Fatty acid transport in articular cartilage. *Arch. Biochem. Biophys.* **2006**, *456*, 71–78. [CrossRef]

71. Silva, M.O.; Gregory, J.L.; Ansari, N.; Stok, K.S. Molecular Signaling Interactions and Transport at the Osteochondral Interface: A Review. *Front. Cell Dev. Biol.* **2020**, *8*, 750. [CrossRef] [PubMed]
72. Pouran, B.; Arbabi, V.; Bleys, R.L.; van Weeren, P.R.; Zadpoor, A.A.; Weinans, H. Solute transport at the interface of cartilage and subchondral bone plate: Effect of micro-architecture. *J. Biomech.* **2017**, *52*, 148–154. [CrossRef] [PubMed]
73. Sekar, S.; Shafie, S.R.; Prasadam, I.; Crawford, R.; Panchal, S.K.; Brown, L.; Xiao, Y. Saturated fatty acids induce development of both metabolic syndrome and osteoarthritis in rats. *Sci. Rep.* **2017**, *7*, 46457. [CrossRef] [PubMed]
74. Park, S.; Baek, I.J.; Ryu, J.H.; Chun, C.H.; Jin, E.J. PPARalpha-ACOT12 axis is responsible for maintaining cartilage homeostasis through modulating de novo lipogenesis. *Nat. Commun.* **2022**, *13*, 3. [CrossRef] [PubMed]
75. Wu, C.L.; Jain, D.; McNeill, J.N.; Little, D.; Anderson, J.A.; Huebner, J.L.; Kraus, V.B.; Rodriguiz, R.M.; Wetsel, W.C.; Guilak, F. Dietary fatty acid content regulates wound repair and the pathogenesis of osteoarthritis following joint injury. *Ann. Rheum Dis.* **2015**, *74*, 2076–2083. [CrossRef] [PubMed]
76. Jones, S.F.; Infante, J.R. Molecular Pathways: Fatty Acid Synthase. *Clin. Cancer Res.* **2015**, *21*, 5434–5438. [CrossRef]
77. Abu-Elheiga, L.; Matzuk, M.M.; Abo-Hashema, K.A.; Wakil, S.J. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* **2001**, *291*, 2613–2616. [CrossRef]
78. Ichimura, A.; Hasegawa, S.; Kasubuchi, M.; Kimura, I. Free fatty acid receptors as therapeutic targets for the treatment of diabetes. *Front. Pharmacol.* **2014**, *5*, 236. [CrossRef]
79. Miao, H.; Chen, L.; Hao, L.; Zhang, X.; Chen, Y.; Ruan, Z.; Liang, H. Stearic acid induces proinflammatory cytokine production partly through activation of lactate-HIF1alpha pathway in chondrocytes. *Sci. Rep.* **2015**, *5*, 13092. [CrossRef]
80. Alvarez-Garcia, O.; Rogers, N.H.; Smith, R.G.; Lotz, M.K. Palmitate has proapoptotic and proinflammatory effects on articular cartilage and synergizes with interleukin-1. *Arthritis Rheumatol.* **2014**, *66*, 1779–1788. [CrossRef]
81. Medina-Luna, D.; Santamaria-Olmedo, M.G.; Zamudio-Cuevas, Y.; Martinez-Flores, K.; Fernandez-Torres, J.; Martinez-Nava, G.A.; Clavijo-Cornejo, D.; Hernandez-Diaz, C.; Olivos-Meza, A.; Gomez-Quiroz, L.E.; et al. Hyperlipidemic microenvironment conditionates damage mechanisms in human chondrocytes by oxidative stress. *Lipids Health Dis.* **2017**, *16*, 114. [CrossRef] [PubMed]
82. Calder, P.C. Mechanisms of Action of (n-3) Fatty Acids. *J. Nutr.* **2012**, *142*, 592S–599S. [CrossRef]
83. Martindale, R.G.; Warren, M.M.; McClave, S.A. Does the use of specialized proresolving molecules in critical care offer a more focused approach to controlling inflammation than that of fish oils? *Curr. Opin. Clin. Nutr. Metab Care* **2016**, *19*, 151–154. [CrossRef] [PubMed]
84. Patterson, E.; Wall, R.; Fitzgerald, G.F.; Ross, R.; Stanton, C. Health Implications of High Dietary Omega-6 Polyunsaturated Fatty Acids. *J. Nutr. Metab.* **2012**, *2012*, 539426. [CrossRef] [PubMed]
85. Adkisson, H.D., 4th; Risener Jr, F.S.; Zarrinkar, P.P.; Walla, M.D.; Christie, W.W.; Wuthier, R.E. Unique fatty acid composition of normal cartilage: Discovery of high levels of n-6 polyunsaturated fatty acids. *FASEB J.* **1991**, *5*, 344–353. [CrossRef] [PubMed]
86. Ioan-Facsinay, A.; Kloppenburg, M. Bioactive lipids in osteoarthritis: Risk or benefit? *Curr. Opin. Rheumatol.* **2018**, *30*, 108–113.
87. Attur, M.; Krasnokutsky, S.; Statnikov, A.; Samuels, J.; Li, Z.; Friese, O.; Le Graverand-Gastineau, M.-P.H.; Rybak, L.; Kraus, V.B.; Jordan, J.M.; et al. Low-Grade Inflammation in Symptomatic Knee Osteoarthritis: Prognostic Value of Inflammatory Plasma Lipids and Peripheral Blood Leukocyte Biomarkers. *Arthritis Rheumatol.* **2015**, *67*, 2905–2915. [CrossRef]
88. Miyamoto, M.; Ito, H.; Mukai, S.; Kobayashi, T.; Yamamoto, H.; Kobayashi, M.; Maruyama, T.; Akiyama, H.; Nakamura, T. Simultaneous stimulation of EP2 and EP4 is essential to the effect of prostaglandin E2in chondrocyte differentiation. *Osteoarthr. Cartil.* **2003**, *11*, 644–652. [CrossRef]
89. Nishitani, K.; Ito, H.; Hiramitsu, T.; Tsutsumi, R.; Tanida, S.; Kitaori, T.; Yoshitomi, H.; Kobayashi, M.; Nakamura, T. PGE2 inhibits MMP expression by suppressing MKK4-JNK MAP kinase-c-JUN pathway via EP4 in human articular chondrocytes. *J. Cell Biochem.* **2010**, *109*, 425–433. [CrossRef]
90. Alvarez-Soria, M.A.; Largo, R.; Sanchez-Pernaute, O.; Calvo, E.; Egido, J.; Herrero-Beaumont, G. Prostaglandin E2 receptors EP1 and EP4 are up-regulated in rabbit chondrocytes by IL-1beta, but not by TNFalpha. *Rheumatol. Int.* **2007**, *27*, 911–917. [CrossRef]
91. Tuure, L.; Hämäläinen, M.; Nummenmaa, E.; Moilanen, T.; Moilanen, E. Downregulation of microsomal prostaglandin E synthase-1 (mPGES-1) expression in chondrocytes is regulated by MAP kinase phosphatase-1 (MKP-1). *Int. Immunopharmacol.* **2019**, *71*, 139–143. [CrossRef] [PubMed]
92. Soccio, R.E.; Breslow, J.L. Intracellular Cholesterol Transport. *Arter. Thromb. Vasc. Biol.* **2004**, *24*, 1150–1160. [CrossRef] [PubMed]
93. Cerqueira, N.M.F.S.A.; Oliveira, E.F.; Gesto, D.S.; Santos-Martins, D.; Moreira, C.; Moorthy, H.N.; Ramos, M.J.; Fernandes, P.A. Cholesterol Biosynthesis: A Mechanistic Overview. *Biochemistry* **2016**, *55*, 5483–5506. [CrossRef] [PubMed]
94. Hu, J.; Zhang, Z.; Shen, W.-J.; Azhar, S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr. Metab.* **2010**, *7*, 47. [CrossRef] [PubMed]
95. Vallim, T.Q.D.A.; Tarling, E.J.; Edwards, P.A. Pleiotropic Roles of Bile Acids in Metabolism. *Cell Metab.* **2013**, *17*, 657–669. [CrossRef]
96. Schafer, W.R.; Trueblood, C.E.; Yang, C.-C.; Mayer, M.P.; Rosenberg, S.; Poulter, C.D.; Kim, S.-H.; Rine, J. Enzymatic Coupling of Cholesterol Intermediates to a Mating Pheromone Precursor and to the Ras Protein. *Science* **1990**, *249*, 1133–1139. [CrossRef]
97. Incardona, J.P.; Eaton, S. Cholesterol in signal transduction. *Curr. Opin. Cell Biol.* **2000**, *12*, 193–203. [CrossRef]
98. Girão, H.; Pereira, P.; Ramalho, J.; Quinlan, R.; Prescott, A. Cholesterol oxides mediated changes in cytoskeletal organisation involves Rho GTPases. *Exp. Cell Res.* **2003**, *291*, 502–513. [CrossRef]

99. Wu, S.; De Luca, F. Role of cholesterol in the regulation of growth plate chondrogenesis and longitudinal bone growth. *J. Biol. Chem.* **2004**, *279*, 4642–4647. [CrossRef]
100. Goldstein, J.L.; Brown, M.S. The LDL receptor. *Arterioscler Thromb Vasc. Biol.* **2009**, *29*, 431–438. [CrossRef]
101. Parthasarathy, S.; Raghavamenon, A.; Garelnabi, M.O.; Santanam, N. Oxidized low-density lipoprotein. *Methods Mol. Biol.* **2010**, *610*, 403–417. [PubMed]
102. Choi, M.-C.; Jo, J.; Park, J.; Kang, H.K.; Park, Y. NF-κB Signaling Pathways in Osteoarthritic Cartilage Destruction. *Cells* **2019**, *8*, 734. [CrossRef] [PubMed]
103. Nishimura, S.; Akagi, M.; Yoshida, K.; Hayakawa, S.; Sawamura, T.; Munakata, H.; Hamanishi, C. Oxidized low-density lipoprotein (ox-LDL) binding to lectin-like ox-LDL receptor-1 (LOX-1) in cultured bovine articular chondrocytes increases production of intracellular reactive oxygen species (ROS) resulting in the activation of NF-kappaB. *Osteoarthr. Cartil.* **2004**, *12*, 568–576. [CrossRef]
104. Kishimoto, H.; Akagi, M.; Zushi, S.; Teramura, T.; Onodera, Y.; Sawamura, T.; Hamanishi, C. Induction of hypertrophic chondrocyte-like phenotypes by oxidized LDL in cultured bovine articular chondrocytes through increase in oxidative stress. *Osteoarthr. Cartil.* **2010**, *18*, 1284–1290. [CrossRef] [PubMed]
105. Pearle, A.D.; Scanzello, C.R.; George, S.; Mandl, L.S.; DiCarlo, E.F.; Peterson, M.; Sculco, T.P.; Crow, M.K.C. Elevated high-sensitivity C-reactive protein levels are associated with local inflammatory findings in patients with osteoarthritis. *Osteoarthr. Cartil.* **2007**, *15*, 516–523. [CrossRef]
106. Tsezou, A.; Iliopoulos, D.; Malizos, K.N.; Simopoulou, T. Impaired expression of genes regulating cholesterol efflux in human osteoarthritic chondrocytes. *J. Orthop. Res.* **2010**, *28*, 1033–1039. [CrossRef]
107. Yvan-Charvet, L.; Wang, N.; Tall, A.R. Role of HDL, ABCA1, and ABCG1 Transporters in Cholesterol Efflux and Immune Responses. *Arter. Thromb. Vasc. Biol.* **2010**, *30*, 139–143. [CrossRef]
108. De Seny, D.; Cobraiville, G.; Charlier, E.; Neuville, S.; Esser, N.; Malaise, D.; Malaise, O.; Calvo, F.Q.; Relic, B.; Malaise, M.G. Acute-Phase Serum Amyloid A in Osteoarthritis: Regulatory Mechanism and Proinflammatory Properties. *PLoS ONE* **2013**, *8*, e66769. [CrossRef]
109. Gentili, C.; Tutolo, G.; Pianezzi, A.; Cancedda, R.; Descalzi Cancedda, F. Cholesterol secretion and homeostasis in chondrocytes: A liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein A1 expression. *Matrix Biol.* **2005**, *24*, 35–44. [CrossRef]
110. de Seny, D.; Cobraiville, G.; Charlier, E.; Neuville, S.; Lutteri, L.; Le Goff, C.; Malaise, D.; Malaise, O.; Chapelle, J.P.; Relic, B.; et al. Apolipoprotein-A1 as a damage-associated molecular patterns protein in osteoarthritis: Ex vivo and in vitro pro-inflammatory properties. *PLoS ONE* **2015**, *10*, e0122904. [CrossRef]
111. Farnaghi, S.; Crawford, R.; Xiao, Y.; Prasadam, I. Cholesterol metabolism in pathogenesis of osteoarthritis disease. *Int. J. Rheum. Dis.* **2017**, *20*, 131–140. [CrossRef] [PubMed]
112. De Munter, W.; Blom, A.B.; Helsen, M.M.; Walgreen, B.; Van Der Kraan, P.M.; Ab Joosten, L.; Berg, W.B.V.D.; Van Lent, P.L. Cholesterol accumulation caused by low density lipoprotein receptor deficiency or a cholesterol-rich diet results in ectopic bone formation during experimental osteoarthritis. *Arthritis Res. Ther.* **2013**, *15*, R178. [CrossRef] [PubMed]
113. Choi, W.S.; Lee, G.; Song, W.H.; Koh, J.T.; Yang, J.; Kwak, J.S.; Kim, H.E.; Kim, S.K.; Son, Y.O.; Nam, H.; et al. The CH25H-CYP7B1-RORalpha axis of cholesterol metabolism regulates osteoarthritis. *Nature* **2019**, *566*, 254–258. [CrossRef] [PubMed]
114. Sun, M.M.-G.; Beier, F. Liver X Receptor activation regulates genes involved in lipid homeostasis in developing chondrocytes. *Osteoarthr. Cartil. Open* **2020**, *2*, 100030. [CrossRef]
115. Kostopoulou, F.; Gkretsi, V.; Malizos, K.N.; Iliopoulos, D.; Oikonomou, P.; Poultsides, L.; Tsezou, A. Central Role of SREBP-2 in the Pathogenesis of Osteoarthritis. *PLoS ONE* **2012**, *7*, e35753. [CrossRef]
116. Kostopoulou, F.; Malizos, K.N.; Papathanasiou, I.; Tsezou, A. MicroRNA-33a regulates cholesterol synthesis and cholesterol efflux-related genes in osteoarthritic chondrocytes. *Arthritis Res. Ther.* **2015**, *17*, 42. [CrossRef]
117. Smith, A.E.; Sigurbjörnsdóttir, E.S.; Steingrímsson, E.; Sigurbjörnsdóttir, S. Hedgehog signalling in bone and osteoarthritis: The role of Smoothened and cholesterol. *FEBS J.* **2022**, *19*, 16440. [CrossRef]
118. Bernstein, P.; Sticht, C.; Jacobi, A.; Liebers, C.; Manthey, S.; Stiehler, M. Expression pattern differences between osteoarthritic chondrocytes and mesenchymal stem cells during chondrogenic differentiation. *Osteoarthr. Cartil.* **2010**, *18*, 1596–1607. [CrossRef]
119. Fagone, P.; Jackowski, S. Membrane phospholipid synthesis and endoplasmic reticulum function. *J. Lipid Res.* **2009**, *50*, S311–S316. [CrossRef]
120. Vance, J.E. Phospholipid Synthesis and Transport in Mammalian Cells. *Traffic* **2015**, *16*, 1–18. [CrossRef]
121. Zhai, G.; Pelletier, J.-P.; Liu, M.; Aitken, D.; Randell, E.; Rahman, P.; Jones, G.; Martel-Pelletier, J. Activation of the Phosphatidylcholine to Lysophosphatidylcholine Pathway is Associated with Osteoarthritis Knee Cartilage Volume Loss Over Time. *Sci. Rep.* **2019**, *9*, 019–46185. [CrossRef] [PubMed]
122. Tabe, S.; Hikiji, H.; Ariyoshi, W.; Hashidate-Yoshida, T.; Shindou, H.; Shimizu, T.; Okinaga, T.; Seta, Y.; Tominaga, K.; Nishihara, T. Lysophosphatidylcholine acyltransferase 4 is involved in chondrogenic differentiation of ATDC5 cells. *Sci. Rep.* **2017**, *7*, 16701. [CrossRef] [PubMed]
123. Hannun, Y.A.; Obeid, L.M. Sphingolipids and their metabolism in physiology and disease. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 175–191. [CrossRef] [PubMed]

124. Kim, M.K.; Lee, H.Y.; Kwak, J.Y.; Park, J.I.; Yun, J.; Bae, Y.S. Sphingosine-1-phosphate stimulates rat primary chondrocyte proliferation. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 67–73. [CrossRef]
125. Garfinkel, R.J.; Dilisio, M.F.; Agrawal, D.K. Vitamin D and Its Effects on Articular Cartilage and Osteoarthritis. *Orthop. J. Sports Med.* **2017**, *5*. [CrossRef]
126. Prabhu, A.V.; Luu, W.; Sharpe, L.J.; Brown, A.J. Cholesterol-mediated Degradation of 7-Dehydrocholesterol Reductase Switches the Balance from Cholesterol to Vitamin D Synthesis. *J. Biol. Chem.* **2016**, *291*, 8363–8373. [CrossRef]
127. Asano, L.; Watanabe, M.; Ryoden, Y.; Usuda, K.; Yamaguchi, T.; Khambu, B.; Takashima, M.; Sato, S.-I.; Sakai, J.; Nagasawa, K.; et al. Vitamin D Metabolite, 25-Hydroxyvitamin D, Regulates Lipid Metabolism by Inducing Degradation of SREBP/SCAP. *Cell Chem. Biol.* **2017**, *24*, 207–217. [CrossRef]
128. Martínez-Sena, T.; Soluyanova, P.; Guzmán, C.; Valdivielso, J.M.; Castell, J.V.; Jover, R. The Vitamin D Receptor Regulates Glycerolipid and Phospholipid Metabolism in Human Hepatocytes. *Biomolecules* **2020**, *10*, 493. [CrossRef]
129. Rai, V.; Dietz, N.E.; Dilisio, M.F.; Radwan, M.M.; Agrawal, D.K. Vitamin D attenuates inflammation, fatty infiltration, and cartilage loss in the knee of hyperlipidemic microswine. *Arthritis Res. Ther.* **2016**, *18*, 203. [CrossRef]
130. Pascual-Garrido, C.; Angeline, M.E.; Ma, R.; Chahla, J.; Voigt, C.; Deng, X.H.; Nguyen, J.; Warren, R.F.; Rodeo, S.A. Low Levels of Vitamin D have a Deleterious Effect on the Articular Cartilage in a Rat Model. *HSS J. Musculoskelet. J. Hosp. Spéc. Surg.* **2016**, *12*, 150–157. [CrossRef]
131. Li, S.; Niu, G.; Dong, X.N.; Liu, Z.; Song, C.; Leng, H. Vitamin D Inhibits Activities of Metalloproteinase-9/-13 in Articular Cartilage. *J. Nutr. Sci. Vitaminol.* **2019**, *65*, 107–112. [CrossRef] [PubMed]
132. Jhun, J.; Woo, J.S.; Kwon, J.Y.; Na, H.S.; Cho, K.-H.; Kim, S.A.; Kim, S.J.; Moon, S.-J.; Park, S.-H.; Cho, M.-L. Vitamin D Attenuates Pain and Cartilage Destruction in OA Animals via Enhancing Autophagic Flux and Attenuating Inflammatory Cell Death. *Immune Netw.* **2022**, *22*, e34. [CrossRef] [PubMed]
133. Rai, V.; Radwan, M.M.; Agrawal, D.K. IL-33, IL-37, and Vitamin D Interaction Mediate Immunomodulation of Inflammation in Degenerating Cartilage. *Antibodies* **2021**, *10*, 41. [CrossRef]
134. Mobasheri, A. Glucose: An energy currency and structural precursor in articular cartilage and bone with emerging roles as an extracellular signaling molecule and metabolic regulator. *Front. Endocrinol.* **2012**, *3*, 00153. [CrossRef] [PubMed]
135. Lee, S.-Y.; Abel, E.D.; Long, F. Glucose metabolism induced by Bmp signaling is essential for murine skeletal. *Nat. Commun.* **2018**, *9*, 4831. [CrossRef]
136. Rosa, S.C.; Gonçalves, J.; Judas, F.; Mobasheri, A.; Lopes, C.; Mendes, A.F. Impaired glucose transporter-1 degradation and increased glucose transport and oxidative stress in response to high glucose in chondrocytes from osteoarthritic versus normal human cartilage. *Arthritis Res. Ther.* **2009**, *11*, R80. [CrossRef]
137. Rogatzki, M.J.; Ferguson, B.S.; Goodwin, M.L.; Gladden, L.B. Lactate is always the end product of glycolysis. *Front. Neurosci.* **2015**, *9*, 22. [CrossRef]
138. Mookerjee, S.A.; Gerencser, A.A.; Nicholls, D.G.; Brand, M.D. Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J. Biol. Chem.* **2017**, *292*, 7189–7207. [CrossRef]
139. Martin, J.; Martini, A.; Molinari, A.; Morgan, W.; Ramalingam, W.; Buckwalter, J.; McKinley, T. Mitochondrial electron transport and glycolysis are coupled in articular cartilage. *Osteoarthr. Cartil.* **2012**, *20*, 323–329. [CrossRef]
140. Otte, P. Basic cell metabolism of articular cartilage. Manometric studies. *Z. Rheumatol.* **1991**, *50*, 304–312.
141. Alfano, B.; Barretta, L.; Del Giudice, A.; De Vito, S.; Di Francia, G.; Esposito, E.; Formisano, F.; Massera, E.; Miglietta, M.L.; Polichetti, T. A Review of Low-Cost Particulate Matter Sensors from the Depvelopers' Perspectives. *Sensors* **2020**, *20*, 6819. [CrossRef] [PubMed]
142. Adams, S.B., Jr.; Setton, L.A.; Kensicki, E.; Bolognesi, M.P.; Toth, A.P.; Nettles, D.L. Global metabolic profiling of human osteoarthritic synovium. *Osteoarthr. Cartil.* **2012**, *20*, 64–67. [CrossRef]
143. Leistad, L.; Feuerherm, A.; Faxvaag, A.; Johansen, B. Multiple phospholipase A2 enzymes participate in the inflammatory process in osteoarthritic cartilage. *Scand. J. Rheumatol.* **2011**, *40*, 308–316. [CrossRef]
144. Tootsi, K.; Kals, J.; Zilmer, M.; Paapstel, K.; Ottas, A.; Märtson, A. Medium- and long-chain acylcarnitines are associated with osteoarthritis severity and arterial stiffness in end-stage osteoarthritis patients: A case-control study. *Int. J. Rheum. Dis.* **2018**, *21*, 1211–1218. [CrossRef]
145. Yang, X.; Chen, W.; Zhao, X.; Chen, L.; Li, W.; Ran, J.; Wu, L. Pyruvate Kinase M2 Modulates the Glycolysis of Chondrocyte and Extracellular Matrix in Osteoarthritis. *DNA Cell Biol.* **2018**, *37*, 271–277. [CrossRef] [PubMed]
146. Pitcher, T.; Sousa-Valente, J.; Malcangio, M. The Mon iodoacetate Model of Osteoarthritis Pain in the Mouse. *J. Vis. Exp.* **2016**, e53746. [CrossRef]
147. Das, R.; van Osch, G.; Kreukniet, M.; Oostra, J.; Weinans, H.; Jahr, H. Effects of individual control of pH and hypoxia in chondrocyte culture. *J. Orthop. Res.* **2009**, *28*, 537–545. [CrossRef] [PubMed]
148. Choi, W.-S.; Yang, J.-I.; Kim, W.; Kim, H.-E.; Kim, S.-K.; Won, Y.; Son, Y.-O.; Chun, C.-H.; Chun, J.-S. Critical role for arginase II in osteoarthritis pathogenesis. *Ann. Rheum. Dis.* **2019**, *78*, 421–428. [CrossRef]
149. Farnaghi, S.; Prasadam, I.; Cai, G.; Friis, T.; Du, Z.; Crawford, R.; Mao, X.; Xiao, Y. Protective effects of mitochondria-targeted antioxidants and statins on cholesterol-induced osteoarthritis. *FASEB J.* **2017**, *31*, 356–367. [CrossRef]
150. Wang, Z.; Ni, S.; Zhang, H.; Fan, Y.; Xia, L.; Li, N. Silencing SGK1 alleviates osteoarthritis through epigenetic regulation of CREB1 and ABCA1 expression. *Life Sci.* **2020**, *268*, 118733. [CrossRef]

151. He, H.; Lu, M.; Shi, H.; Yue, G.; Luo, H. Vaspin regulated cartilage cholesterol metabolism through miR155/LXRα and participated in the occurrence of osteoarthritis in rats. *Life Sci.* **2021**, *269*, 119096. [CrossRef] [PubMed]
152. Wei, Y.; Yan, L.; Luo, L.; Gui, T.; Jang, B.; Amirshaghaghi, A.; You, T.; Tsourkas, A.; Qin, L.; Cheng, Z. Phospholipase A_2 inhibitor–loaded micellar nanoparticles attenuate inflammation and mitigate osteoarthritis progression. *Sci. Adv.* **2021**, *7*, eabe6374. [CrossRef] [PubMed]
153. Stradner, M.H.; Gruber, G.; Angerer, H.; Huber, V.; Setznag, D.; Kremser, M.-L.; Moazedi-Fürst, F.C.; Windhager, R.; Graninger, W.B. Sphingosine 1-phosphate counteracts the effects of interleukin-1β in human chondrocytes. *Arthritis Rheum.* **2013**, *65*, 2113–2122. [CrossRef] [PubMed]
154. Xiang, C.; Yang, K.; Liang, Z.; Wan, Y.; Cheng, Y.; Ma, D.; Zhang, H.; Hou, W.; Fu, P. Sphingosine-1-phosphate mediates the therapeutic effects of bone marrow mesenchymal stem cell-derived microvesicles on articular cartilage defect. *Transl. Res.* **2017**, *193*, 42–53. [CrossRef] [PubMed]
155. Hunter, D.J.; Bierma-Zeinstra, S. Osteoarthritis. *Lancet* **2019**, *393*, 1745–1759. [CrossRef]

nutrients

MDPI

Article

Comparison of the Coincidence of Osteoporosis, Fracture, Arthritis Histories, and DEXA T-Score between Monozygotic and Dizygotic Twins: A Cross-Sectional Study Using KoGES HTS Data

Hyo Geun Choi [1,†], So Young Kim [2,†], Bong Cheol Kwon [3], Ho Suk Kang [4], Hyun Lim [4], Joo-Hee Kim [5], Ji Hee Kim [6], Seong Jin Cho [7], Eun Sook Nam [7], Kyueng Whan Min [8], Ha Young Park [9], Nan Young Kim [10], Younghee Choi [11,12] and Mi Jung Kwon [13,*]

1 Department of Otorhinolaryngology-Head & Neck Surgery, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
2 Department of Otorhinolaryngology-Head & Neck Surgery, CHA Bundang Medical Center, College of Medicine, CHA University, Seongnam 13488, Korea
3 Department of Orthopedic Surgery, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
4 Division of Gastroenterology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
5 Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
6 Department of Neurosurgery, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
7 Department of Pathology, Kangdong Sacred Heart Hospital, College of Medicine, Hallym University, Seoul 05355, Korea
8 Department of Pathology, Hanyang University Guri Hospital, College of Medicine, Hanyang University, Guri 11923, Korea
9 Department of Pathology, Busan Paik Hospital, College of Medicine, Inje University, Busan 47392, Korea
10 Hallym Institute of Translational Genomics and Bioinformatics, Medical Center, Hallym University, Anyang 14068, Korea
11 Department of Pathology, Hallym University Dongtan Sacred Heart Hospital, College of Medicine, Hallym University, Hwaseong 18450, Korea
12 Research Institute for Complementary & Alternative Medicine, Hallym University, Anyang 14068, Korea
13 Department of Pathology, Hallym University Sacred Heart Hospital, College of Medicine, Hallym University, Anyang 14068, Korea
* Correspondence: mulank@hanmail.net
† These authors contributed equally to this work.

Citation: Choi, H.G.; Kim, S.Y.; Kwon, B.C.; Kang, H.S.; Lim, H.; Kim, J.-H.; Kim, J.H.; Cho, S.J.; Nam, E.S.; Min, K.W.; et al. Comparison of the Coincidence of Osteoporosis, Fracture, Arthritis Histories, and DEXA T-Score between Monozygotic and Dizygotic Twins: A Cross-Sectional Study Using KoGES HTS Data. *Nutrients* **2022**, *14*, 3836. https://doi.org/10.3390/nu14183836

Academic Editor: Qian Zhang

Received: 12 August 2022
Accepted: 7 September 2022
Published: 16 September 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: We explored the genetic and environmental inter-relationships among osteoporosis, fracture, arthritis, and bone mineral density concordance in monozygotic twins compared to those in dizygotic twins. This cross-sectional research assessed data of 1032 monozygotic and 242 dizygotic twin pairs aged >20 years included in the Healthy Twin Study data of the Korean Genome and Epidemiology Study between 2005 and 2014. Outcomes of interest included illness concordance and absolute differences in dual-energy X-ray absorptiometry (DEXA) T-scores. We found comparable concordances of osteoporosis, fractures, osteoarthritis, and rheumatoid arthritis between monozygotic and dizygotic twins. Medical histories of osteoporosis, fractures caused by accident or falling, osteoarthritis, and rheumatoid arthritis were not distinct between monozygotic and dizygotic twins. Accidental fracture occurrence in both monozygotic twins showed significantly lower odds than that in dizygotic twins. Genetic influence on liability to fracture risk might thus be maintained. DEXA T-scores for bone mineral density indicated more comparable tendencies within monozygotic twin pairs than within dizygotic ones, suggesting the relative importance of genetic contribution to bone mineral density. The relative importance of genetic factors in bone mineral density is sustained between monozygotic twins; overt disease expression of osteoporosis, fractures, or arthritis may be affected by environmental factors.

Keywords: dizygotic twins; monozygotic twins; aging; bone mineral density; osteoporosis; fracture; osteoarthritis; rheumatoid arthritis; genetic factors; environmental factors

1. Introduction

Osteoporosis, fractures, osteoarthritis (OA), and rheumatoid arthritis (RA) are highly prevalent diseases that are associated with considerable morbidity, mortality, and healthcare costs [1–5]. Age-related bone and joint diseases are closely interrelated and characterized by multifaceted interactions between genetic and environmental factors [6–9]. While aging is one of the main risk factors for the development of osteoporosis, fractures, OA, and RA [9–11], other factors such as genetic predisposition, obesity, inflammation, sex and hormones, or metabolic syndrome contribute to the development of these diseases [8,12–14].

Osteoporosis is characterized by a reduction in bone mineral density and quality, with a consequent increase in bone fragility and risk of fracture [15,16]. Updates to the epidemiology and pathophysiology of osteoporosis have proposed an inverse relationship with OA and a positive correlation with RA [17]: higher bone mineral density and lower fracture risk in patients with OA [2,3], and reduced bone mineral density and high frequency of osteoporosis in patients with RA compared with those in the general population [1]. In other rheumatic diseases such as ankylosing spondylitis, a high prevalence of bone mineral density and osteoporosis is observed in patients with early-stage disease [18]. In this context, subclinical radiologic changes related to bone mineral density might be a clinically important intermediate process in overt diseases, in which a potential indicator for disease expression might be expected.

Twin studies may offer a valuable approach for estimating the relative significance of genetic and environmental contributions to these bone and joint diseases and complex traits, given the genetic similarity and shared family and rearing environment between twins [19,20]. A higher correspondence between monozygotic twin pairs than between dizygotic ones may be ascribed to genetic factors, while a comparable degree of similitude between monozygotic and dizygotic twin groups may be attributed to an environmental influence [19]. A genetic predisposition to age-related bone diseases was suggested in individual epidemiologic studies, including a few twin studies, with the possible individual heritability of bone mineral density (60–80%) [21], osteoporosis (36–85%) [22,23], fracture (68%) [7,8], falls (35%) [24], frailty (43%) [25], OA (39–65%) [14], and RA (52–60%) [26–28]. However, combined analyses of aging-related bone and joint diseases and their relevance to bone mineral density, with full adjustments for lifestyle factors, have not been determined in validated twin studies. Since osteoporosis, fractures, OA, and RA appear to share possible risk factors and reciprocal associations, further studies adjusting for possible mutual confounders are needed.

This study aimed to evaluate the putative effects of environmental factors on the genetic predisposition toward aging-related bone disease by comparing twin cohorts. To explore this issue, we investigated medical histories and concordances of aging-related bone diseases and absolute differences in dual-energy X-ray absorptiometry (DEXA) T-scores regarding bone mineral density between monozygotic twins (genetic influence) and dizygotic twins (environmental contribution over genetic influence) after adjusting for lifestyle factors.

2. Materials and Methods

2.1. Study Population and Data Collection

The ethics committee of Hallym University (2021-03-004) approved the use of the data. The requirement for written informed consent was waived by the institutional review board. This cohort study was based on the Healthy Twin Study (HTS), a nationwide cross-sectional survey that is a part of the prospective Korean Genome Epidemiology Study (KoGES), that recruited Korean same-sex twin pairs aged over 20 years who primarily resided in

Seoul or Busan, the two largest urban areas in Korea [29]. The participants were voluntarily recruited through advertisements and media at health-related governmental agencies and participating hospitals since 2005 [30]. The baseline data of KoGES HTS were obtained from 2005 to 2013 and follow-up data from 2008 to 2014. Zygosity was assessed at baseline using genetic analysis, including 16 short tandem repeat markers (Amp*Fl*STR Identifier Kit; Perkin Elmer, Waltham, MA, USA) [31]. Two-thirds of the participants who completed the baseline examination were followed up, and their medical histories were updated. The study data were described in detail in previous studies [29,30,32,33].

2.2. Participants Selection

Among 1300 twin participants, those who did not have the records of DEXA data measuring bone mineral density (n = 22) and sleep time (n = 4) were excluded. A total of 1032 monozygotic twins (516 pairs of twins) and 242 dizygotic twins (121 pairs of twins) were enrolled (Figure 1). We then analyzed the concordance of their disease histories and DEXA T-scores between the monozygotic and dizygotic twin participants.

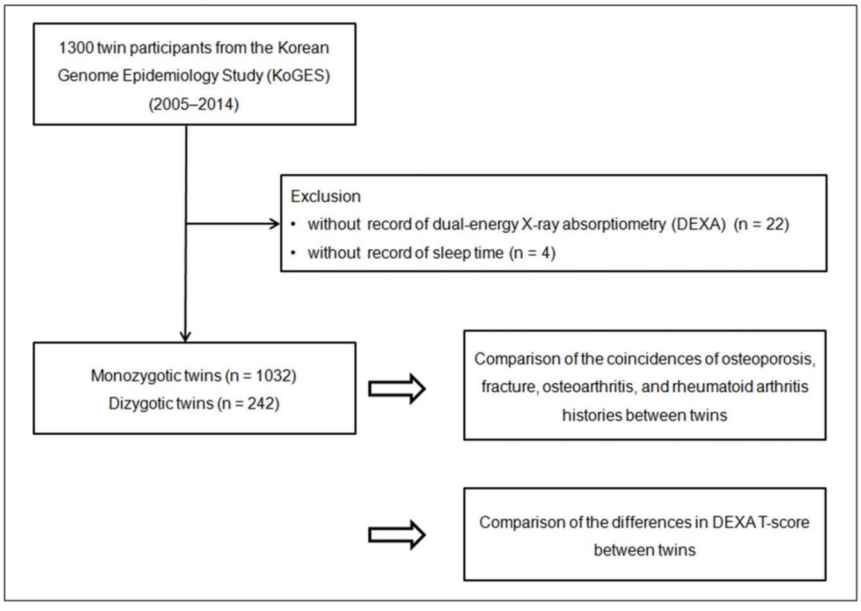


Figure 1. The study design of the present study; 1032 monozygotic twins and 242 dizygotic twins were compared for concordance of several aging-related bone and joint diseases, including osteoporosis, fracture, osteoarthritis, and rheumatoid arthritis between twins. DEXA T-scores for bone mineral density were also compared.

2.3. Survey

Participants completed the interviewer-administered KoGES Baseline Core Questionnaire officially designed by the Korean National Institute of Health to collect information on demographic characteristics and lifestyle, including dietary habits, health conditions, and medical history; the questionnaire is publicly available on the Korean National Institute of Health website (https://nih.go.kr/contents.es?mid=a50401010300) (accessed on 1 January 2022). Face-to-face interviews by trained interviewers were performed to clarify incomplete or ambiguous responses [29,34]. The listed questionnaires and data are regularly validated and updated. Interviewer-administered questionnaires, anthropometric measurements, and biochemical tests were conducted at the officially designated University hospitals and medical institutions, as previously described in detail [29].

Trained interviewers asked the participants about their medical history of osteoporosis, fracture by accident, fracture by fall, OA, or RA. Bone mineral density was measured using dual-energy DEXA (Lunar Radiation, Madison, WI, USA; and Delphi W; Hologic, Boston, MA, USA) for the whole body [20]. We used the mean bone mineral density of the whole body. All twins underwent bone mineral density measurements using the same DEXA machine at the same center. These devices were maintained using the standard quality control procedures recommended by the manufacturer to assure that the calibration of bone mineral density remained constant, and the coefficients of variation for bone mineral density measurement were 1.0% for the two machines. The results were expressed as T-scores. The T-score is a measure of deviation from the expected population mean value of the peak young adult bone mass [13]. Clinically, it is used to predict fracture risk. All DEXA data were expressed as mean ± standard deviation (SD).

The income group was divided into low income (<USD2000 per month), middle income (~USD2000–USD3999 per month), and high income (≥USD4000 per month) groups based on household income. Education was grouped as under high school, graduated from high school, dropped out of college, or graduated from college. Marital status was assessed as unmarried, married, divorced, or other. Physical activity levels were assessed using hard, moderate, walking, and sitting times. Physical activity was measured both at home and at the workplace. Body mass index was calculated as kg/m^2 using the health checkup data. Smoking history was classified as nonsmoker (<100 cigarettes in entire life), past smoker (quit more than 1 year ago), and current smoker. Drinking alcohol habits were categorized as non-drinkers, ≤1 time per month, 2–4 times per month, and ≥2 times per week. Sleep time was calculated as 5/7 weekdays plus 2/7 weekends.

2.4. Exposure

Monozygotic and dizygotic twins were considered independent variables in this study. All the participants were twins. There were no triplets, quadruplets, or higher-order multiples.

2.5. Outcomes

We calculated the coincidence of medical histories between matched twin participants. These data were categorized as positive–positive, positive–negative, or negative–negative.

Additionally, we calculated the absolute difference in the DEXA T-scores between the matched twin participants. For example, one of the twin participants had a T-score of 2, and the other had a T-score of 1; thus, the absolute difference in T-score was calculated as 1.

2.6. Statistical Analyses

A Chi-square test (categorical variables) or Wilcoxon rank sum test (continuous variables) was performed to compare the general characteristics of the participants between different patient groups.

We calculated the odds ratios (OR) with 95% confidence intervals (CI) of the coincidence of disease histories. First, we calculated the OR of monozygotic twins ([positive–positive or negative–negative]/[positive–negative]) compared to that of dizygotic twins using a binomial logistic regression model. Second, we calculated the OR of monozygotic twins ([positive–positive]/[positive–negative]/[negative–negative]/) compared with that of dizygotic twins using a multinomial logistic regression model.

We calculated the estimated values (EV) with a 95% CI of the absolute difference in the T-score. EV was measured as the absolute difference between monozygotic twins minus the absolute difference between dizygotic twins, using a linear regression model.

Crude, adjusted model 1 (age, sex, income, education, marital status, physical activity, obesity, smoking habit, frequency of alcohol consumption, and sleep time) and adjusted model 2 (model 1 plus history of each disease [osteoporosis, fracture by accident, fracture by falling, OA, and RA]) were used.

Two-tailed analyses were conducted and a p-value < 0.05 was considered statistically significant. The results were statistically analyzed using SPSS software (version 24.0; IBM, Armonk, NY, USA).

3. Results

The baseline features of the monozygotic and dizygotic twins are summarized in Table 1. The medical histories of osteoporosis, fractures by accident or falling, OA, and RA or DEXA T-scores were not dissimilar between monozygotic and dizygotic twins (all $p > 0.05$). The distribution of age groups, sex ratio, and hard-level physical activity showed differences between the two groups ($p = 0.003$, $p = 0.021$, and $p = 0.019$, respectively). Other components of household income, education, marriage, overall physical activity except for hard level, obesity, smoking, alcohol consumption, and sleeping hours did not differ between monozygotic and dizygotic twins (all $p > 0.05$).

Table 1. Baseline features of the monozygotic and dizygotic twins.

Characteristics	Total Participants		
	Monozygotic Twins	Dizygotic Twins	*p*-Value
Age (years, *n*, %)			0.003 *
20–24	6 (0.6)	0 (0)	
25–29	64 (6.2)	4 (1.7)	
30–34	352 (34.1)	85 (35.1)	
35–39	242 (23.4)	65 (26.9)	
40–44	139 (13.5)	36 (14.9)	
45–49	129 (12.5)	20 (8.3)	
50–54	82 (7.9)	22 (9.1)	
55–59	12 (1.2)	10 (4.1)	
60–64	4 (0.4)	0 (0)	
65+	2 (0.2)	0 (0)	
Sex (n, %)			0.021 *
Males	378 (36.6)	108 (44.6)	
Females	654 (63.4)	134 (55.4)	
Income (n, %)			0.959
<2 million (won)	342 (33.1)	81 (33.5)	
2 to <3 million (won)	276 (26.7)	68 (28.1)	
3 to <4 million (won)	210 (20.3)	48 (19.8)	
≥4 million (won)	204 (19.8)	45 (18.6)	
Education (n, %)			0.798
Under high school	119 (11.5)	25 (10.3)	
Graduated from high school	365 (35.4)	90 (37.2)	
Commercial college—Dropped out of college	121 (11.7)	32 (13.2)	
Graduated from college	427 (41.4)	95 (39.3)	
Marriage (n, %)			0.362
Unmarried	242 (23.4)	50 (20.7)	
Married	723 (70.1)	171 (70.7)	
Divorced or others	67 (6.5)	21 (8.7)	
Physical Activity			
Hard (hour/1 week, mean, SD)	3.1 (6.8)	4.6 (9.7)	0.019 *
Moderate (hour/1 week, mean, SD)	5.8 (10.5)	6.2 (10.2)	0.666
Walk (hour/1 week, mean, SD)	6.1 (9.6)	6.9 (10.9)	0.287
Sit (hour/1 week, mean, SD)	40.2 (22)	37.8 (20.8)	0.118
Obesity (n, %)			0.232
Underweight (BMI < 18.5)	27 (2.6)	5 (2.1)	
Normal (BMI ≥ 18.5 to <23)	497 (48.2)	112 (46.3)	
Overweight (BMI 23 to <25)	217 (21)	67 (27.7)	
Obese I (BMI ≥ 25 to <30)	259 (25.1)	52 (21.5)	
Obese II (BMI ≥ 30)	32 (3.1)	6 (2.5)	

Table 1. *Cont.*

Characteristics	Total Participants		
	Monozygotic Twins	**Dizygotic Twins**	***p*-Value**
Smoking status (n, %)			0.172
Nonsmoker	678 (65.7)	145 (59.9)	
Past smoker	106 (10.3)	33 (13.6)	
Current smoker	248 (24)	64 (26.4)	
Frequency of drinking alcohol (n, %)			0.360
Nondrinker	302 (29.3)	64 (26.4)	
≤1 time per month	232 (22.5)	46 (19)	
2–4 times per month	295 (28.6)	79 (32.6)	
≥2 times per week	203 (19.7)	53 (21.9)	
Sleeping hours (n, %)			0.367
≤5 h	53 (5.1)	16 (6.6)	
6–7 h	610 (58.7)	146 (59.8)	
8–9 h	349 (33.6)	72 (29.5)	
≥10 h	28 (2.7)	10 (4.1)	
Osteoporosis (n, %)	27 (2.6)	4 (1.7)	0.491
Fracture by accident (n, %)	80 (7.8)	25 (10.3)	0.189
Fracture by falling down (n, %)	128 (12.4)	23 (9.5)	0.226
Osteoarthritis (n, %)	37 (3.6)	7 (2.9)	0.699
Rheumatoid arthritis (n, %)	20 (1.9)	3 (1.2)	0.598
DEXA T-score (mean, SD)	0 (1.6)	0 (1.8)	0.932

* Significance at $p < 0.05$. Chi-square test (categorical variables) or Wilcoxon rank sum test (continuous variables) was performed. Abbreviation: BMI, body mass index; SD, standard deviation; DEXA, dual-energy X-ray absorptiometry.

With full adjustment analyses, we determined any possible associations of the concordance rates in terms of the presence or absence of osteoporosis, fractures by accident or by falling down, OA, and RA within monozygotic compared to those within dizygotic twins (Table 2). Crude or adjusted ORs for concordances of these diseases within monozygotic twins were not significantly higher than those within dizygotic twins (all $p > 0.05$). However, we observed a trend toward an increased likelihood of concordant association of fracture by falling in monozygotic twins with borderline significance (adjusted OR, 1.44 [95% CI, 0.97–2.15]; $p = 0.070$).

Table 2. Analysis of odds ratios with 95% confidence interval of coincidence of bone diseases in monozygotic twins compared to that in dizygotic twins (reference: positive/negative of diseases between twins).

Coincidence of Diseases	Monozygotic Twins	Dizygotic Twins	Odds Ratios (95% Confidence Interval)					
	***n* (%)**	***n* (%)**	**Crude**	***p***	**Model 1 ***	***p***	**Model 2 †**	***p***
Osteoporosis								
Concordant	998/1032 (96.7)	238/242 (98.3)	2.03 (0.71–5.77)	0.185	1.97 (0.60–6.41)	0.261	1.99 (0.60–6.58)	0.263
Discordant	34/1032 (3.3)	4/242 (1.7)	1		1		1	
Fracture by accident								
Concordant	888/1032 (86)	204/242 (84.3)	1.15 (0.78–1.69)	0.484	1.14 (0.74–1.75)	0.563	1.09 (0.71–1.68)	0.688
Discordant	144/1032 (14)	38/242 (15.7)	1		1			
Fracture by falling down								
Concordant	820/1032 (79.5)	204/242 (84.3)	1.39 (0.95–2.03)	0.089	1.45 (0.98–2.15)	0.066	1.44 (0.97–2.15)	0.070
Discordant	212/1032 (20.5)	38/242 (15.7)	1		1			
Osteoarthritis								
Concordant	966/1032 (93.6)	228/242 (94.2)	1.11 (0.61–2.02)	0.725	1.19 (0.59–2.41)	0.622	1.09 (0.54–2.19)	0.821
Discordant	66/1032 (6.4)	14/242 (5.8)	1		1		1	
Rheumatoid arthritis								
Concordant	998/1032 (96.7)	237/242 (97.9)	1.62 (0.63–4.17)	0.322	1.74 (0.63–4.79)	0.287	1.49 (0.53–4.20)	0.452
Discordant	34/1032 (3.3)	5/242 (2.1)	1		1		1	

* Adjusted for age, sex, income, education, marriage status, physical activity, obesity, smoking habit, frequency of drinking alcohol, and sleep time. † Model 1 plus histories of each disease (osteoporosis, fracture by accident, fracture by falling down, osteoarthritis, and rheumatoid arthritis).“Concordant” means concordant positive–positive or negative–negative result between monozygotic twins or between dizygotic twins, whereas “discordant” means discordant positive and negative results between monozygotic twins or between dizygotic twins.

We further investigated whether the occurrence of at least one incident disease (in terms of osteoporosis, fractures by an accident or falling down, OA, or RA) in monozygotic twin pairs was more frequent than that in dizygotic twin pairs (Table 3). The occurrence of fractures by accident in both monozygotic twin pairs showed lower odds than in dizygotic twin pairs after full adjustment (adjusted OR 0.25 [95% CI, 0.08–0.79]; $p = 0.018$). The occurrence of at least one other bone disease was not higher in monozygotic than in dizygotic twins (all $p > 0.05$).

Table 3. Analysis of odds ratios with 95% confidence interval of occurrence of at least one bone disease in monozygotic twins compared to that in dizygotic twins (reference: negative/negative of diseases between twins).

Coincidence of Diseases	Monozygotic Twins	Dizygotic Twins	Odds Ratios (95% Confidence Interval)					
	n (%)	*n* (%)	Crude	*p*	Model 1 †	*p*	Model 2 ‡	*p*
Osteoporosis								
Positive–positive	10/1032 (1)	2/242 (0.8)	1.19 (0.26–5.49)	0.819	N/A	N/A	N/A	N/A
Positive–negative	34/1032 (3.3)	4/242 (1.7)	2.03 (0.71–5.78)	0.184	1.94 (0.61–6.11)	0.260	1.81 (0.57–5.71)	0.315
Negative–negative	988/1032 (95.7)	236/242 (97.5)	1		1		1	
Fracture by accident								
Positive–positive	8/1032 (0.8)	6/242 (2.5)	0.30 (0.10–0.87)	0.027 *	0.29 (0.09–0.88)	0.029 *	0.25 (0.08–0.79)	0.018 *
Positive–negative	144/1032 (14)	38/242 (15.7)	0.85 (0.58–1.26)	0.422	0.99 (0.65–1.49)	0.946	0.95 (0.63–1.44)	0.809
Negative–negative	880/1032 (85.3)	198/242 (81.8)	1		1		1	
Fracture by falling down								
Positive–positive	22/1032 (2.1)	4/242 (1.7)	1.38 (0.47–4.05)	0.559	1.67(0.53–5.26)	0.380	1.68 (0.52–5.43)	0.390
Positive–negative	212/1032 (20.5)	38/242 (15.7)	1.40 (0.96–2.04)	0.083	1.42 (0.96–2.09)	0.077	1.41 (0.95–2.08)	0.085
Negative–negative	798/1032 (77.3)	200/242 (82.6)	1		1		1	
Osteoarthritis								
Positive–positive	4/1032 (0.4)	0/242 (0)	N/A	N/A	N/A	N/A	N/A	N/A
Positive–negative	66/1032 (6.4)	14/242 (5.8)	1.12 (0.62–2.03)	0.720	1.18 (0.61–2.26)	0.625	1.10 (0.57–2.11)	0.781
Negative–negative	962/1032 (93.2)	228/242 (94.2)	1		1		1	
Rheumatoid arthritis								
Positive–positive	2/1032 (0.2)	0/242 (0)	N/A	N/A	N/A	N/A	N/A	N/A
Positive–negative	34/1032 (3.3)	5/242 (2.1)	1.62 (0.63–4.18)	0.320	1.76 (0.67–4.64)	0.251	1.52 (0.57–4.05)	0.404
Negative–negative	996/1032 (96.5)	237/242 (97.9)	1		1		1	

* Significance at $p < 0.05$. † Adjusted for age, sex, income, education, marriage status, physical activity, obesity, smoking habit, frequency of drinking alcohol, and sleep time. ‡ Model 1 plus histories of each disease (osteoporosis, fracture by accident, fracture by falling down, osteoarthritis, and rheumatoid arthritis).

We calculated differences in DEXA T-scores between the matched monozygotic and dizygotic twin pairs to analyze the EV (Table 4). The difference in the DEXA T-score was significantly higher within dizygotic than within monozygotic twins (EV, 0.62 [95% CI, 0.45–0.79]; $p < 0.001$), indicating a similar tendency within monozygotic twins.

Table 4. Analysis of estimated values of absolute value of difference between the matched twins (reference: absolute value of difference between monozygotic twins).

Difference of Clinical Examination	Monozygotic Twins	Dizygotic Twins	Estimated Values of Absolute Difference between Twin (95% CI)					
	Mean (SD)	Mean (SD)	Crude	*p*	Model 1 †	*p*	Model 2 ‡	*p*
Difference in DEXA T-score	0.5 (1.1)	1.1 (1.7)	0.64 (0.47–0.81)	<0.001 *	0.62 (0.45–0.79)	<0.001 *	0.62 (0.45–0.79)	<0.001 *

Abbreviation: DEXA, dual-energy X-ray absorptiometry; 95% CI, 95% confidence interval; SD, standard deviation. * Significance at $p < 0.05$. † Adjusted for age, sex, income, education, marriage status, physical activity, obesity, smoking habit, frequency of drinking alcohol, and sleep time. ‡ Model 1 plus histories of each disease (osteoporosis, fracture by accident, fracture by falling down, osteoarthritis, and rheumatoid arthritis).

4. Discussion

In this cross-sectional study based on validated twin cohorts, we could not identify any significant concordances of age-related bone and joint diseases or any increased likelihood of osteoporosis, fractures, OA, or RA within monozygotic twins compared to those within dizygotic twins through concurrent adjustments for comprehensive confounding (e.g., lifestyle and socioeconomic) factors. Nonetheless, the relationship between DEXA T-scores and bone density changes indicated a highly comparable trend within monozygotic twins compared to that within dizygotic twin pairs. Owing to the rarity of qualified twin

cohort data, our current epidemiological study might promote the understanding of the environmental influence on genetic contribution in overt osteoporosis, fractures, OA, or RA, which are critical global issues and a healthcare burden in an aging society.

The combined analysis of osteoporosis, fractures, OA, and RA with full adjustments for lifestyle and environmental factors has not been conducted in twin studies. We found that there were no higher concordances of incident osteoporosis or fractures, OA, or RA within monozygotic twins compared to those within dizygotic twins. However, a trend toward concordant association of fracture by falling in monozygotic twins with borderline significance was observed: the occurrence of fractures by accident in both monozygotic twin pairs showed lower odds than that in dizygotic twin pairs. Because of the relatively minor excess in concordance in monozygotic twins compared with that in dizygotic twins, these findings might carefully indicate the low genetic influence on liability to fracture risk, consistent with a previous twin study [22,35]. Anatomically identical stress fractures are repeatedly described in monozygotic twins [35–37]. However, the indistinct or vague concordance in the phenotype of these diseases within monozygotic twins in the present study might be attributed to the possible contribution of environmental factors relevant to dissimilar acquired lifestyle behaviors between monozygotic twin pairs who share an identical genetic background, sex, and age [9]. Molecular studies, including epigenome-wide association studies, might provide one of the possible explanations for the discordant disease expressions in monozygotic twins, indicating the complex interplay between genetic, environmental, and epigenetic influences on osteoporosis, fractures, OA, and RA [12,38,39]. While twins are epigenetically indistinguishable during the early phase of life, older monozygotic twins harbor prominent disparities in the genomic distribution of 5-methylcytosine DNA and histone acetylation [7], which might lead to differential gene expression signatures and disease phenotypes with age [40]. Similarly, one prospective study on Finnish twins indicated that environmental factors are more likely to make an adult population susceptible to fractures [23]. Genetic variations related to osteoporosis and fracture may depend on fracture type and age [41]. The overall age-adjusted fracture accounted for a genetic contribution of <20% [41]. Since exclusively adult populations were included in this study, different environmental or acquired influences might lessen the concordance likelihood of overt bone and joint diseases in monozygotic twin pairs compared to those in dizygotic twin pairs.

Although bone mineral density is associated with the risk of osteoporosis, fractures, OA, and RA, a comparative study using the DEXA T-score in these bone and joint diseases has not been assessed in twin studies. The clinical significance of baseline radiologic parameters, such as the DEXA T-score, appears to be underestimated in the evaluation of genetic contribution when comparing twin pairs. In our study, despite the discordance of disease expression within monozygotic twins, we have shown a highly similar tendency of DEXA-T-scores between monozygotic twins compared to that between dizygotic twins (EV = 0.62, 95% CI, 0.45–0.79, $p < 0.001$), which seems to suggest the relative importance of genetic contribution in bone mineral density at the subclinical level over environmental factors. Our findings support the results of a previous Korean twin study indicating the high heritability of bone mineral density [20]. Furthermore, the present results are also in line with those of several European population-twin studies that have shown fewer differences in bone mass between monozygotic and dizygotic twins, suggesting that there may be substantial heritability (50–85%) and genetic traits for bone mineral density [26,27]. Genome-wide association studies have revealed several genetic polymorphisms associated with bone mineral density, osteoporosis, and fracture [8]. However, these genotype determinations have failed to identify individuals at an increased risk of osteoporosis [42], which might imply additional triggers mediated by extrinsic factors. Gene–environment interactions, such as smoking, alcohol consumption, diet, physical activity, and epigenetic differences, might result in discordance in disease expression between monozygotic twin pairs [27]. Thus, the relative genetic and environmental significance of bone mineral density

using DEXA T-scores seems to be comparable with those of previous twin studies mainly based on European populations [26,27].

The strength of this study was based on prospective twin cohort data with follow-up data composed of both monozygotic and dizygotic twins, KoGES HTS, with qualified data quality regularly validated by national statisticians, which made our findings more reliable. We comprehensively considered potential confounders of lifestyle factors, including physical activity, obesity, smoking, alcohol consumption, sleep duration, and socioeconomic status, including income level, education level, and marital status, comparing twin pairs. Adjustments for lifestyle factors (income, alcohol consumption, smoking status, physical activity, marital status, and obesity) may be additional strengths because they were listed as relevant risk factors for bone and joint diseases [43]. To the best of our knowledge, this study is the first combined analysis covering osteoporosis, fracture, OA, and RA with full adjustment for comprehensive lifestyle and socioeconomic factors.

Our study has some limitations that should be addressed. First, even though substantial variables were adjusted in this study, unmeasured confounders may remain, and they could not be completely excluded. Second, the causal relationship between twins and bone and joint diseases could not be confirmed by a cross-sectional study design. Third, the relatively small number of concordant diseases between both twin pairs may be limited in this study, despite a large number of twin participants with bone and joint diseases. Fourth, the absence of genetic data on related bone and joint diseases or no information on diet may be another limitation. Finally, we did not calculate the bone mineral density from the thoracic and lumbar spine, which is a common site of fractures due to osteoporosis.

5. Conclusions

In conclusion, while the relative importance of genetic factors in bone mineral density may be sustained between monozygotic twins, the disease expression of osteoporosis, fractures, OA, or RA might be affected by environmental factors. Our results provide supportive evidence that common bone and joint diseases may be preventable.

Author Contributions: M.J.K., investigation, funding acquisition, writing—original draft, review and editing; H.G.C. and S.Y.K., funding acquisition, project administration; H.S.K., H.L., S.J.C. and E.S.N., methodology; J.-H.K., J.H.K. and Y.C., formal analysis; K.W.M. and H.Y.P., software; B.C.K. and N.Y.K., validation. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation (NRF) of Korea from the Korean Ministry of Science and ICT, grant No. NRF-2021-R1C1C1004986 to H.G.C. and grant No. NRF-2022R1F1A1065335 to M.J.K. The APC was funded by NRF-2022R1F1A1065335.

Institutional Review Board Statement: The ethics committee of Hallym University (2021-03-004) approved the use of these data. The requirement for written informed consent was waived by the Institutional Review Board.

Informed Consent Statement: Patient consent was waived due to the fact that the study utilized secondary data.

Data Availability Statement: Restrictions apply to the availability of these data. Data were obtained from the Korean Genome and Epidemiology Study (KoGES) and are available at https://www.nih.go.kr/contents.es?mid=a50401010100#1 (accessed on 1 January 2022).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lee, S.-G.; Park, Y.-E.; Park, S.-H.; Kim, T.-K.; Choi, H.-J.; Lee, S.-J.; Kim, S.-I.; Lee, S.-H.; Kim, G.-T.; Lee, J.-W.; et al. Increased frequency of osteoporosis and BMD below the expected range for age among South Korean women with rheumatoid arthritis. *Int. J. Rheum. Dis.* **2012**, *15*, 289–296. [CrossRef] [PubMed]
2. Cauley, J.A.; Fullman, R.L.; Stone, K.L.; Zmuda, J.M.; Bauer, D.C.; Barrett-Connor, E.; Ensrud, K.; Lau, E.M.C.; Orwoll, E.S. Factors associated with the lumbar spine and proximal femur bone mineral density in older men. *Osteoporos. Int.* **2005**, *16*, 1525–1537. [CrossRef] [PubMed]
3. Vestergaard, P.; Rejnmark, L.; Mosekilde, L. Osteoarthritis and Risk of Fractures. *Calcif. Tissue Int.* **2009**, *84*, 249–256. [CrossRef] [PubMed]
4. Choi, H.G.; Kang, H.S.; Lim, H.; Kim, J.-H.; Kim, J.H.; Cho, S.-J.; Nam, E.S.; Min, K.-W.; Park, H.Y.; Kim, N.Y.; et al. Potential Cancer Risk in Patients with Rheumatoid Arthritis: A Longitudinal Korean Population-Based Analysis. *J. Pers. Med.* **2022**, *12*, 965. [CrossRef] [PubMed]
5. Kwon, M.J.; Kim, J.-H.; Kim, J.H.; Park, H.-R.; Kim, N.Y.; Hong, S.; Choi, H.G. Incident Rheumatoid Arthritis Following Statin Use: From the View of a National Cohort Study in Korea. *J. Pers. Med.* **2022**, *12*, 559. [CrossRef]
6. Terao, C.; Ikari, K.; Nakayamada, S.; Takahashi, Y.; Yamada, R.; Ohmura, K.; Hashimoto, M.; Furu, M.; Ito, H.; Fujii, T.; et al. A twin study of rheumatoid arthritis in the Japanese population. *Mod. Rheumatol.* **2016**, *26*, 685–689. [CrossRef]
7. Vogt, G.; Huber, M.; Thiemann, M.; Van Den Boogaart, G.; Schmitz, O.J.; Schubart, C.D. Production of different phenotypes from the same genotype in the same environment by developmental variation. *J. Exp. Biol.* **2008**, *211*, 510–523. [CrossRef]
8. Bathum, L.; Hjelmborg, J.V.B.; Christiansen, L.; Madsen, J.S.; Skytthe, A.; Christensen, K. Evidence for an association of methylene tetrahydrofolate reductase polymorphism C677T and an increased risk of fractures: Results from a population-based Danish twin study. *Osteoporos. Int.* **2004**, *15*, 659–664. [CrossRef]
9. Moayyeri, A.; Hart, D.J.; Snieder, H.; Hammond, C.J.; Spector, T.D.; Steves, C.J. Aging Trajectories in Different Body Systems Share Common Environmental Etiology: The Healthy Aging Twin Study (HATS). *Twin Res. Hum. Genet.* **2016**, *19*, 27–34. [CrossRef]
10. Lopes, K.G.; Rodrigues, E.L.; Lopes, M.R.d.S.; Nascimento, V.A.D.; Pott, A.; Guimarães, R.D.C.A.; Pegolo, G.E.; Freitas, K.D.C. Adiposity Metabolic Consequences for Adolescent Bone Health. *Nutrients* **2022**, *14*, 3260. [CrossRef]
11. Koppula, S.; Akther, M.; Haque, E.; Kopalli, S.R. Potential Nutrients from Natural and Synthetic Sources Targeting Inflammaging—A Review of Literature, Clinical Data and Patents. *Nutrients* **2021**, *13*, 4058. [CrossRef]
12. Svendsen, A.J.; Kyvik, K.O.; Houen, G.; Junker, P.; Christensen, K.; Christiansen, L.; Nielsen, C.; Skytthe, A.; Hjelmborg, J. On the Origin of Rheumatoid Arthritis: The Impact of Environment and Genes—A Population Based Twin Study. *PLoS ONE* **2013**, *8*, e57304. [CrossRef]
13. Li, X.; Chen, S.; Kantorovich, V.; Cheng, L.S.; Cohn, D.H.; Rotter, J.I.; Yang, H.; Adams, J.S. Genetic determinants of osteoporosis susceptibility in a female Ashkenazi Jewish population. *Genet. Med.* **2004**, *6*, 33–37. [CrossRef]
14. Spector, T.D.; Cicuttini, F.; Baker, J.; Loughlin, J.; Hart, D. Genetic influences on osteoarthritis in women: A twin study. *BMJ* **1996**, *312*, 940–943. [CrossRef]
15. Kim, S.Y.; Yoo, D.M.; Kwon, M.J.; Kim, J.H.; Kim, J.-H.; Byun, S.-H.; Park, B.; Lee, H.-J.; Choi, H.G. Increased Risk of Temporomandibular Joint Disorder in Osteoporosis Patients: A Longitudinal Study. *Front. Endocrinol.* **2022**, *13*, 835923. [CrossRef]
16. Park, C.; Ha, Y.-C.; Jang, S.; Jang, S.; Yoon, H.-K.; Lee, Y.-K. The incidence and residual lifetime risk of osteoporosis-related fractures in Korea. *J. Bone Miner. Metab.* **2011**, *29*, 744–751. [CrossRef]
17. Clayton, E.S.; Hochberg, M.C. Osteoporosis and Osteoarthritis, Rheumatoid Arthritis and Spondylarthropathies. *Curr. Osteoporos. Rep.* **2013**, *11*, 257–262. [CrossRef]
18. Van der Weijden, M.A.C.; Claushuis, T.A.M.; Nazari, T.; Lems, W.F.; Dijkmans, B.A.C.; van der Horst-Bruinsma, I.E. High prevalence of low bone mineral density in patients within 10 years of onset of ankylosing spondylitis: A systematic review. *Clin. Rheumatol.* **2012**, *31*, 1529–1535. [CrossRef]
19. Hawkes, C.H. Twin studies in diabetes mellitus. *Diabet. Med.* **1997**, *14*, 347–352. [CrossRef]
20. Park, J.-H.; Song, Y.-M.; Sung, J.; Lee, K.; Kim, Y.S.; Park, Y.S. Genetic influence on bone mineral density in Korean twins and families: The healthy twin study. *Osteoporos. Int.* **2012**, *23*, 1343–1349. [CrossRef]
21. Piroska, M.; Tarnoki, D.; Szabo, H.; Jokkel, Z.; Meszaros, S.; Horvath, C.; Tarnoki, A. Strong Genetic Effects on Bone Mineral Density in Multiple Locations with Two Different Techniques: Results from a Cross-Sectional Twin Study. *Medicina* **2021**, *57*, 248. [CrossRef]
22. MacGregor, A.J.; Snieder, H.; Spector, T.D. Genetic factors and osteoporotic fractures in elderly people. *BMJ* **2000**, *320*, 1669. [CrossRef]
23. Kannus, P.; Palvanen, M.; Kaprio, J.; Parkkari, J.; Koskenvuo, M. Genetic factors and osteoporotic fractures in elderly people: Prospective 25 year follow up of a nationwide cohort of elderly Finnish twins. *BMJ* **1999**, *319*, 1334–1337. [CrossRef]
24. Pajala, S.; Era, P.; Koskenvuo, M.; Kaprio, J.; Viljanen, A.; Rantanen, T. Genetic Factors and Susceptibility to Falls in Older Women. *J. Am. Geriatr. Soc.* **2006**, *54*, 613–618. [CrossRef]
25. Dato, S.; Montesanto, A.; Lagani, V.; Jeune, B.; Christensen, K.; Passarino, G. Frailty phenotypes in the elderly based on cluster analysis: A longitudinal study of two Danish cohorts. Evidence for a genetic influence on frailty. *AGE* **2012**, *34*, 571–582. [CrossRef]
26. Peacock, M.; Turner, C.H.; Econs, M.; Foroud, T. Genetics of Osteoporosis. *Endocr. Rev.* **2002**, *23*, 303–326. [CrossRef]

27. Pocock, N.A.; Eisman, J.A.; Hopper, J.L.; Yeates, M.G.; Sambrook, P.N.; Eberl, S. Genetic determinants of bone mass in adults. A twin study. *J. Clin. Investig.* **1987**, *80*, 706–710. [CrossRef]
28. Speed, D.; Hemani, G.; Johnson, M.R.; Balding, D.J. Improved Heritability Estimation from Genome-wide SNPs. *Am. J. Hum. Genet.* **2012**, *91*, 1011–1021. [CrossRef]
29. Sung, J.; Cho, S.-I.; Lee, K.; Ha, M.; Choi, E.-Y.; Choi, J.-S.; Kim, H.; Kim, J.; Hong, K.S.; Kim, Y.; et al. Healthy Twin: A Twin-Family Study of Korea—Protocols and Current Status. *Twin Res. Hum. Genet.* **2006**, *9*, 844–848. [CrossRef]
30. Sung, J.; Lee, K.; Song, Y.-M.; Lee, M.K.; Lee, D.-H. Heritability of Eating Behavior Assessed Using the DEBQ (Dutch Eating Behavior Questionnaire) and Weight-related Traits: The Healthy Twin Study. *Obesity* **2010**, *18*, 1000–1005. [CrossRef] [PubMed]
31. Song, Y.-M.; Lee, D.-H.; Lee, M.K.; Lee, K.; Lee, H.J.; Hong, E.J.; Han, B.; Sung, J. Validity of the Zygosity Questionnaire and Characteristics of Zygosity-Misdiagnosed Twin Pairs in the Healthy Twin Study of Korea. *Twin Res. Hum. Genet.* **2010**, *13*, 223–230. [CrossRef] [PubMed]
32. Kim, Y.; Han, B.-G.; The KoGES Group. Cohort Profile: The Korean Genome and Epidemiology Study (KoGES) Consortium. *Int. J. Epidemiol.* **2017**, *46*, 1350. [CrossRef] [PubMed]
33. Kim, S.Y.; Yoo, D.M.; Kwon, M.J.; Kim, J.H.; Kim, J.-H.; Bang, W.J.; Choi, H.G. Comparison of the Differences in State-Trait Anxiety Inventory Scores and Insomnia Histories between Monozygotic and Dizygotic Twins: A Cross-Sectional Study Using KoGES HTS Data. *J. Clin. Med.* **2022**, *11*, 4011. [CrossRef] [PubMed]
34. Sung, J.; Lee, K.; Song, Y.-M. Heritabilities of Alcohol Use Disorders Identification Test (AUDIT) scores and alcohol biomarkers in Koreans: The KoGES (Korean Genome Epi Study) and Healthy Twin Study. *Drug Alcohol Depend.* **2011**, *113*, 104–109. [CrossRef]
35. Beamish, A.J.; Roberts, G.L.; Cnudde, P. A case of patellar fractures in monozygotic twin gymnasts. *Sports Med. Arthrosc. Rehabil. Ther. Technol.* **2012**, *4*, 20. [CrossRef]
36. Van Meensel, A.-S.; Peers, K. Navicular stress fracture in high-performing twin brothers: A case report. *Acta Orthop. Belg.* **2010**, *76*, 407.
37. Singer, A.; Ben-Yehuda, O.; Ben-Ezra, Z.; Zaltzman, S. Multiple identical stress fractures in monozygotic twins. Case report. *J. Bone Jt. Surg.* **1990**, *72*, 444–445. [CrossRef]
38. Ceribelli, A.; Selmi, C. Epigenetic Methods and Twin Studies. *Adv. Exp. Med. Biol.* **2020**, *1253*, 95–104. [CrossRef]
39. Carry, P.M.; Terhune, E.A.; Trahan, G.D.; Vanderlinden, L.A.; Wethey, C.I.; Ebrahimi, P.; McGuigan, F.; Åkesson, K.; Hadley-Miller, N. Severity of Idiopathic Scoliosis Is Associated with Differential Methylation: An Epigenome-Wide Association Study of Monozygotic Twins with Idiopathic Scoliosis. *Genes* **2021**, *12*, 1191. [CrossRef]
40. Fraga, M.F.; Ballestar, E.; Paz, M.F.; Ropero, S.; Setien, F.; Ballestar, M.L.; Heine-Suñer, D.; Cigudosa, J.C.; Urioste, M.; Benitez, J.; et al. From The Cover: Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10604–10609. [CrossRef]
41. Michaëlsson, K.; Melhus, H.; Ferm, H.; Ahlbom, A.; Pedersen, N.L. Genetic Liability to Fractures in the Elderly. *Arch. Intern. Med.* **2005**, *165*, 1825–1830. [CrossRef]
42. Prentice, A. The relative contribution of diet and genotype to bone development. *Proc. Nutr. Soc.* **2001**, *60*, 45–52. [CrossRef]
43. An, S.; Ahn, C.; Jang, J.; Lee, J.; Kang, D.; Lee, J.-K.; Park, S.K. Comparison of the Prevalence of Cardiometabolic Disorders and Comorbidities in Korea and the United States: Analysis of the National Health and Nutrition Examination Survey. *J. Korean Med. Sci.* **2022**, *37*, e149. [CrossRef]

nutrients

MDPI

Article

Friedelin Alleviates the Pathogenesis of Collagenase-Induced Tendinopathy in Mice by Promoting the Selective Autophagic Degradation of p65

Huaji Jiang [1,2,†], Xuemei Lin [3,†], Wei Liang [2], Yiqiang Li [3,*] and Xiao Yu [1,4,*]

[1] Department of Immunology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China; gukejianghuaji@163.com
[2] Department of Orthopaedics, Yuebei People's Hospital Affiliated to Medical College of Shantou University, Shaoguan 512026, China; beiji3feng@163.com
[3] Department of Pediatric Orthopedics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, China; lynn_xm@163.com
[4] Guangdong Provincial Key Lab of Single Cell Technology and Application, Southern Medical University, Guangzhou 510515, China
* Correspondence: liyiq@gwcmc.org (Y.L.); xiaoyu523@smu.edu.cn (X.Y.)
† These authors contributed equally to this work.

Abstract: With the development of an aging population, tendinopathy has become a common musculoskeletal disease in the elderly with a high recurrence rate and no curative treatment. The inflammation mediated by NF-κB signaling plays an important role in tendon senescence and degeneration. Friedelin (FR) is a triterpenoid derived from green plants, which has a variety of pharmacological functions, such as analgesia, anti-inflammation, antioxidation, and anti-tumor functions. However, the role and mechanism of FR in tendinopathy are unclear. Here, we found that FR improved the mechanical strength of the Achilles tendon, restored the orderly arrangement of collagen fibers, reduced inflammatory cell infiltration, and promoted tenogenesis, thereby blocking the progression of tendinopathy. Mechanistically, FR promoted the autophagic degradation of p65 by enhancing the interaction between p62 and p65 and effectively inhibited the activation of the NF-κB pathway, thus alleviating the inflammatory response of tenocytes. In addition, FR recruited E3 ubiquitin enzyme RNF182 to increase the K48-linked ubiquitination of p65 and promoted p62-mediated autophagic degradation. Furthermore, blocking ubiquitination reversed the degradation of p65 by FR. Therefore, these findings identify the new pharmacological mechanism of the anti-inflammatory effect of FR and provide a new candidate drug for the treatment of tendinopathy.

Keywords: tendinopathy; friedelin; autophagic degradation; p65; ubiquitination

Citation: Jiang, H.; Lin, X.; Liang, W.; Li, Y.; Yu, X. Friedelin Alleviates the Pathogenesis of Collagenase-Induced Tendinopathy in Mice by Promoting the Selective Autophagic Degradation of p65. *Nutrients* **2022**, *14*, 1673. https://doi.org/10.3390/nu14081673

Academic Editor: Bradley S. Ferguson

Received: 11 February 2022
Accepted: 13 April 2022
Published: 18 April 2022

1. Introduction

Tendinopathy, a degenerative disease of a tendon, accounts for about 1/3 of musculoskeletal disorders [1,2]. With the development of society and the aging of the population, the incidence of tendinopathy is increasing year by year [3–5]. In terms of the incidence group, tendinopathy tends to occur in the elderly [6,7], and epidemiological investigation shows that the incidence of tendon injury in the elderly is significantly higher than in the young [8,9]. Tendinopathy is common in the Achilles tendon, patellar tendon, and rotator cuff tendons [10] and often presents with localized pain and mobility impairment. Long-term chronic tendinopathy increases the risk of tendon rupture, which can lead to disability in severe cases [11–13]. Currently, tendinopathy is mainly treated with conservative therapy, including non-steroidal anti-inflammatory drugs, shockwave therapy, ultrasound-guided percutaneous electrolysis, and exercise rehabilitation therapy [14,15]. However, these treatments can only relieve symptoms but not stop the progression of tendinopathy. Furthermore, non-steroidal anti-inflammatory drugs have many complications, such as

peptic ulcers, cardiovascular disease, and renal impairment [16]. Patients with tendon rupture or giant tendon calcification usually need surgical treatment. However, the surgical trauma is significant, and the recurrence rate is high (40%) [2], causing a heavy blow to the patient's body and mind. Therefore, it is urgent to explore novel potential therapies for tendinopathy.

It is currently believed that tendinopathy begins after an acute reactive tendon injury, with subsequent disordered or poor repair progressing to chronic tendinopathy [17]. The etiology is complex and multifactorial, and aging is one of the important risk factors of tendinopathy [18,19], especially when the immune system undergoes complex remodeling with age, in which senescent cells are more prone to developing chronic inflammatory responses. In addition, decreased immune function in elderly patients is associated with disturbances in the gut microbiota, which may be related to pain in bone and joint diseases [20,21]. Recent studies have shown that inflammation plays a key role in the aging of tendons [22–24]. Inflammatory mediators (IL-1β, IL-6 and TNF-α) have been reported to be highly expressed in tendon diseases and accelerate the progression of tendinopathy [22,23]. Meanwhile, inflammatory mediators can also induce tendon fibrosis and reduce the mechanical properties of tendons [25]. In addition, inflammatory factors inhibit the tenogenic differentiation of tendon-derived stem cells, thus preventing tendon healing [25]. Several studies have also shown that the classical inflammatory pathway NF-κB signaling is activated during the pathogenesis of tendon senescence [24,26,27]. While inhibiting the NF-κB pathway reduces the release of pro-inflammatory mediators, tendinopathy progression can be delayed [27]. Therefore, targeting the NF-κB pathway to inhibit inflammation could be a feasible and effective method for treating tendinopathy.

Friedelin (FR) is a triterpenoid compound existing in many plants, which can be derived from Aristotelia chilensis leaves (Elaeocarpaceae), Cannabis roots, and Maytenus ilicifolia leaves [28]. Especially, FR is most abundant in the cork of trees [29]. FR has a wide range of biological activities, such as anti-inflammatory [30], analgesic, antioxidant [31], antitumor [32], and antibacterial effects [33]. Thus, it can be used to treat a variety of diseases, such as ulcerative colitis [28], gastric ulcer [34], and leukemia. Compared with non-steroidal anti-inflammatory drugs, the gastric protective function of FR has significant advantages [34]. Importantly, in the acute toxicity test in rats, FR showed good drug safety performance, and the maximum dosage was up to 80 mg/kg [34].

In this study, we found that FR ameliorated the structural disorder of the Achilles tendon, improved the biomechanical properties of the Achilles tendon, and attenuated inflammatory infiltration, thereby delaying the progression of tendinopathy in mice. Mechanistically, FR increases K48-linked ubiquitination of p65 by recruiting RNF182, promotes the binding of p62 and p65, and subsequently accelerates autophagic degradation of p65, thus inhibiting the NF-κB signaling pathway to reduce the tendon's inflammatory response. Therefore, this study identifies that FR could be a potential drug for the prevention and treatment of tendinopathy.

2. Materials and Methods

2.1. Reagents and Antibodies

Reagents and antibodies in this study are shown in Table 1.

Table 1. The resource of Reagents and Antibodies.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-F4/80	ABclonal	A18637
Anti-IL-6	Cell Signaling Technology	#12912
Anti-p-IKK	Cell Signaling Technology	#2697
Anti-IκBa	Cell Signaling Technology	#4814

Table 1. *Cont.*

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-p-p65	Cell Signaling Technology	#3039
Anti-p65	Santa Cruz Biotechnology	sc-8008
Anti-Actin	ABclonal	AC026
Anti-Flag agarose gels	Sigma-Aldrich	A2220
Horseradish peroxidase (HRP)-anti-Flag (M2)	Sigma-Aldrich	A8592
Anti-HA	Roche Applied Science	12013819001
Anti-Beclin1	Cell Signaling Technology	#3738
Anti-ATG5	Cell Signaling Technology	#12994
Anti-p62	Santa Cruz Biotechnology	sc-48402
Anti-Ub	Santa Cruz Biotechnology	sc-8017
Anti-K48 Ub	ABclonal	A3606
Anti-RNF182	Novus Biologicals	NBP1-82707
Anti-ING4	ABclonal	A5833
Anti-PPARγ	ABclonal	A11183
Goat anti-rabbit IgG (H&L)	Beijing Ray Antibody Biotech	RM3002
Goat anti-mouse IgG (H&L)	Beijing Ray Antibody Biotech	RM3001
Goat anti-rabbit IgG (H&L) Alexa Fluor 488	Immunoway	RS23220
Goat anti-rabbit IgG (H&L) Alexa Fluor 594	Immunoway	RS23420
Anti-IL-1β (ELISA)	Elabscience Biotechnology	#E-EL-M0037c
Anti-IL-6 (ELISA)	Elabscience Biotechnology	#E-EL-M0044c
Anti-TNF-α (ELISA)	Elabscience Biotechnology	#E-EL-M1084c
Reagents		
Friedelin	MedChemExpress	HY-N4110
LPS	Sigma-Aldrich	L2630
Recombinant IL-1β	Abcam	ab9723
Recombinant TNFα	Biovision	1051-1000
MG-132	Sigma-Aldrich	C-2211
Chloroquine	Glpbio	1954/5/7
Cycloheximide	Sigma-Aldrich	C7698
3-methyladenine	Sigma-Aldrich	5142-23-4
MLN7243	Sigma-Aldrich	HY-100487
DMSO	Sigma-Aldrich	D2650
Paraformaldehyde	Sigma-Aldrich	P6148
Lipofectamine 2000	ThermoFisher	11668019
Protein G beads	GenScript	L00209
Type I collagenase	Wako	031-17601
Hematoxylin and eosin solution	Beyotime	C0105S
Masson's trichrome kit	Solarbio	G1340
TRIZOL	Beyotime	R0016
Fetal bovine serum	Sigma-Aldrich	12103C
Penicillin/streptomycin	Gibco	15140122
Amphotericin B	Gibco	15290026
Trypsinization	Gibco	R001100
Cell Counting Kit-8	Beyotime	C0037
Lipofectamine 2000	Invitrogen	11668030
Lipofectamine RNAiMAX	Invitrogen	13778150
Enhanced chemiluminescence kit	Cell Signaling Technology	#12630

2.2. Animals and Treatment

This type of study belongs to basic experimental research. Eight-week-old C57BL/6 male mice were purchased from the Animal Center of Guangdong, Guangzhou, China. Care and use of all animals conformed to the guidelines set forth by the Chinese National Institutes of Health. Ethical approval for this study was obtained from the Medical Ethics Committee of the Medical College of Shantou University (No. SUMC2021-480). The

experimental mice were randomly divided into four groups: a sham group, a collagenase-induced tendinopathy (CIT) group, a CIT model + FR group, and an FR group. The model of CIT was induced as previously described [35]. In short, 20 μL type I collagenase (1%) was injected around the right Achilles tendon of mice. One week after establishing the CIT model, the mice were treated with corresponding treatments. Specifically, the CIT + FR group was treated with a local injection of FR (40 μM, 20 μL) near the right Achilles tendon in CIT mice. Mice in the sham group and CIT group were injected with the same dose of saline. FR group mice were treated with a local injection of FR (40 μM, 20 μL) near the right Achilles tendon of normal mice. Four weeks after treatment, mice were euthanized and sampled, and the right Achilles tendons connected with the tibia and calcaneus were obtained for subsequent experimental study.

2.3. Biomechanical Assay

We performed biomechanical testing as in a previous study [36]. Briefly, the collected Achilles tendon tissue (retaining the calcaneus and the lower 1/3 muscle of the triceps of the lower leg) was taken out for the biomechanical test. Then, the tensile test was carried out on the Instron 5943 dynamic and static test system of universal electronic materials (Instron Corporation, Canton, MA, USA). Both ends of the Achilles tendon were wrapped with saline gauze and then placed on the test apparatus fixture. After the operating parameters were entered, the biomechanical testing started.

2.4. Histological Assessments

The collected Achilles tendons were washed twice with PBS and then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 24 h. Next, the samples were dehydrated, paraffin-embedded, and finally sliced with a thickness of 5 μm. The protocol of hematoxylin and eosin (HE) staining was carried out with reference to the previous research method. Briefly, Achilles tendon sections were dewaxed and hydrated, followed by hematoxylin staining for 5 min. After washing with PBS, eosin staining was performed for 3 min, and finally dried and sealed for storage. We referenced the established histological scoring system to analyze the changes in total histological scores on HE-stained slides after treatment [37]. The score of the intact group was defined as 20 points.

The staining instructions for the commercial Masson's trichrome kit were followed for Masson staining. That is, Masson's trichrome staining is used to visualize the original high-intensity collagen (red) and the newly synthesized low-intensity collagen (blue) [38].

For immunofluorescence staining, the Achilles tendon slices were first dewaxed and rehydrated. After washing with PBS three times, the sections were subjected to antigen retrieval with sodium citrate solution, followed by blocking with goat serum for 1 h. Then, primary antibodies were added and incubated overnight at 4 °C. On the second day, sections were incubated with the second antibody in the dark for 1 h and finally preserved by DAPI sealing. The scanning tissue microscope obtained different histological images (Olympus BX51, Tokyo, Japan). All histological images were obtained randomly at least 3 times.

2.5. Quantitative Reverse Transcription PCR (qRT-PCR) Assay

Total RNA was extracted from tissues or cells by TRIZOL. Next, we use reverse transcriptase to generate complementary cDNA. Real-time PCR was carried out using the ABI Q6 analyzer using the SYBR GreenER qRT-PCR SuperMix Universal and specific primers. Quantification of the gene expressions was assessed by fold changes normalized to the housekeeping gene GAPDH. The primers used in this study are listed in Table 2.

2.6. Cell Culture and Treatment

We extracted tenocytes according to previously researched methods [27]. Briefly, mice were first euthanized. Next, the bilateral Achilles tendons of mice were isolated, and the adipose tissue and muscle tissue around the Achilles tendon were carefully removed.

After washing the Achilles tendon with PBS three times, the Achilles tendon was cut into pieces with ophthalmic scissors and placed in a DMEM medium. Subsequently, type I collagenase was added to the chopped Achilles tendon for digestion for 3 h. The digested tissue was passed through a 100 μm cell filter and then centrifuged at 400× *g* for 5 min. The supernatant was discarded, and the cells were suspended in a complete medium (DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B) and cultured in an incubator at 37 °C, 5% carbon dioxide and 95% humidity. The medium was changed every three days, and the cells were passaged after trypsinization. When the cells were cultured to the third passage, they were used for subsequent experimental research.

Table 2. Primers for real-time RT-PCR used in this study.

Gene	Sequences	Species
Dcn	Forward: AGACTCACAGCCGAGTAGGA Reverse: ACATTCGCATCTCAGACACC	Mouse
Scx	Forward: CCTTCTGCCTCAGCAACCAG Reverse: GGTCCAAAGTGGGGCTCTCCGTGACT	Mouse
Mkx	Forward: CCCCGGACATCGGATCTACTA Reverse: CTCTTAGGATGAGGATTTAGGTA	Mouse
Tnmd	Forward: GGGTGGTCCCGCAAGTGAAGGTG Reverse: GCCTCGACGACAGTAAATACAACAGT	Mouse
Il-1b	Forward: GCAACTGTTCCTGAACTCAACT Reverse: GTGCTCATGTCCTCATCCTG	Mouse
Il-6	Forward: CTCTGGGAAATCGTGGAAAT Reverse: CCAGTTTGGTAGCATCCATC	Mouse
Tnfa	Forward: GACGTGGAACTGGCAGAAGAG Reverse: TTGGTGGTTTGTGAGTGTGAG	Mouse
Gapdh	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA	Mouse

For the stimulation of tenocytes, the cells were plated in 6-well plates at a density of 2×10^6 cells per well, and then 1.5 mL of complete medium was added. After 24 h of culture, the cells were pretreated with different concentrations of FR for 3 h. Subsequently, the cells were treated with IL-1β (10 ng/mL; R&D Systems, Minneapolis, MN, USA) for another 24 h. Finally, the cell supernatant was collected, and cellular RNA and protein were extracted for subsequent experiments.

2.7. Cell Viability Assay

We used a Cell Counting Kit-8 (CCK-8) to evaluate cell proliferation and viability. In short, tenocytes were seeded in 96-well plates at a density of 1×10^3/well. After the cells adhered, they were treated with different concentrations of FR for 24 h. 10 μL The CCK-8 working solution was added to each well and cultured in the cell incubator for 4 h. Finally, the absorbance value was measured in a microplate reader with a wavelength of 450 nm.

2.8. Plasmids and Transfection

Plasmids were cloned into the pcDNA3.1 vector for transient expression. HEK293T transfection was performed using Lipofectamine 2000 according to procedures recommended by the manufacturer. Chemically synthesized 21-nucleotide siRNA duplexes were obtained from TranSheepBio and transfected using Lipofectamine RNAiMAX according to the manufacturer's instructions. The sequences of target siRNAs are shown in Table 3.

2.9. Protein Degradation Inhibition Assays

MG132 (10 μM) was used to inhibit proteasome-mediated protein degradation. Both 3-MA (10 mM) and CQ (50 μM) were used to inhibit autolysosome- or lysosome-mediated protein degradation.

Table 3. The sequences of target siRNAs in this study.

Gene	Sequences	Species
p62 siRNA	5′-GCUGAAACAUGGACACUUUTT-3′ 3′-AAAGUGUCCAUGUUUCAGCTT-5′	Mouse
RNF182 siRNA	5′-GCGCCAAAUGCCUCUACAATT-3′ 3′-UUGUAGAGGCAUUUGGCGCTT-5′	Mouse
RNF182 siRNA	5′-GACAACAACAUCCUUGUAATT-3′ 3′-UUACAAGGAUGUUGUUGUCTT-5′	Human
ING4 siRNA	5′-GAUCCCAACGAACCCACAUTT-3′ 3′-AUGUGGGUUCGUUGGGAUCTT-5′	Mouse
PPARγ siRNA	5′-GCAAGAGAUCACAGAGUAUTT-3′ 3′-AUACUCUGUGAUCUCUUGCTT-5′	Mouse

2.10. ELISA

Cell supernatants and serum were detected by the mouse IL-1β, IL-6, and TNF-α ELISA kit (#E-EL-M0037c, #E-EL-M0044c, #E-EL-M1084c; Elabscience Biotechnology Co., Ltd., Wuhan, China). Absorbance was detected at 450 nm by the Multiskan FC (Thermo Fisher, Waltham, MA, USA).

2.11. Western Blotting

Proteins from cells and tissues were extracted with cell lysates (Tris-HCI, pH7.5, 1 M; EDTA 0.5 M; 10% SDS; NP-40; sodium deoxycholate; CHAPS Triton X-100). The lysed protein liquid was then placed in the EP tube and centrifuged at 13,000 rpm at 4 °C for 5 min. Next, we took the protein supernatant, added the loading buffer, and boiled it in a metal bath for 15 min to denaturate the protein. After the protein concentration was determined, it was packed and stored at −20 °C. Then the electrophoretic gel was prepared, and the protein samples were added to the gel for electrophoresis. The gel was taken out and placed in PVDF membrane for transfer reaction (100 V, 90 min). The PVDF membrane was placed in 5% skim milk and sealed for 1 h. Then, the primary antibodies were added and incubated overnight. The next day, the secondary antibodies were added and incubated for 1 h. Finally, signals were revealed using an enhanced chemiluminescence kit.

2.12. Statistical Analysis

The data were graphed using GraphPad Prism software version 8.0 (GraphPad Software Inc., La Jolla, CA, USA). We used a one-way analysis of variance followed by Student's t-test to determine statistical differences between treatment groups. Error bars represent the standard error of the mean in the cell experiment and the standard deviation in the animal experiment. Differences between the two groups were considered significant when the *p*-value was less than 0.05.

3. Results

3.1. FR Alleviates the Progression of Tendinopathy in Mice

To explore the effect of FR on the progression of tendinopathy, we first constructed a mouse model of CIT. The chemical structural formula of FR is shown in Figure 1A. In the CIT group, the biomechanical indexes decreased, the histological structure was disordered, and the transcription level of tenogenic factors decreased, indicating that the CIT mouse model was successful. Compared with the CIT group, FR significantly increased the failure load (Figure 1B) and ultimate stress (Figure 1C) in the CIT + FR group. However, the biomechanical indexes of tendons in the FR group were not affected by FR alone (Figure 1B,C). In addition, the HE staining indicated that FR effectively alleviated the structural disorder of tendinopathy, reduced inflammatory cell infiltration, restored the normal arrangement of collagen fibers, and reduced neovascularization (Figure 1E). Similarly, histological scoring results showed a consistent trend (Figure 1D). In addition, the results of Masson staining showed that a large number of low-strength collagen fibers

(blue part) were produced in the CIT group, while more high-strength collagen fibers (red part) were produced in the CIT + FR group (Figure 1F). Moreover, FR alone did not affect the formation of collagen fibers, which indicates that FR functions during the progression of tendinopathy. To further clarify the effect of FR on tenogenesis in tendinopathy, we detected the transcriptional levels of tendon forming factors (*Dcn*, *Scx*, *Mkx* and *Tnmd*) and found that FR effectively reversed the mRNA expression of *Dcn*, *Scx*, *Mkx* and *Tnmd* in mice with tendinopathy (Figure 1G–J). Therefore, these data demonstrate that FR effectively alleviates the progress of tendinopathy in mice.

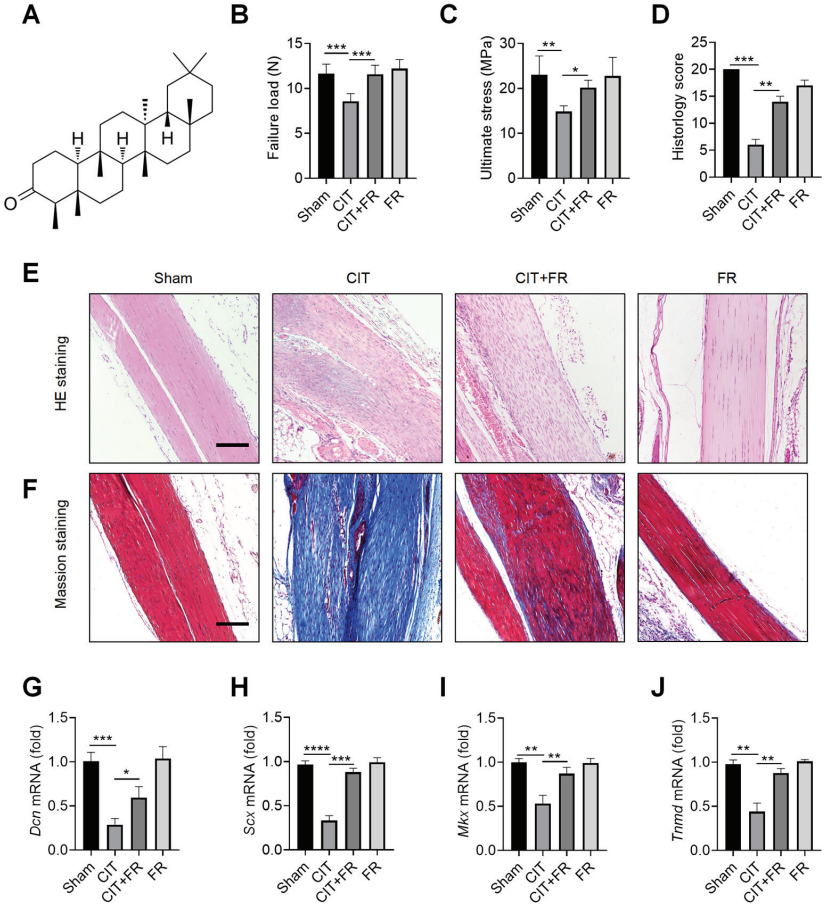


Figure 1. Progression of tendinopathy is alleviated by FR in mice. (**A**) The chemical structural formula of FR. Four weeks after treatment in mice, the Achilles tendons were collected for experimental detection. Biomechanical detection of the effect of FR on Achilles tendon healing: failure load (**B**), ultimate stress (**C**). (**D**) Histological score of HE staining. (**E**) HE staining was used to detect the effect of FR on the histological healing of the Achilles tendon in mice. (**F**) Masson's staining was used to detect the effect of FR on the formation of collagen fibers in the process of tendinopathy in mice. (**G**–**J**) The effect of FR on the expression of tendon-forming factors (*Dcn*, *Scx*, *Mkx*, and *Tnmd*) was detected by qRT-PCR. Scale bar: 100 μm. Data are expressed as the means ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2. FR Attenuates the Infiltration of Inflammatory Factors during Tendinopathy

Inflammation is known to play an important role in the progression of tendinopathy [39]. Next, we investigated the effect of FR on the inflammatory response of tendinopathy in mice. qRT-PCR results showed that the expression of inflammatory cytokines *Il-1b*, *Il-6* and *Tnfa* significantly increased during tendinopathy, and this enhancement was reversed by FR treatment in the CIT group (Figure 2A–C). Macrophages, the key immune cells that mediate inflammation, play an important pathogenic role in the progression of tendinopathy [40]. Then, we explored whether FR could regulate the infiltration of macrophages in the process of tendinopathy. As shown in Figure 2D, a large number of F4/80$^+$ macrophages were infiltrated in the CIT group, while the number of F4/80$^+$ macrophages was dramatically reduced in the CIT + FR group. Consistent with this, the immunofluorescence result showed that the protein expression of IL-6 in the CIT + FR group was significantly lower than in the CIT group (Figure 2E). Moreover, FR alone did not affect the expression of F4/80$^+$ macrophages and IL-6 in normal mice. These data suggest that FR attenuates the infiltration of inflammatory cytokines and cells during tendinopathy in mice, thereby attenuating the progression of tendinopathy.

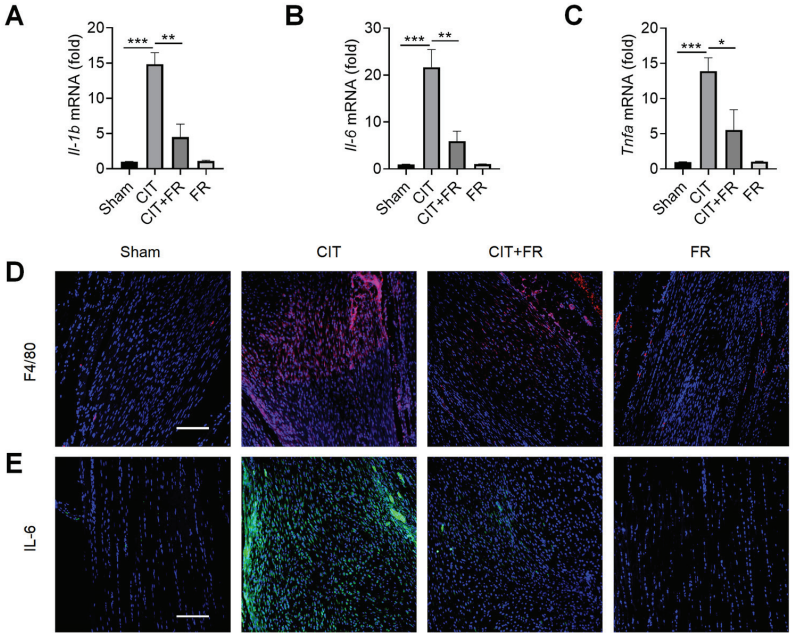


Figure 2. FR alleviates the inflammatory response of tendinopathy in mice. (**A**–**C**) Four weeks after treatment in mice, the effect of FR on the expression of inflammatory cytokine (*Il-1b*, *Il-6*, and *Tnfa*) mRNA was detected by qRT-PCR. The expressions of macrophage marker F4/80 (**D**) and inflammatory factor IL-6 (**E**) were detected by immunofluorescence. Scale bar: 100 μm. Data are expressed as the means ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. FR Targets p65 to Regulate NF-κB Signaling to Inhibit the Inflammatory Response in Tenocytes

To investigate the mechanism of FR in inhibiting inflammation, we then treated the tenocytes with FR under the condition of inflammatory stimulation. Firstly, the CCK-8 result suggests that the concentrations of 0–40 μM of FR have no effect on the proliferation of tenocytes (Figure S1A), which indicates that this is the safe concentration range for the subsequent experiments. It is reported that IL-1β is highly expressed in tendinopathy, which is a key inflammatory mediator to mediate the inflammatory reaction and accelerate

tendinopathy [39,41]. We then explored the effect of FR on the IL-1β-mediated inflammatory response of tenocytes and found that FR reduces the mRNA expression of *Il-6* and *Tnfa* induced by IL-1β (Figure 3A,B). Meanwhile, FR also inhibited the protein expression of IL-6 and TNF-α proteins mediated by IL-1β (Figure 3C,D). These data indicated that FR effectively inhibits the IL-1β-mediated release of inflammatory factors in tenocytes. In addition, we also examined the effect of FR on the inflammatory responses mediated by other inflammatory factors. Both LPS- and TNF-α-mediated inflammation can be effectively alleviated by FR (Figure S1B–E). Therefore, FR can effectively inhibit the inflammatory response of tenocytes induced by various inflammatory mediators.

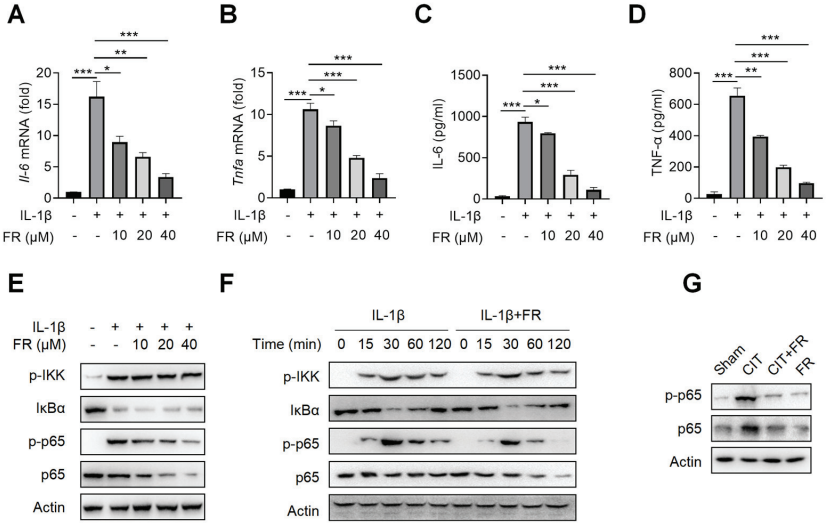


Figure 3. FR targets p65 to inhibit NF-κB signaling *in vitro* and in vivo. Tenocytes were stimulated with IL-1β (10 ng/mL) and FR (0–40 μM) for 24 h. The cell supernatant, total cell RNA, and total protein were collected for experimental detection. qRT-PCR was used to detect the effect of FR on the mRNA expression of *Il-6* (**A**) and *Tnfa* (**B**) mediated by IL-1β. ELISA was used to detect the effect of FR on the supernatant protein expression of IL-6 (**C**) and TNF-α (**D**) induced by IL-1β. (**E**) The expression of NF-κB pathway-related proteins (p-IKK, IκBα, p-p65, and p65) treated with different concentrations of FR was detected by WB. (**F**) Tenocytes were treated with IL-1β (10 ng/mL) and FR (40 μM) for 0–120 min. The expression of NF-κB pathway-related proteins (p-IKK, IκBα, p-p65, p65) was detected by WB. (**G**). Four weeks after treatment, the protein expression of p-p65 and p65 in Achilles tendons was detected by WB. The data are representative of three independent experiments. Error bars show the means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

It is known that NF-κB signaling is not only one of the most classical pathways to mediate the inflammatory response but also the key pathway to aggravating the deterioration of tendinopathy [24]. We further investigated whether the anti-inflammatory effect of FR is achieved by targeting the NF-κB pathway. As shown in Figure 3E, FR inhibited the expression and phosphorylation of p65 in a concentration-dependent manner but did not affect the expression of p-IKK and IkBα. Similarly, FR also inhibited the expression of p65 and p-p65 without affecting the expression of p-IKK and IkBα after different times of stimulation (Figure 3F). Consistent results were also obtained in the condition of LPS or TNF-α mediated inflammation (Figure S1F–H). Furthermore, it was confirmed in vivo that FR inhibited the expression of p65 and p-p65 (Figure 3G). These data suggest that FR targets the NF-κB pathway and plays an anti-inflammatory role in tenocytes at the molecular level of p65.

3.4. FR Promotes the Degradation of p65 through the Autophagy-Lysosome Pathway

Next, we further explore the mechanism of FR in inhibiting the expression of p65. We found that FR inhibited the expression of p65 protein (Figure 4A) but did not affect the transcription level of p65 (Figure 4B). These data suggest that FR may inhibit the expression of p65 by regulating the degradation of p65. To further confirm that FR promotes the degradation of p65, we investigated the effect of FR on the degradation of p65 under the condition of protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 4C, FR accelerated the degradation of p65 in the presence of CHX, suggesting that FR indeed plays a role in promoting the degradation of p65.

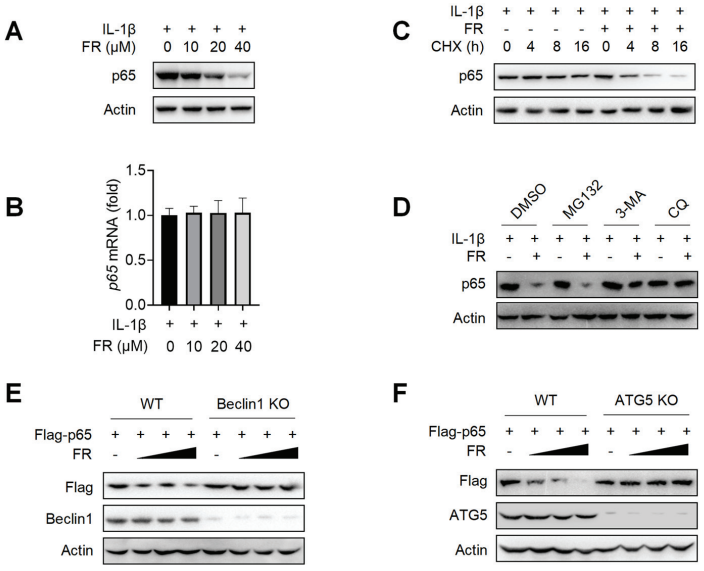


Figure 4. FR degrades p65 through an autophagy-lysosome pathway. Tenocytes were treated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h. (**A**) The expression of p65 protein was detected by WB. (**B**) The mRNA expression of *p65* was detected by qRT-PCR. (**C**) Tenocytes were treated with CHX (50 μg/mL), IL-1β (10 ng/mL) and FR (40 μM) for 0–16 h. The protein expression of p65 was detected by WB. (**D**). Tenocytes were pretreated with MG132 (10 μM), or 3-MA (5 mM), or CQ (10 μM) for 6 h, followed by the addition of IL-1β (10 ng/mL) and FR (40 μM) for 24 h, and then the p65 protein was detected by WB. (**E**) WT and Beclin1-knockout HEK293T cells were transfected with Flag-p65 plasmid for 24 h, followed by 8 h treatment with FR (0–40 μM). Finally, the protein expression of Flag-p65 and Beclin1 were detected by WB. (**F**) WT and ATG5-knockout HEK293T cells were transfected with Flag-p65 plasmid for 24 h, followed by 8 h treatment with FR (0–40 μM). Then, the protein expression of Flag-p65 and ATG5 were detected by WB. Data are expressed as the means ± SD from three independent experiments.

Since there are at least three protein degradation systems (the proteasome, lysosome, and autolysosome pathways) [42], we next determined for which pathway FR induces the degradation of p65. The degradation of p65 by FR was reversed when the autophagy inhibitor 3-MA and the lysosomal inhibitor CQ were used, but not by the proteasome inhibitor MG132 (Figure 4D). To further confirm that FR degrades p65 protein through the autophagy-dependent pathway, we used Beclin1 deficiency or ATG5 deficiency HEK293T cells for verification. In WT HEK293T cells, FR significantly promoted the degradation of exogenous p65 protein. However, when Beclin1 or ATG5 was knocked out in HEK293T, the degradation of p65 by FR disappeared (Figure 4E,F). These results demonstrated that FR promotes the degradation of p65 through an autophagy-lysosome pathway.

3.5. FR Mediates Selective Autophagic Degradation of p65 via p62-Dependent Pathway

p62 is a selective autophagy adaptor protein, which plays an important role in mediating the autophagic degradation of proteins [42,43]. Next, we explored whether FR mediates the selective autophagic degradation of p65 through p62. The results showed that FR could not degrade p65 in p62-knockout HEK293T cells (Figure 5A). Moreover, silencing p62 in tenocytes could also reverse the degradation of p65 by FR (Figure 5B). Therefore, FR promoted the degradation of p65 via p62-mediated selective autophagy. Subsequently, we further explored whether FR affected the degradation of p65 by influencing the interaction between p62 and p65. In the exogenous IP experiment, FR promoted the interaction between p62 and p65 (Figure 5C). In addition, FR also enhanced the association of endogenous p62 and p65 (Figure 5D). These results suggest that FR promotes the selective autophagic degradation of p65 by increasing the association of p62 and p65.

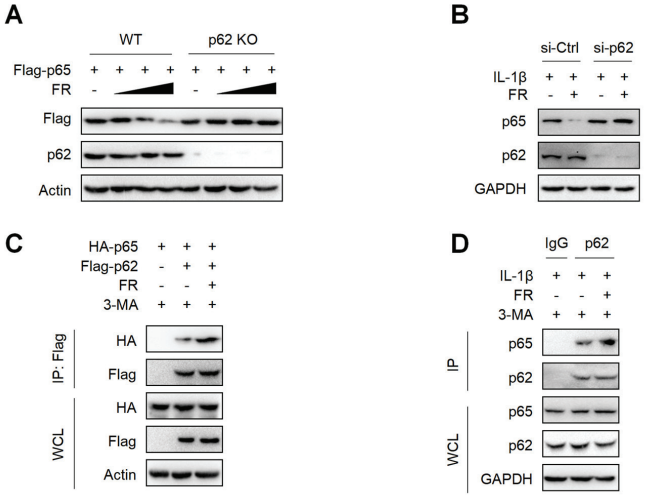


Figure 5. FR promotes the degradation of p65 via p62-mediated selective autophagy. (**A**) The Flag-p65 plasmid was transfected into WT and p62 knockout HEK293T cells for 24 h, and FR (0–40 μM) was added for another 8 h. Finally, WB was used to detect the protein expression of Flag-p65 and p62. (**B**) Tenocytes were transfected with p62 siRNA for 24 h and then treated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h. Finally, the protein expression of p65 and p62 was detected by WB. (**C**) HEK293T cells were transfected with HA-p65 and Flag-p62 plasmids for 24 h, then treated with 3-MA (5 mM) for 6 h, and then treated with FR (40 μM) for another 8 h. Finally, cell proteins were collected for immunoprecipitation (IP). The expressions of HA-p65 and Flag-p62 proteins in IP samples and whole-cell lysates (WCL) were detected by WB. (**D**) Tenocytes were pretreated with 3-MA (5 mM) for 6 h, then stimulated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h, and the cellular proteins were collected for IP. WB was used to detect the protein expression of p65 and p62 in IP samples and WCL. Data are expressed as the means ± SD from three independent experiments.

3.6. FR Increases the K48-Linked Ubiquitination of p65 to Promote Its Autophagic Degradation

It is reported that p62 directs ubiquitinated proteins to autophagolysosomes for selective degradation mainly through its C-terminal ubiquitin-associated domain [42,43]. Next, we investigated whether FR could affect the ubiquitination of p65 and found that FR remarkably increased the poly-ubiquitination of endogenous p65 (Figure 6A). Subsequently, we found that FR specifically increased K48-linked (K48-only ubiquitin mutant) poly-ubiquitination of p65, but not the ubiquitination of p65 with other ubiquitin linkages in an overexpression system (Figure 6B). Likewise, FR also promoted the K48-linked ubiquitination of endogenous p65 (Figure 6C). In addition, the degradation of p65 by FR was reversed in the presence of ubiquitin inhibitor MLN7243 (Figure 6D,E). These data sug-

gest that FR accelerates p62-mediated selective degradation by promoting the K48-linked ubiquitination of p65.

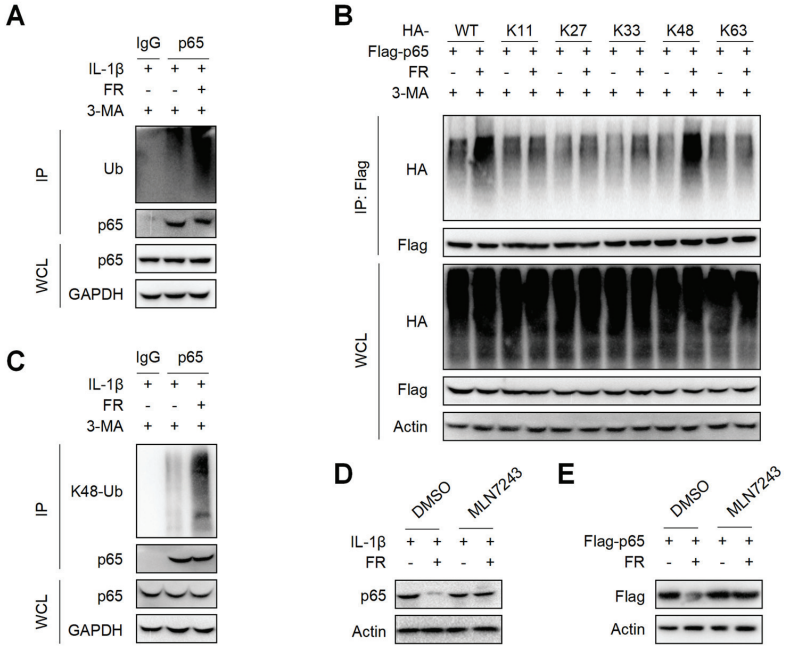


Figure 6. FR promotes the K48-linked ubiquitination of p65 in vitro. (**A**) Tendon cells were pretreated with 3-MA (5 mM) for 6 h, then stimulated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h, and cell proteins were collected for IP. WB was used to detect the protein expression of p65 and Ub. (**B**) Flag-p65 and HA-labeled ubiquitinated plasmids (WT, K11, K27, K33, K48, K63) were transfected into HEK293T cells for 24 h, then treated with 3-MA (5 mM) for 6 h, and then treated with FR (40 μM) for 8 h. Finally, the proteins were collected for IP. WB was used to detect the protein expression with HA tag and Flag tag in IP samples and WCL. (**C**) Tenocytes were pretreated with 3-MA (5 mM) for 6 h, then stimulated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h, and cell proteins were collected for IP. WB was used to detect the protein expression of p65 and K48 Ub in IP samples and WCL. (**D**) Tenocytes were pretreated with ubiquitination inhibitor MLN7243 (5 μM) for 6 h, then treated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h, and the expression of p65 protein was detected by WB. (**E**) Flag-p65 plasmid was transfected into HEK293T cells for 24 h, then MLN7243 (5 μM) was added for 6 h, and FR (40 μM) was added for 8 h. WB was used to detect the protein expression of Flag-p65. Data are expressed as the means ± SD from three independent experiments.

3.7. FR Mediates K48-Linked Ubiquitination of p65 via E3 Ubiquitination Enzyme RNF182

It is reported that E3 ubiquitinase is a key enzyme mediating protein ubiquitination [44]. Although FR promotes the ubiquitination of p65, it is not clear which E3 ubiquitin enzyme plays a key role in the FR-induced ubiquitination of p65. It is reported that RNF182, ING4, and PPARγ are E3 ubiquitination enzymes that mediate the K48-linked ubiquitination of p65 [45–47]. Next, we determined which E3 ubiquitin enzyme plays its role in the FR-mediated ubiquitination of p65. We silenced the expression of RNF182, ING4, and PPARγ in tenocytes by the siRNAs (Figure S2A–C) and found that silencing RNF182 reversed the degradation of p65 by FR (Figure 7A), whereas silencing ING4 and PPARγ did not reverse the degradation of p65 (Figure 7B,C), which indicates that FR mediates the degradation of p65 through the RNF182-dependent pathway. Next, we examined whether FR mediated the K48-linked ubiquitination of p65 through RNF182 and found that FR failed to promote the K48-linked ubiquitination of p65 when RNF182 was silenced in the overexpression system (Figure 7D). Meanwhile, similar

results were obtained in the endogenous IP experiment (Figure 7E). In addition, in the case of overexpression of RNF182, FR could further promote the degradation of p65 (Figure 7F). These data suggest that FR mediates the K48-linked ubiquitination of p65 by recruiting RNF182, promoting autophagic degradation. Furthermore, we also found that FR effectively increased the interaction between RNF182 and p65 in the overexpression and endogenous systems (Figure 7G,H). Therefore, these data suggest that FR promotes the K48-linked ubiquitination of p65 by promoting the association of RNF182 and p65, thus accelerating the autophagic degradation of p65.

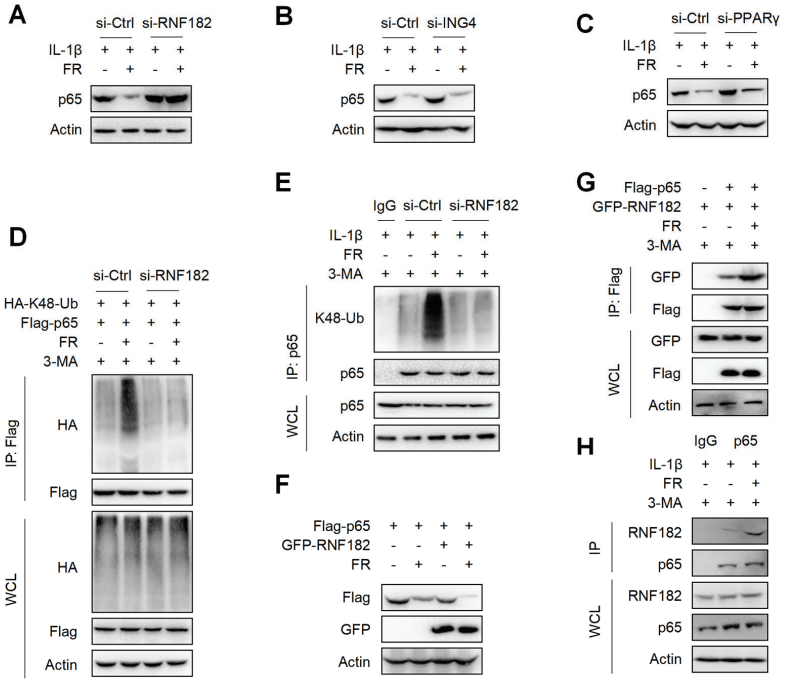


Figure 7. FR promotes the K48-linked ubiquitination of p65 by recruiting the E3 ubiquitin enzyme RNF182. Tenocytes were silenced with RNF182 siRNA (**A**), ING4 siRNA (**B**), or PPARγ siRNA (**C**) for 24 h and then treated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h. Finally, the protein expression of p65 was detected by WB. (**D**) HEK293T cells were treated with RNF182 siRNA for 24 h, then transfected with HA-K48-Ub and Flag-p65 plasmids for 12 h, and next stimulated with 3-MA (5 mM) for 6 h, and finally treated with FR (40 μM) for 8 h. Cell lysates were collected for IP and WB detection. (**E**) Tenocytes were transfected with RNF182 siRNA for 24 h, then treated with 3-MA (5 mM) for 6 h, and finally treated with IL-1β (10 ng/mL) and FR (40 μM) for 24 h. Cell proteins were collected for IP. The expressions of p65 and K48-Ub in IP and WCL samples were detected by WB. (**F**) Flag-p65 and GFP-RNF182 plasmids were transfected into HEK293T cells for 24 h and then treated with FR (40 μM) for 8 h. Finally, WB was used to detect the protein of the Flag tag and GFP tag. (**G**) HEK293T cells were transfected with Flag-p65 and GFP-RNF182 plasmids for 24 h, then 3-MA (5 mM) was added for 6 h and then treated with FR (40 μM) for another 8 h. Finally, cell proteins were collected for IP, and proteins with Flag and GFP labels were detected by WB. (**H**) Tenocytes were pretreated with 3-MA (5 mM) for 6 h, followed by the addition of IL-1β (10 ng/mL) and FR (40 μM) for 24 h. Cell proteins were collected for IP. WB detected the protein expressions of p65 and RNF182 in IP and WCL samples. Data are expressed as the means ± SD from three independent experiments.

4. Discussion

Tendinopathy is a degenerative disease that occurs after tendon injury, which often causes local pain and limited mobility and leads to disability in severe cases. With the development of aging, the incidence of tendinopathy is increasing year by year [3–5]. However, there is currently no cure for tendinopathy, which brings significant challenges to the prevention and treatment of tendinopathy. FR is a triterpenoid compound with a wide range of sources, high safety, and a variety of medicinal values [28,48,49]. Although FR plays a certain role in treating inflammatory diseases, there is a lack of in-depth research on the target and mechanisms of its disease resistance. Here, we found that FR alleviated the degeneration of tendinopathy, reduced inflammatory infiltration, and improved the biomechanical strength, which plays an important role in the prevention and treatment of tendinopathy. Mechanically, FR inhibited the NF-κB pathway by promoting the autophagic degradation of p65 and decreasing the inflammatory response, thereby alleviating the progression of tendinopathy. Furthermore, FR increased the K48-linked ubiquitination of p65 by recruiting the E3 ubiquitinase RNF182, promoting the association of p65 and cargo protein p62 and thus mediating the selective autophagic degradation of p65 (Figure 8).

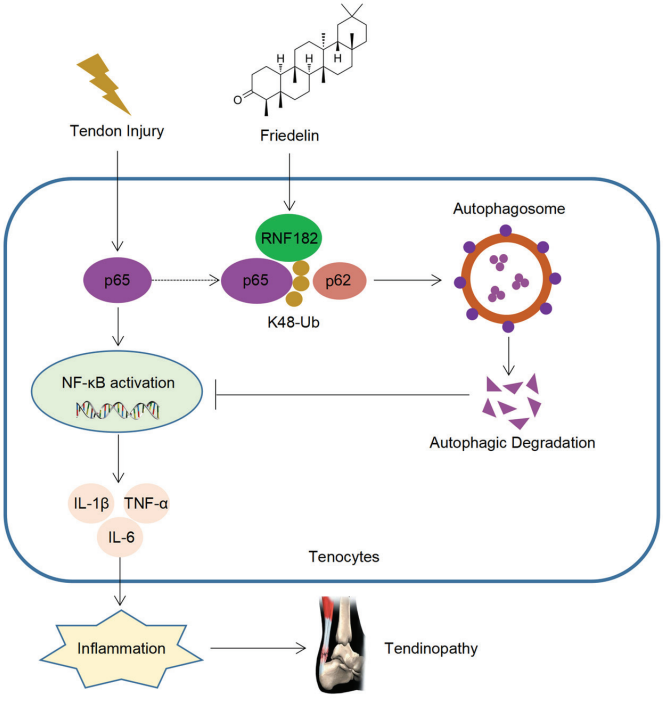


Figure 8. Schematic diagram of the action of FR. FR promotes the K48-linked ubiquitination of p65 through recruiting E3 ubiquitinase RNF182 and then enhances p62-mediated selective autophagic degradation of p65, thereby inhibiting NF-κB signaling and alleviating the progression of tendinopathy.

After tendon trauma, a large number of immune cells can be recruited to the local area, in which macrophages release a variety of inflammatory factors to mediate the chronic inflammation of tendinopathy [13,40,50]. It is reported that a small number of inflammatory factors could mediate the regeneration of tendon tissue, but long-term chronic inflammation will lead to tendon tissue degeneration [13,40,50]. In addition, chronic inflammation can further activate relevant immune cells, produce various pathogenic factors, and hinder the repair and healing of tendon tissue [17,23,51]. In the long run, normal tendon tissue is replaced by hyperplastic tissue and even calcification [52,53]. Therefore, targeted

elimination of inflammation is a good strategy for preventing and treating tendinopathy. However, drugs that target inflammation and their mechanisms remain unknown, limiting the development of tendinopathy treatments. Here, our results show that FR improves the biomechanical strength of the tendon and promotes the generation of high-strength muscle fibers, which are important for preventing tendon rupture. Meanwhile, FR reduces tendon fibrosis, restores the orderly arrangement of collagen fibers, and decreases angiogenesis in tendinopathy. FR effectively inhibits the expression of inflammatory factors and reduces the infiltration of $F4/80^{+}$ macrophages. More importantly, FR can also promote the expression of tendon-forming factors (*Scx*, *Tnmd* and *Mkx*). In addition, the use of FR alone does not cause pathological changes in the normal tendon. These findings show that FR is a safe and effective candidate for treating tendon diseases.

Several studies have shown that the NF-κB pathway is a classical pathway that mediates inflammatory cascade [27,54,55]. During the progression of tendinopathy, the NF-κB pathway is significantly activated, leading to an inflammatory storm and accelerating the progression of tendinopathy [27,54,55]. It is reported that the progression of tendinopathy is weakened in IKKβ-deficient mice, indicating targeted inhibition of the NF-κB pathway is a good strategy for the prevention and treatment of tendinopathy [27]. Our study found that FR can inhibit the NF-κB pathway, thus inhibiting the release of inflammatory factors. Unlike other anti-inflammatory drugs, FR acts on p65 molecules downstream of the NF-κB pathway without affecting the upstream pathway, which shows good selectivity and targeting. To clarify the pharmacological mechanism of the anti-inflammatory effect of FR, we conducted experiments in tenocytes and HEK293T cells. The results showed that FR promoted the autophagy-dependent degradation of p65 but did not affect the transcription of p65. This indicates that FR inhibits the NF-κB pathway by regulating the post-translational modification of p65. Further results showed that FR mediated the autophagic degradation of p65 through autophagy cargo p62. Moreover, FR promotes the interaction of p65 and p62. Therefore, these results demonstrate that FR promotes autophagic degradation by increasing the combination of p62 and p65.

Ubiquitination is a form of post-translational modification of proteins by coupling ubiquitin proteins to substrate proteins [56–58]. Ubiquitin is a protein containing 76 amino acids that bind to the lysine residue of the substrate protein by an isopeptide bond [56–58]. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and one methionine [56–58]. Since p62 needs to be combined with ubiquitinated substrates [42], we further explored how FR regulates the ubiquitination of p65. Our results showed that FR could promote the K48-linked ubiquitination of p65. In addition, after blocking ubiquitination, p65 could not be degraded by FR, which indicated that the degradation of p65 by FR was dependent on ubiquitination. Therefore, targeted regulation of ubiquitination is also one of the effective strategies for the treatment of tendinopathy. Ubiquitination occurs through the sequential activities of three enzymes, namely ubiquitin enzyme (E1), ubiquitin-binding enzyme (E2), and ubiquitin ligase (E3). Several E3 ubiquitin enzymes have been reported to mediate the K48-linked ubiquitination of p65 [45–47]. Here, we confirmed that FR mediates the K48-linked ubiquitin of p65 through the E3 ubiquitin enzyme RNF182 but is independent of ING4 and PPARγ. Moreover, the interaction between RNF182 and p65 can be enhanced by FR. Therefore, the targeted degradation of p65 by FR is closely related to p62 and RNF182, which provides a theoretical basis for applying FR in the treatment of tendinopathy and other inflammatory diseases.

Although the present study demonstrates that FR has a good alleviating effect on tendinopathy, some limitations still need to be further studied. Due to ethical issues, this study only used mouse tenocytes for experiments. However, further study is needed to determine whether FR has the same effect on human tenocytes. Additionally, although we have proven that FR shows a good anti-inflammatory effect on tendinopathy, whether it has a similar effect on other inflammatory bone diseases remains to be explored.

5. Conclusions

In conclusion, FR reduces the inflammatory response of tenocytes, improves the biological properties of the tendon, and promotes tendon healing by targeting the degradation of p65, thus effectively alleviating the progress of tendinopathy. To our knowledge, this study describes for the first time that FR alleviates tendinopathy by regulating ubiquitin-autophagy degradation. Therefore, FR is a promising new drug for treating tendinopathy and inflammation-related diseases. Of course, further clinical trials are needed to prove its safety and efficacy in human tendinopathy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14081673/s1, Figure S1: FR inhibits NF-κB signaling in tenocytes. (A) Tenocytes were treated with FR (0-40 μM) for 24 h, and then cell viability was detected by CCK8 assay. Tenocytes were treated with FR (0-40 μM) combined with LPS (100 ng/ml) or TNF-α (100 ng/ml) for 24 h, and then the cellular RNA, protein, and cell supernatant were collected for the following experiments. (B and C) The mRNA expression of IL-6 was detected by qRT-PCR. (D and E) The expression of IL-6 protein in cell supernatant was detected by ELISA. (F and H) The expression of NF-κB pathway-related proteins (p-IKK, IκBα, p-p65, and p65) were detected by WB. (G) Tenocytes were treated with FR (40 μM) and LPS (100 ng/ml) for 0-120 min, and then the expression of NF-κB pathway-related proteins (p-IKK, IκBα, p-p65, and p65) were detected by WB. The data are representative of three independent experiments. Error bars show the means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Figure S2. Silencing efficiency of siRNA. Tenocytes were transfected with RNF182 siRNA, ING4 siRNA, and PPARγ siRNA for 24 h, and the proteins expression of RNF182 (A), ING4 (B), and PPARγ (C) were detected by WB.

Author Contributions: Conceptualization: H.J., X.L., Y.L. and X.Y.; Data curation: X.L. and Y.L.; Formal analysis: H.J.; Funding acquisition: X.L. and X.Y.; Investigation: H.J., X.L. and W.L.; Methodology: H.J.; Project administration: Y.L. and X.Y.; Resources: Y.L. and X.Y.; Software: W.L.; Supervision: X.Y.; Validation: H.J., X.L. and W.L.; Visualization: H.J.; Writing—original draft: H.J. and X.L.; Writing—review & editing: Y.L. and X.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the National Natural Science Foundation of China (82171741 and 81801579), Guangdong Basic and Applied Basic Research Foundation (2019B1515120033 and 2021A1515012140), Guangdong Zhujiang Youth Scholar funding, the Science and Technology Planning Project of Guangzhou (201904010064) to Xiao Yu, and the Start-up Fund for High-level Hospital of Guangzhou Women and Children's Medical Center to Xuemei Lin.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Medical College of Shantou University (Protocol code SUMC2021-480).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Jun Cui (Sun Yat-sen University) for providing p62, ATG5, and Beclin1 KO 293T cells.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Riley, G. The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology* **2004**, *43*, 131–142. [CrossRef] [PubMed]
2. Morita, W.; Snelling, S.J.; Dakin, S.G.; Carr, A.J. Profibrotic mediators in tendon disease: A systematic review. *Arthritis Res. Ther.* **2016**, *18*, 269. [CrossRef] [PubMed]
3. Choi, R.; Smith, M.; Clarke, E.; Little, C. Cellular, matrix, and mechano-biological differences in load-bearing versus positional tendons throughout development and aging: A narrative review. *Connect. Tissue Res.* **2018**, *59*, 483–494. [CrossRef] [PubMed]
4. Smith, R.K.W.; Birch, H.L.; Goodman, S.; Heinegard, D.; Goodship, A.E. The influence of ageing and exercise on tendon growth and degeneration—hypotheses for the initiation and prevention of straininduced tendinopathies. *Comp. Biochem. Physiol.* **2002**, *133*, 1039–1350. [CrossRef]

5. Birch, H.L.; Peffers, M.J.; Clegg, P.D. Influence of Ageing on Tendon Homeostasis. *Adv. Exp. Med. Biol.* **2016**, *920*, 247–260. [CrossRef]
6. Byl, N.; Wilson, F.; Merzenich, M.; Melnick, M.; Scott, P.; Oakes, A.; McKenzie, A. Sensory Dysfunction Associated With Repetitive Strain Injuries of Tendinitis and Focal Hand Dystonia: A Comparative Study. *J. Orthop. Sports Phys. Ther.* **1996**, *23*, 234–244. [CrossRef]
7. Gupta, A.K.; Chalmers, P.N.; Klosterman, E.L.; Harris, J.D.; Bach, B.R., Jr.; Verma, N.N.; Cole, B.J.; Romeo, A.A. Subpectoral biceps tenodesis for bicipital tendonitis with SLAP tear. *Orthopedics* **2015**, *38*, e48–e53. [CrossRef]
8. Dressler, M.R.; Butler, D.L.; Boivin, G.P. Age-related changes in the biomechanics of healing patellar tendon. *J. Biomech.* **2006**, *39*, 2205–2212. [CrossRef]
9. Gumina, S.; Carbone, S.; Campagna, V.; Candela, V.; Sacchetti, F.M.; Giannicola, G. The impact of aging on rotator cuff tear size. *Musculoskelet. Surg.* **2013**, *97* (Suppl. 1), 69–72. [CrossRef]
10. Riley, G. Tendinopathy–from basic science to treatment. *Nat. Clin. Pract. Rheumatol.* **2008**, *4*, 82–89. [CrossRef]
11. Jarvinen, T.A.; Kannus, P.; Paavola, M.; Jarvinen, T.L.; Jozsa, L.; Jarvinen, M. Achilles tendon injuries. *Curr. Opin. Rheumatol.* **2001**, *13*, 150–155. [CrossRef] [PubMed]
12. Kane, S.F.; Olewinski, L.H.; Tamminga, K.S. Management of Chronic Tendon Injuries. *Am. Fam. Physician.* **2019**, *100*, 147–157. [PubMed]
13. Jomaa, G.; Kwan, C.K.; Fu, S.C.; Ling, S.K.; Chan, K.M.; Yung, P.S.; Rolf, C. A systematic review of inflammatory cells and markers in human tendinopathy. *BMC Musculoskelet. Disord.* **2020**, *21*, 78. [CrossRef] [PubMed]
14. Silbernagel, K.G.; Hanlon, S.; Sprague, A. Current Clinical Concepts: Conservative Management of Achilles Tendinopathy. *J. Athl. Train.* **2020**, *55*, 438–447. [CrossRef]
15. Fernandez, A.C.M.; Carballar, C.B.; Villafane, J.H.; Perez, S.M.; Perez, J.L.A.; Diaz-Meco, R.; Jimenez, D.G.; Romero, E.A.S. A new ultrasound-guided percutaneous electrolysis and exercise treatment in patellar tendinopathy: Three case reports. *Front. Biosci.* **2021**, *26*, 1166–1175. [CrossRef]
16. Bindu, S.; Mazumder, S.; Bandyopadhyay, U. Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. *Biochem. Pharmacol.* **2020**, *180*, 114147. [CrossRef]
17. Sharma, P.; Maffulli, N. Biology of tendon injury: Healing, modeling and remodeling. *J. Musculoskelet. Neuronal. Interact.* **2006**, *6*, 181–190.
18. Li, H.Y.; Hua, Y.H. Achilles Tendinopathy: Current Concepts about the Basic Science and Clinical Treatments. *Biomed. Res. Int.* **2016**, *2016*, 6492597. [CrossRef]
19. Leong, H.T.; Fu, S.C.; He, X.; Oh, J.H.; Yamamoto, N.; Hang, S. Risk factors for rotator cuff tendinopathy: A systematic review and meta-analysis. *J. Rehabil. Med.* **2019**, *51*, 627–637. [CrossRef]
20. Sanchez Romero, E.A.; Melendez Oliva, E.; Alonso Perez, J.L.; Martin Perez, S.; Turroni, S.; Marchese, L.; Villafane, J.H. Relationship between the Gut Microbiome and Osteoarthritis Pain: Review of the Literature. *Nutrients* **2021**, *13*, 716. [CrossRef]
21. Ticinesi, A.; Nouvenne, A.; Cerundolo, N.; Catania, P.; Prati, B.; Tana, C.; Meschi, T. Gut Microbiota, Muscle Mass and Function in Aging: A Focus on Physical Frailty and Sarcopenia. *Nutrients* **2019**, *11*, 1633. [CrossRef] [PubMed]
22. Ackerman, J.E.; Best, K.T.; Muscat, S.N.; Loiselle, A.E. Metabolic Regulation of Tendon Inflammation and Healing Following Injury. *Curr. Rheumatol. Rep.* **2021**, *23*, 15. [CrossRef] [PubMed]
23. Arvind, V.; Huang, A.H. Reparative and Maladaptive Inflammation in Tendon Healing. *Front. Bioeng. Biotechnol.* **2021**, *9*, 719047. [CrossRef] [PubMed]
24. Wang, C.; Zhou, Z.; Song, W.; Cai, Z.; Ding, Z.; Chen, D.; Xia, F.; He, Y. Inhibition of IKKbeta/NF-kappaB signaling facilitates tendinopathy healing by rejuvenating inflamm-aging induced tendon-derived stem/progenitor cell senescence. *Mol. Ther. Nucleic Acids* **2022**, *27*, 562–576. [CrossRef]
25. Wang, Y.; He, G.; Tang, H.; Shi, Y.; Kang, X.; Lyu, J.; Zhu, M.; Zhou, M.; Yang, M.; Mu, M.; et al. Aspirin inhibits inflammation and scar formation in the injury tendon healing through regulating JNK/STAT-3 signalling pathway. *Cell Prolif.* **2019**, *52*, e12650. [CrossRef]
26. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a001651. [CrossRef]
27. Abraham, A.C.; Shah, S.A.; Golman, M.; Song, L.; Li, X.; Kurtaliaj, I.; Akbar, M.; Millar, N.L.; Abu-Amer, Y.; Galatz, L.M.; et al. Targeting the NF-kB signaling pathway in chronic tendon disease. *Sci. Transl. Med.* **2019**, *11*, eaav4319. [CrossRef]
28. Shi, B.; Liu, S.; Huang, A.; Zhou, M.; Sun, B.; Cao, H.; Shan, J.; Sun, B.; Lin, J. Revealing the Mechanism of Friedelin in the Treatment of Ulcerative Colitis Based on Network Pharmacology and Experimental Verification. *Evid. Based Complement. Altern. Med.* **2021**, *2021*, 4451779. [CrossRef]
29. Han, J.Y.; Ahn, C.H.; Adhikari, P.B.; Kondeti, S.; Choi, Y.E. Functional characterization of an oxidosqualene cyclase (PdFRS) encoding a monofunctional friedelin synthase in Populus davidiana. *Planta* **2019**, *249*, 95–111. [CrossRef]
30. Antonisamy, P.; Duraipandiyan, V.; Ignacimuthu, S. Anti-inflammatory, analgesic and antipyretic effects of friedelin isolated from Azima tetracantha Lam. in mouse and rat models. *J. Pharm. Pharmacol.* **2011**, *63*, 1070–1077. [CrossRef]
31. Sunil, C.; Duraipandiyan, V.; Ignacimuthu, S.; Al-Dhabi, N.A. Antioxidant, free radical scavenging and liver protective effects of friedelin isolated from Azima tetracantha Lam. leaves. *Food Chem.* **2013**, *139*, 860–865. [CrossRef] [PubMed]

32. Subash-Babu, P.; Li, D.K.; Alshatwi, A.A. In vitro cytotoxic potential of friedelin in human MCF-7 breast cancer cell: Regulate early expression of Cdkn2a and pRb1, neutralize mdm2-p53 amalgamation and functional stabilization of p53. *Exp. Toxicol. Pathol.* **2017**, *69*, 630–636. [CrossRef] [PubMed]
33. Mokoka, T.A.; McGaw, L.J.; Mdee, L.K.; Bagla, V.P.; Iwalewa, E.O.; Eloff, J.N. Antimicrobial activity and cytotoxicity of triterpenes isolated from leaves of Maytenus undata (Celastraceae). *BMC Complement. Altern. Med.* **2013**, *13*, 111. [CrossRef] [PubMed]
34. Antonisamy, P.; Duraipandiyan, V.; Aravinthan, A.; Al-Dhabi, N.A.; Ignacimuthu, S.; Choi, K.C.; Kim, J.H. Protective effects of friedelin isolated from Azima tetracantha Lam. against ethanol-induced gastric ulcer in rats and possible underlying mechanisms. *Eur. J. Pharmacol.* **2015**, *750*, 167–175. [CrossRef] [PubMed]
35. Kokubu, S.; Inaki, R.; Hoshi, K.; Hikita, A. Adipose-derived stem cells improve tendon repair and prevent ectopic ossification in tendinopathy by inhibiting inflammation and inducing neovascularization in the early stage of tendon healing. *Regen. Ther.* **2020**, *14*, 103–110. [CrossRef]
36. Lin, X.; Huang, M.; Yin, G.; Zhang, J.; Zhang, Z.; Lai, P.; Yan, B.; Chen, Y.; Jin, D.; Wang, L. Characterization of a Novel Calcific Achilles Tendinopathy Model in Mice: Contralateral Tendinopathy Induced by Unilateral Tenotomy. *Calcif. Tissue Int.* **2018**, *103*, 698–707. [CrossRef]
37. Wang, Y.; He, G.; Tang, H.; Shi, Y.; Zhu, M.; Kang, X.; Bian, X.; Lyu, J.; Zhou, M.; Yang, M.; et al. Aspirin promotes tenogenic differentiation of tendon stem cells and facilitates tendinopathy healing through regulating the GDF7/Smad1/5 signaling pathway. *J. Cell Physiol.* **2020**, *235*, 4778–4789. [CrossRef] [PubMed]
38. Wu, Y.T.; Wu, Y.T.; Huang, T.C.; Su, F.C.; Jou, I.M.; Wu, C.C. Sequential inflammation model for Achilles tendinopathy by elastin degradation with treadmill exercise. *J. Orthop. Translat.* **2020**, *23*, 113–121. [CrossRef]
39. Dakin, S.G.; Newton, J.; Martinez, F.O.; Hedley, R.; Gwilym, S.; Jones, N.; Reid, H.A.B.; Wood, S.; Wells, G.; Appleton, L.; et al. Chronic inflammation is a feature of Achilles tendinopathy and rupture. *Br. J. Sports Med.* **2018**, *52*, 359–367. [CrossRef]
40. Sunwoo, J.Y.; Eliasberg, C.D.; Carballo, C.B.; Rodeo, S.A. The role of the macrophage in tendinopathy and tendon healing. *J. Orthop. Res.* **2020**, *38*, 1666–1675. [CrossRef]
41. September, A.V.; Nell, E.M.; O'Connell, K.; Cook, J.; Handley, C.J.; van der Merwe, L.; Schwellnus, M.; Collins, M. A pathway-based approach investigating the genes encoding interleukin-1beta, interleukin-6 and the interleukin-1 receptor antagonist provides new insight into the genetic susceptibility of Achilles tendinopathy. *Br. J. Sports Med.* **2011**, *45*, 1040–1047. [CrossRef] [PubMed]
42. Liu, T.; Tang, Q.; Liu, K.; Xie, W.; Liu, X.; Wang, H.; Wang, R.F.; Cui, J. TRIM11 Suppresses AIM2 Inflammasome by Degrading AIM2 via p62-Dependent Selective Autophagy. *Cell Rep.* **2016**, *16*, 1988–2002. [CrossRef] [PubMed]
43. Dikic, I. Proteasomal and Autophagic Degradation Systems. *Annu. Rev. Biochem.* **2017**, *86*, 193–224. [CrossRef] [PubMed]
44. Buetow, L.; Huang, D.T. Structural insights into the catalysis and regulation of E3 ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* **2016**, *17*, 626–642. [CrossRef] [PubMed]
45. Cao, Y.; Sun, Y.; Chang, H.; Sun, X.; Yang, S. The E3 ubiquitin ligase RNF182 inhibits TLR-triggered cytokine production through promoting p65 ubiquitination and degradation. *FEBS Lett.* **2019**, *593*, 3210–3219. [CrossRef] [PubMed]
46. Hou, Y.; Zhang, Z.; Xu, Q.; Wang, H.; Xu, Y.; Chen, K. Inhibitor of growth 4 induces NFkappaB/p65 ubiquitin-dependent degradation. *Oncogene* **2014**, *33*, 1997–2003. [CrossRef]
47. Hou, Y.; Moreau, F.; Chadee, K. PPARgamma is an E3 ligase that induces the degradation of NFkappaB/p65. *Nat. Commun.* **2012**, *3*, 1300. [CrossRef]
48. Toledo, C.R.; Pereira, V.V.; Duarte, L.P.; Sousa, G.F.; Silva-Cunha, A. Anti-angiogenic activity and safety of intraocular application of triterpenes. *Doc. Ophthalmol.* **2021**, *143*, 259–270. [CrossRef]
49. Sunil, C.; Irudayaraj, S.S.; Duraipandiyan, V.; Alrashood, S.T.; Alharbi, S.A.; Ignacimuthu, S. Friedelin exhibits antidiabetic effect in diabetic rats via modulation of glucose metabolism in liver and muscle. *J. Ethnopharmacol.* **2021**, *268*, 113659. [CrossRef]
50. Millar, N.L.; Akbar, M.; Campbell, A.L.; Reilly, J.H.; Kerr, S.C.; McLean, M.; Frleta-Gilchrist, M.; Fazzi, U.G.; Leach, W.J.; Rooney, B.P.; et al. IL-17A mediates inflammatory and tissue remodelling events in early human tendinopathy. *Sci. Rep.* **2016**, *6*, 27149. [CrossRef]
51. Chisari, E.; Rehak, L.; Khan, W.S.; Maffulli, N. Tendon healing in presence of chronic low-level inflammation: A systematic review. *Br. Med. Bull.* **2019**, *132*, 97–116. [CrossRef] [PubMed]
52. Gravanis, M.B.; Gaffney, E.F. Idiopathic calcifying tenosynovitis. Histopathologic features and possible pathogenesis. *Am. J. Surg. Pathol.* **1983**, *7*, 357–361. [CrossRef] [PubMed]
53. Sato, T.; Hori, N.; Nakamoto, N.; Akita, M.; Yoda, T. Masticatory muscle tendon-aponeurosis hyperplasia exhibits heterotopic calcification in tendons. *Oral. Dis.* **2014**, *20*, 404–408. [CrossRef] [PubMed]
54. Dakin, S.G.; Martinez, F.O.; Yapp, C.; Wells, G.; Oppermann, U.; Dean, B.J.F.; Smith, R.D.J.; Wheway, K.; Watkins, B.; Roche, L.; et al. Inflammation activation and resolution in human tendon disease. *Sci. Transl. Med.* **2015**, *7*, 311ra173. [CrossRef] [PubMed]
55. Moqbel, S.A.A.; Xu, K.; Chen, Z.; Xu, L.; He, Y.; Wu, Z.; Ma, C.; Ran, J.; Wu, L.; Xiong, Y. Tectorigenin Alleviates Inflammation, Apoptosis, and Ossification in Rat Tendon-Derived Stem Cells via Modulating NF-Kappa B and MAPK Pathways. *Front. Cell Dev. Biol.* **2020**, *8*, 568894. [CrossRef] [PubMed]
56. Shaid, S.; Brandts, C.H.; Serve, H.; Dikic, I. Ubiquitination and selective autophagy. *Cell Death Differ.* **2013**, *20*, 21–30. [CrossRef]
57. Behl, T.; Chadha, S.; Sachdeva, M.; Kumar, A.; Hafeez, A.; Mehta, V.; Bungau, S. Ubiquitination in rheumatoid arthritis. *Life Sci.* **2020**, *261*, 118459. [CrossRef]
58. Cockram, P.E.; Kist, M.; Prakash, S.; Chen, S.H.; Wertz, I.E.; Vucic, D. Ubiquitination in the regulation of inflammatory cell death and cancer. *Cell Death Differ.* **2021**, *28*, 591–605. [CrossRef]

MDPI AG
Grosspeteranlage 5
4052 Basel
Switzerland
Tel.: +41 61 683 77 34

Nutrients Editorial Office
E-mail: nutrients@mdpi.com
www.mdpi.com/journal/nutrients

Academic Open
Access Publishing

mdpi.com

ISBN 978-3-7258-2548-6