CLAIMED EFFECTS, OUTCOME VARIABLES AND METHODS OF MEASUREMENT FOR HEALTH CLAIMS PROPOSED UNDER EUROPEAN COMMUNITY REGULATION 1924/2006 IN THE FRAMEWORK OF BONE HEALTH.

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DECLARATIONS OF INTEREST

None.

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LIST OF ABBREVIATIONS:

ALP: Alkaline Phosphatase; BMC: Bone Mineral Content; BMD :Bone Mineral Density; CPII: C-terminal type II procollagen peptide; CT: Computed Tomography; CTXII: C-terminal crosslinking telopeptide; DXA: Dual energy X-ray Absorptiometry; ECLIA: Electrochemiluminescence Immunoassay; ECM: Extra-Cellular Matrix; ELISA: Enzyme-Linked Immunosorbent Assay; FRAX: Fracture Risk Assessment Tool; HPLC: High Pressure Liquid Cromatography; MRI: Magnetic Resonance Imaging; OA: Osteoarthritis; PICP: C-terminal type I procollagen peptide; PIINP: N-terminal type II procollagen peptide; PINP: N-terminal type I procollagen peptide; pQCT: Peripheal Quantitative Computer Tomography; PTH: Pararathyroid Hormone: QCT: Quantitative Computer Tomography; RCTs: Randomized Controlled Trials; SPA: Single Photon Absorptiometry; VAS: Visual Analogue Scale; VFA: Vertebral Fracture Assessment; WHO: World Health Organization; WOMAC: Western Ontario and McMaster Universities.

ABSTRACT

Several foods or food components have been the object of application for authorization of health claims on bone health, pursuant to Regulation EC 1924/2006. For most of them, the European Food Safety Authority (EFSA) has issued negative opinions mainly for reasons pertaining to an insufficient substantiation of the claim, incluiding the choice of not appropriate outcome variables (OVs) and methods of measurement (MMs). The present manuscript refers to the collection, collation and critical analysis of OVs and MMs related to bone health compliant with the Regulation. The definition of the keywords, the PubMed search strategies and the creation of databases of references were performed to critically analyse the OVs and their MMs on the basis of the literature review. The assessment of each OV and related MM was defined according to its appropriatness in relation to the claimed effects proposed. The results obtained are relevant for the choice of the best OVs and MMs to be used in randomized controlled trials to substantiatiate the claims on bone health. Moreover, the results can be used by EFSA during the update of guidance for the scientific requirements of health claims on bone health.

Keywords: health claim; outcome variable; methods; bone.

1 INTRODUCTION

Bone health is an important factor in determining an adequate quality of life. In fact, in spite very few people die as a direct result of bone disease, these diseases can have a significant impact on the everyday lives of those who suffer from the disease, other than being responsible for high healthcare costs[1]. Among bone diseases, defined as conditions that result in the impairment of normal bone function and can make bones weak, the most common is osteoporosis, characterized by low bone mass and deterioration of bone structure, which predisposes to an increased risk of fractures especially in the elderly and mostly in postmenopausal women[2]. It has been estimated that osteoporosis causes up to 9 million fractures annually worldwide[3].

Following fractures, like hip fractures in the elderly, most people are not able to return to their activities of daily living, with a loss of independence that can have negative consequences on the emotional domains of the quality of life for both the individuals who suffer them and for their families [4,5].

In spite bone health can be influenced by genetic factors, controllable lifestyle factors such as diet and physical activity are responsible for a notable portion of bone mass and structure[6].

Regarding nutrition, it has been shown that a balanced diet can help increase or preserve bone mass. In particular, calcium and vitamin D intake are now known to be major contributors to bone health, even if also other nutrients can play a role in this scenario. That is why most of the dietary guidelines recommend the daily consumption of calcium and vitamin D-rich sources such as dairy foods[7,8].

In this scenario, many foods or food component have been the object of applications for authorisation of health claims pursuant to Regulation EC 1924/2006. Some of these applications have received a positive opinion by the European Food Safety Authority while other received negative opinions due to different reasons. These may include an insufficient characterization of the food/food component, the choice of a not appropriate claimed effect or an insufficient substantiation of the claim (e.g. for reasons belonging to sample size, statistical analysis, characteristics of the

168 subjects, or to the not appropriate choice of outcome variables (OVs) and/or methods of
169 measurement (MMs)).
170 In this scenario, a project has been developed with the aim of improving the quality of applications
171 provided by applicants to EFSA, through an appropriate choice of OVs and MMs, as described in
172 Martini et al. (2017)[9]. This manuscript referes to the collection, collation and critical analysis of
173 OVs and MMs related to bone health.

174 2 MATERIALS AND METHODS: SEARCH STRATEGY

175 OVs and MMs were collected from the relative Guidance document (EFSA 2011), from the
176 applications for authorization of health claims under Articles 13.5 and 14 of Regulation 1924/2006
177 related to bone health, as well as from comments received during public consultations. As described
178 in Martini et al. (2017)[9], the OVs and their MMs were included only if the food/food constituent(s)
179 was sufficiently characterized and the claimed effect was considered to be beneficial. Following this
180 decision tree, 3 claimed effects with 8 OVs were evaluated under Article 13.5, whereas 2 disease
181 risk reduction claims and 1 claimed effect referred to children development were selected under the
182 Article 14. For each OV, a database of references was created on PubMed and was used for the
183 critical analysis of the OVs and the MMs. Each OV and related MM was ranked in one of the
184 following categories: (i) appropriate; (ii) appropriate only/better if in combination with other OV or
185 MM; (iii) not appropriate per se; (iv) not appropriate in relation to the specific claimed effect
186 proposed by the applicant(s), (v) not appropriate alone, but useful as supportive evidence for the
187 scientific substantiation of the claimed effect.

3 RESULTS: CRITICAL EVALUATION OF OUTCOME VARIABLES AND METHODS OF MEASUREMENT

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3.1.1 IMPROVEMENT/MAINTENANCE OF BONE MASS

3.1.1.1 BONE MINERAL DENSITY

It is well assessed that bone is a metabolically active tissue and its mass results from the co-existing activity of osteoblasts and osteoclasts, leading to a balance between bone deposition and resorption during adult life. Thus, the bone mass is the total amount of trabecular and cortical bone, the last representing 20% of total bone in the body[10]. Bone mass is considered as a synonym of bone mineral density (BMD); indeed, based on the evaluation methodology, bone mass amounts the sum of two components: areal BMD, which is a two-dimensional measurement, expressed in g/cm^2, usually obtained through Dual energy X-ray Absorptiometry (DXA) scans, and volumetric BMD, expressed in g/cm^3, which is a 3D measure given by Quantitative Computer Tomography (QCT). Volumetric BMD can discriminate between cortical and trabecular bone, thus emerging as qualitative, other than quantitative medical tool only. Physiologically, BMD reaches its peak in the early adulthood in both males and females and subsequently declines with ages from the fifth decade[11], even if lifestyle (e.g. cigarette smoking, excessive alcohol consumption, prolonged immobilization) or genetic factors can accelerate this process. At the opposite, bone mass increases in response to increased mechanical stimuli (e.g. physical activity and gravity), that are able to at least maintain bone homeostasis. Bone mass is also influenced by ethnic differences and sex[12]. BMD distribution describes the local mineral content of the bone matrix, reflecting mineralization kinetics, bone turnover, and average bone matrix age. Any deviations from normal BMD distribution has significant biological and clinical relevance.

To evaluate the appropriateness of BMD as OV of improvement/maintenance of bone mass, the literature deriving from database #1 was critically evaluated (Table 1).

BMD measurement is widely carried out both in physiologic and in pathologic context to evaluate bone strength and a well consolidated tool for fracture risk assessment and management [13]. The peak bone mass (i.e. the total amount present in the body at the accomplishment of skeletal growth) is a significant determinant of fracture risk especially in the elderly when risk of falling is an additive risk for fractures. Considering that vertebral fracture is the hallmark of osteoporosis, bone mass, and in particular its component, i.e. areal BMD, is a valuable parameter for diagnosis and follow-up of osteoporosis in the presence or in the absence of pharmacological intervention. Sites where BMD is frequently measured are hip, lumbar spine and femoral neck[12]. BMD analysis is recommended in case of previous fractures in adult life occurring spontaneously, history of parental hip fractures, current smoking, glucocorticoids exposure, daily alcohol intake malnutrition, premature menopause (< 45 years) and pathologies as rheumatoid arthritis, osteoporosis, type I diabetes, chronic liver disease, osteogenesis imperfecta, long-standing untreated hyperthyroidism and hypogonadism. By the fact that the absolute risk of fracture is not the same between women and men and that it is also influenced by age, BMD measurement must be adjusted for gender and age differences. BMD measurements can be expressed quantitatively by comparing the results to those obtained in healthy young adults, or age-matched adults of the same sex. The former comparison defines whether a person has a bone mass reduction or osteopenia, while the latter defines, in part, a person's future fracture risk, relative to a cohort of the same age and sex. Thus, BMD values are expressed as z-scores, the number of standard deviations reflecting how a patient's BMD differs from the average BMD corresponding to their age and sex in the whole population. Currently, WHO score is used to define BMD: a T-score $\geq$ -1 means normal bone, a T-score between -1 and -2,5 denotes osteopenia and a T score $\leq$ -2,5 stands for osteoporosis[14]. Thus, even if the evaluation of BMD alone is sufficient for the assessment of bone mass and bone health status, a combination of BMD and vertebral fracture assessment (VFA) or, even better, a combination of BMD, VFA and FRAX significantly increases the efficacy in identifying individuals who need treatment[13]. In conclusion,

BMD can be used as appropriate outcome variable for the scientific substantiation of health claims in the context of improvement/maintenance of bone mass.

3.1.1.1.1 DXA

DXA, also known as bone densitometry or bone density scanning, can accurately analyze bone and non-bone tissue, providing a quantification of BMD, bone mineral content (BMC), fat mass and soft lean mass. It is considered the gold standard by WHO for measuring bone mass[15]; it has been validated across age groups, from premature infants to older adults, including both normal and overweight subjects. The use of DXA in infants and children is gradually increasing, with the aim to understand the impact of disease on bone health or nutritional impact on body composition. DXA is a peculiar imaging modality which differs from other X ray systems because requires special beam filtering and near perfect spatial registration of two attenuations. Indeed, DXA system creates a two dimensional image that is the combination of low and high energy attenuations. Although density is typically given by mass per unit volume, DXA can only quantify the bone density as a mass per unit area, since it uses planar images and cannot measure the bone depth. By the fact that a two-dimensional output is given, DXA-based bone mass cannot distinguish between bone compartments (i.e. trabecular and cortical tissue)[10]. Nevertheless, regarded as a safe, with a minimal radiation exposure (0.1 μGy), fairly fast (6-7 min for total body, 1-2 min for lumbar spine and 2 minutes for proximal hip assessment), convenient, accurate and non-invasive method, DXA is frequently used in many clinical settings[16]. On the other hand, it is relatively more expensive than others and requires expert skills. Another limitation of DXA scanning is the need to remain perfectly still during the entire scan[17].

Whole body DXA scans is primarily used for bone mass measurements in children and for body composition measurements in adults, while several common measurement sites, including the lumbar spine, the proximal hip and the forearm, are preferred when measuring BMD to diagnose osteoporosis or other bone loss-related diseases, to follow-up osteoporosis treatment and to assess

the risk of bone fractures. Importantly, as for the intervention studies, DXA measurement is made at baseline and then not earlier than 12 months, which is considered the most appropriate follow-up interval to detect (if any) significant changes in BMD and/or BMC.

In summary, DXA is generally an appropriate method to assess BMD in human intervention studies.

3.1.1.2 BONE TURNOVER MARKERS

Bone is a metabolically active organ undergoing a continue remodeling process which leads to approximately 20% of bone tissue renewed annually throughout the entire life. Circulating bone turnover markers are biological factors reflecting either osteoclastic (resorption) or osteoblastic (formation) activity and offer surrogate measures of bone status, including bone density and bone metabolism[18,19]. Type I collagen is the major bone tissue protein and undergoes peculiar post-translational modification in connective tissues so that type I collagen-based markers are specific markers of bone metabolism. The bone turnover markers can be classified into two groups: markers of bone resorption and markers of bone formation. The main markers of bone formation are bone alkaline phosphatase (bALP), osteocalcin, carboxy-terminal propeptide of type 1 procollagen (P1CP) and procollagen type 1 N-terminal propeptide (PINP). The markers of bone resorption include pyridinium cross-links (pyridinoline and deoxypyridinoline) and their associated peptides (collagen type I N-terminal telopeptide, collagen type I C-terminal telopeptide), released during collagen breakdown [18-20].

To evaluate the appropriateness of bone turnover markers as OV of improvement/maintenance of bone mass, the literature deriving from database #2 was critically evaluated (Table 1).

Biochemical markers of bone formation and resorption allow to assess and monitor the status of the skeletal system. In detail, they allow to evaluate the structural and functional conditions and the rate of metabolic processes undergoing in bone tissue [19]. The primary criterion for a useful marker of bone turnover is to reflect the underlying bone changes so as to avoid the need for bone biopsy and to allow clinicians to manage bone disease. Moreover, because the activity of osteoblasts and

osteoclasts is intertwined during normal bone remodeling, bone formation and bone resorption markers must be used together to provide an indication of overall bone turnover. Despite the fact that the above markers are widely used in clinical and research practice, they are not disease-specific also because some of them are produced by non-bony tissues. Thus, the obtained results should always be evaluated taking into account the clinical background, as well as having a firm understanding of the biological sources of each marker. As a matter of fact, bone turnover markers must be evaluated together with other markers of general health [18] in order to ensure that these markers specifically refer to bone-related diseases. For a thorough evaluation, special attention must be paid to type I collagen fragments in blood or urine as they are direct markers of bone formation and indirect markers of bone resorption owing to the fact that type I collagen is the major bone tissue protein. The assessment of selected bone markers allows to investigate the rate of spontaneous bone loss and to monitor the progression of bone disease in both adults and in children. Most bone turnover markers exhibit significant within-subject biological variability but also subject-indipendent variability, both from pre-analytical and analytical factors [20]. In addition to standard factors of assay performance (e.g. choice of sample collection and storage), technical sources of variability are also present. Therefore, knowledge of the sources of variability and of the strategies used to minimize them are mandatory to obtain reliable and meaningful results.

In conclusion, bone turnover markers are not appropriate outcome variables to be used alone for the substantiation of health claims regarding the improvement/maintenance of bone mass and the normal bone growth and development in children. However, they can be used in support of a mechanism through which the food/constituent could exert the claimed effect, in addition to BMD.

3.1.1.2.1 DIRECT COMPETITIVE ELISA

Many methods can be applied to measure bone turnover markers. The most used methods are based on immunological techniques and include RIA, ELISA, electrochemiluminescence immunoassay and immunochemiluminometric assay. Due to the specific features of each marker of bone turnover,

cartilage metabolism and net collagen formation and breakdown (e.g. glycolization and other post-translational modification and structural features), the type of ELISA must be chosen on a case-by-case basis. ELISA is widely used in biomedical research for the detection and quantification of specific antigens or antibodies in blood, serum and urine. ELISA allows the detection of very small quantities of proteins, peptides, hormones, antigens or antibodies in fluids using the basic immunology concept of an antigen binding to its specific antibody. Quantitative or qualitative assessments can be done on the base of a colorimetric reading. ELISA techniques include direct and indirect methods. A further subdivision is between competitive or noncompetitive assays. Direct competitive methods allow to quantify specific antigens such as bALP, C2C, CPII, CTXI, CTXII[21]. The general procedure is as follows. First, the primary antibody is incubated with the antigen of interest and the resulting antibody-antigen complex is added to wells coated with the same antigen. After an incubation period, unbound antibodies are washed off. A secondary enzyme-conjugated antibody is then added, followed by a substrate eliciting a chromogenic or fluorescent signal. Lack of coloration indicates the presence of the antigen in the sample. Competitive ELISA kits include enzyme-conjugated antigens in addition to enzyme-conjugated antibodies. In the former case, the labeled antigen competes with unlabeled sample antigen for the primary antibody-binding site. Thus, the signal is indirectly proportional to the quantity of sample antigen retained in the well. The main advantage of ELISA is its high sensitivity as it detects compositional differences in antigen mixtures (whose purification is not preliminarily required) even when the specific detecting antibody is present in small amounts. However, a limitation of ELISA is that it requires the production of the appropriate antibody (or antigen) for detecting the given antigen (or antibody). Owing to the non-specific binding of the antibody (or antigen) to the plate, false positive results may occur. False positive findings may also occur due to the fact that the enzyme-mediated color change reacts over time and the fact that color intensity inaccurately reflects the amount of primary antibody in the samples. For this reason, the use of a blocking solution is crucial for limiting false positive results.

Despite these limitations, ELISA is presently the most used method because of its relative simplicity, but HPLC is better suited to serve as reference method[22].

In summary, direct competitive ELISA assays seem to be appropriate methods to quantify bone turnover markers, cartilage metabolism markers, as well as net collagen formation and breakdown markers. However, owing to the peculiarities of each biomarker, a case-by-case evaluation is required to choose the most adequate method.

3.1.1.2.2 DIRECT NONCOMPETITIVE ELISA

As described in Section 3.1.1.2.1., many methods can be applied to measure bone turnover markers. Among ELISA methods, this can be classified as competitive and noncompetitive. Sandwich ELISA is a noncompetitive direct technique that can be used to quantify some markers of bone turnover, cartilage metabolism and net collagen formation and breakdown, e.g. CTXI[23,24]. The general procedure is as follows. First, the antigen-specific antibody is blocked onto the well surface and the biological fluid (blood, serum or urine) containing the antigen to be detected is applied to the plate. A specific primary antibody is then added that "sandwiches" the antigen. Enzyme-linked secondary antibodies are applied that bind to the primary antibody. Unbound antibody-enzyme conjugates are washed off. Substrate is added and is enzymatically converted to a color that can be quantified. One advantage of using sandwich ELISA is that it does not need to purify the antigen from the sample, thus simplifying the assay and increasing its specificity and sensitivity. Moreover, sandwich ELISA allows the detection of an antigen/ antibody at very low concentrations. However, a limitation of ELISA is that it requires the production of the appropriate antibody (or antigen) for detecting the given antigen (or antibody). Owing to the non-specific binding of the antibody (or antigen) to the plate, false positive results may occur. False positive findings may also occur due to the fact that the enzyme-mediated color change reacts over time and the fact that color intensity inaccurately reflects the amount of primary antibody in the samples. For this reason, the use of a blocking solution is crucial for limiting false positive results. Despite these limitations, ELISA is presently the most used

method because of its relative simplicity, but HPLC is better suited to serve as reference method[22,24].

In summary, direct noncompetitive ELISA assays seem to be appropriate methods to quantify bone turnover markers, cartilage metabolism markers, as well as net collagen formation and breakdown markers. However, owing to the peculiarities of each biomarker, a case-by-case evaluation is required to choose the most adequate method.

3.1.2 MAINTENANCE OF JOINT FUNCTION

3.1.2.1 JOINT MOBILITY

The statement "joint mobility" refers to the distance and direction to which a joint can be extended. The range of joint motion is a function of the conditions not only of the joint itself but also of the surrounding muscles and connective tissues involved. Starting from this definition, it is clear that only joints implying a certain degree of movement are considered. Indeed, joints are functionally classified in synarthrosis, which permit no or very limited movement, like skull suture, amphiarthrosis, permitting slight mobility and exemplified by intervertebral discs, and diarthrosis which allow a wide range of movement and are usually known as synovial joints. The range of mobility depends on the underlying joint structure, and in particular the degree of collagen cross-linking, which in turn attracts and holds water, leading to increased joint mobility. In older subjects, the range of motion progressive diminishes, due to the loss of water and the progressive intensification of the crosslinking between collagen molecules. There are also clear evidences that, other than age, factors influencing joint mobility are genetic background, gender and ethnical group[25]. In particular, healthy females have been seen to have a higher degree of motion respect to males of the same age. Joint mobility can be affected by such diseases, either increasing (e.g. hypermobility condition, Ehlers-Danlos syndrome)[26] or decreasing it (diabetes mellitus, osteoarthritis, rheumatoid arthritis)[27].

To evaluate the appropriateness of joint mobility as OV of maintenance of joint mobility, the literature deriving from database #3 was critically evaluated (Table 1).

The evaluation and examination of joint mobility is usually carried out by clinician to verify the correct articular development, in children, and state, in adults; moreover, joint mobility is also a useful tool to assess the proper musculoskeletal function. In this regard, joint mobility is considered as a surrogate measure of muscle tone owing to the common difficulty of directly assess the muscle state. Thus, joint mobility is routinely assessed both because it provides information on articular status and with the purpose to integrate it with other parameters of musculoskeletal functioning. In this regard, one study focusing on hypermobility condition elucidates the correlation between joint mobility and motor development in infancy. Another investigation demonstrates the association between impaired joint mobility in children with a higher risk for microvasculature[27]. Despite the showed importance of the correct articular mobility, primarily for the maintenance of joint function and, secondarily, for the general health status, limited research has been so far conducted relating to therapists' ability to reliably identify a joint exhibiting signs of dysfunction. By the fact that no standardization, neither in clinical parameters nor in equipment, has been yet achieved, there is a need to develop a standard protocol for joint mobility assessment, taking age, gender and ethnic origin into consideration, too[25]. Despite these limitations, joint mobility can be used as appropriate outcome variable for the scientific substantiation of health claims in the context of the maintenance of joint function.

3.1.2.1.1 GONIOMETERS

The functional performance and the mobility of different joints (e.g. knee, ankle), clinically defined as range of motion, are traditionally assessed with validated protocols and procedures under well-defined testing conditions using appropriate goniometers. The usual analog goniometer is a simple and easy-to-use instrument and is the most common device used by clinicians and physiotherapists to perform the measurement of joint angle position with recording capability of one degree (1°). The

goniometer must be manually aligned with anatomical landmarks like the lateral epicondyle of the humerus and the tip of the acromion, and this fact is one of the main source of errors. Additionally, literature studies report that range of motion measurement with goniometers can be affected by movement of adjacent joints and variation between patients, all factors decreasing the reliability of the method[28,29]. Nevertheless, due to its easiness and convenience, the analog goniometer has been for a long time considered as the standard method against which to compare and validate alternative devices. Nowadays the advent of digital technologies has overshadowed the analogic goniometer in favor of much modern instrumentation sharing higher precision, validity and reliability, like optoelectric systems, digital inclinometers, gyroscopes, accelerometers, and/or combination of such sensors, e.g. in wireless micromechanical systems. Electrogoniometers using video are a relatively new and precise method to quantitatively measure joint performance and range of motion [29] A limitation of such devices, shared with goniometer, is that the presence of the physiotherapist or technician is required at the time of measurement, impeding the evaluation during non-supervised activity. Another disadvantage of using traditionally goniometer is that the measurements obtained are only referred to the movement of one joint, and collected data could be affected by a different evaluator or operation bias. On the other hand, the systems nowadays available have disadvantages to be labor intensive, time-consuming, expensive, and difficult for clinicians and researchers to use, thus bringing the operator in front of the choice between “practice but inadequate” and “reliable but expensive”.

In summary, goniometers represent an appropriate method to assess joint mobility in human intervention studies.

3.1.2.2 CARTILAGE METABOLISM MARKERS

The generic term “cartilage” encompasses different types of cartilaginous tissues, all sharing the feature of being a supporting, specialized connective tissue. Articular cartilage, also named hyaline cartilage, is one of the three form of cartilage found in the human body, namely the hyaline, elastic

and fibrous cartilage, identifiable by the variation of the combination of the ECM components. Articular cartilage, which is located in diarthrodial joints, is devoid of nerves, blood and lymphatics vessels, and therefore it has limited capacity for intrinsic healing and repair. A cartilage biomarker of ECM is a molecule, or fragment thereof, which is released into biological fluids during tissue biosynthesis and turnover and which can usually be measured by immunoassays. Type II collagen can be targeted as hyaline cartilage biomarker by the fact that is one of the major constituent of cartilage matrix, representing 90-95% of total cartilage collagen[30,31]. Collagen types I, IV, V, VI, IX, and XI are also present but only in a minor proportion and help to form and stabilize the type II collagen fibril network. Metabolic alterations in articular cartilage, mainly due to reiterated wear and mechanical overloading, have a pathological meaning and, for this reason, urinary levels of fragments of type II collagen are clinically assessed. CTXII and the neoepitope C2C which is generated by denaturation of the triple helix domain of type II collagen, are considered biomarkers of cartilage degradation, while PIINP and CPII are fragments targeted as biomarkers of cartilage synthesis[31,32].

To evaluate the appropriateness of cartilage metabolism markers as OV of maintenance of joint mobility, the literature deriving from database #4 was critically evaluated (Table 1).

Hyaline cartilage tissue is characterized by an anaerobe environment with neither blood nor lymphatic vessels and, therefore, the chondrocytes, the cellular components of cartilage secreting the ECM, are in turn primarily dependent on ECM homeostasis for protection and nutrient supply. In this regard, the metabolic state of ECM correlates with the balance between degradation of different macromolecules and their replacement by newly synthetized products. Monitoring matrix molecules as biomarkers is a powerful tool for the assessment of the health condition of the cartilage because their levels allow the evaluation of the structural and functional conditions and also the rate of metabolic processes thus providing a clear insight on the proportion between degradation and neo synthesis[31,32]. Indeed, the primary criterion for a useful biomarker of cartilage metabolism is to

462 reflect underlying tissue changes, hence avoiding the need for cartilage biopsy and enabling
463 clinicians to manage diseases (even when they occur in childhood) based on surrogate measures.
464 Even if a wide range of ECM components (e.g. aggrecans) are available for this purpose, a special
465 attention must be paid to the investigation of type II collagen fragments content in biological fluids
466 (frequently urine because of its accessibility and non-invasiveness) as markers of formation and
467 resorption, because collagen type II is the major ECM protein of the articular cartilage. Several
468 studies found in literature identify the meaningful role of measuring cartilage metabolism markers
469 levels for the early diagnosis and the prediction of progression of joint related diseases like
470 osteoarthritis, rheumatoid arthritis, juvenile inflammatory arthritis, polychondritis etc[30]. The
471 analysis of cartilage metabolism is usually carried out through CTXII and C2C as indicators of
472 degradation, and through PIINP and CPII for synthesis; the ratio among them is also a useful tool
473 that allows an estimate of the potential therapeutic response to the treatment. Nevertheless, changes
474 in their serum or urine levels are only indicative of an altered condition of cartilage metabolism and
475 do not directly demonstrate an alteration of joint function. Therefore, cartilage metabolism markers
476 are not appropriate outcome variables to be used alone to substantiate the health claims regarding
477 the maintenance of joint function. However, they can be used as supportive evidence of a mechanism
478 through which the food/constituent could exert the claimed effect.

479 #### 3.1.2.2.1 DIRECT COMPETITIVE ELISA

480 See Section 3.1.1.2.1

481 #### 3.1.2.2.2 DIRECT NONCOMPETITIVE ELISA

482 See Section 3.1.1.2.2

483 ### 3.1.2.3 WOMAC INDEX

484 The Western Ontario and McMaster Universities (WOMAC) Index, developed in the early 1980s,
485 is a disease specific OV of health status, based on self-reported symptoms assessed through the
486 WOMAC questionnaire. The WOMAC Index has 3 subscales, totally containing 24 items, 5 referred

to pain, 2 referred to stiffness and 17 regarding articular function. It produces partial scores from the evaluation of pain, stiffness and function, which can be considered both separately and summed together to give a total WOMAC score. Specifically, scores range from 0 (least pain) to 20 (highest pain) for pain, from 0 (least stiffness) to 8 (highest stiffness) for stiffness and from 0 (best function) to 68 (worst function) for articular function, leading to a total score ranging from 0 (best health) to 96 (worst health)[33]. Data provided by WOMAC Index are frequently collected in studies of osteoarthritis, rheumatoid arthritis and other chronical inflammatory disease affecting joints[33,34]. WOMAC Index is also widely used to evaluate the effect of therapies in the treatment of arthritic diseases and to follow-up joint replacement surgery. Moreover, several clinical studies demonstrated the validity, reliability and responsiveness of WOMAC Index in orthopedic context when is applied to elderly patients with hip and femoral neck fracture or that underwent joint replacement arthroplasty [35,36].

To evaluate the appropriateness of WOMAC Index as OV of maintenance of joint mobility, the literature deriving from database #5 was critically evaluated (Table 1).

WOMAC Index has been validated for the assessment of joint pain stiffness and loss of function related to osteoarthritis and other joint-related diseases. Self-report instruments, such as WOMAC Index, are usually preferred over physical performance measures. However, it has been shown that these evaluation tools provide complementary information; thus, both are valuable to perceive the multidimensional impact of pain and the construct of physical function in its totality[33]. WOMAC Index is also a reliable tool for monitoring the quality of life in patients with hip or knee joint disease[34]. On the contrary, even if it is disease specific, a lot of working groups tend to collect data, obtained through the administration of the WOMAC questionnaire, from subjects affected by chronic inflammatory disease of joint, such as osteoarthritis or rheumatoid arthritis, in order to then compare these results to the target health population. In this regard, it must be highlighted that WOMAC Index is valuable for health-related researches because it measures relevant parameters

for health status, such as pain and articular mobility, but it must be only referred to a disease-affected target population, thus avoiding prediction errors to be generated in the assessment of individual level of quality of life[34]. Thus, even if the WOMAC Index is a reliable and valid assessment tool in the evaluation of patients with joint diseases, showing excellent reliability and validity properties, it is not appropriate for the scientific substantiation of health claims related to the maintenance of joint function in healthy population. On the contrary, the use of the WOMAC Index fulfills the need to monitor joint-related conditions in patients.

3.1.2.3.1 WOMAC QUESTIONNAIRE

The WOMAC Index is obtained through a disease-specific twenty-four-item questionnaire (scored on a 5-point Likert scale) measuring joint pain, stiffness, and function in patients suffering of arthritic diseases or fractures [37]. It has been extensively validated in patients who underwent knee and hip arthroplasty because of osteoarthritis, too. The 24 items, included in 3 subscales (pain, stiffness and function) are the following 5 items for pain, 2 items for stiffness and 17 items for physical/ articular function:

- pain: during walking, using stairs, in bed, sitting or lying, and standing;
- stiffness: after first waking and later in the day;
- physical function: stair use, rising from sitting, standing, bending, walking, getting in / out of a car, shopping, putting on / taking off socks, rising from bed, lying in bed, getting in / out of bath, sitting, getting on / off toilet, heavy household duties, light household duties.

Composite summary scores are created from each subscale and then used in data analysis studies. The WOMAC total score, generated by filling out the WOMAC questionnaire and summing up the subscale-related scores, is widely employed in both epidemiological and observational studies and also used to monitor changes due to therapeutic treatments including pharmacotherapy, arthroplasty, physical exercise, physical therapy, knee bracing, and acupuncture. The WOMAC questionnaire has

been adapted, translate and cross-cultural validated for different countries [35]. Multinational studies have shown that the resulting WOMAC Index has strong disease-related properties, being the most widely used measure for assessing self-reported pain, stiffness, and function in patients with fractures, osteoarthritis and rheumatoid arthritis. Moreover, literature studies have validated the administration of the WOMAC questionnaire to subjects with different conditions such as low back pain, Systemic Lupus Erythematosus and fibromyalgia. The WOMAC questionnaire has the advantages of being noninvasive, easy comprehensible, thus quick to complete and easy to administer[33]. Nevertheless, some limitations in using composite summary scores are to be accounted, such as the fact that the significance on the components does not necessarily imply significance of the composite (e.g. one intervention having a positive effect on one component but a negative effect on another component results in a non-statistically significant composite). Other that, bias can also be generated whether the relative importance of the components differs: in this case it is advisable to consider most severe events per se, rather than as a part of the composite.

Finally, all these aspects considered and despite the disadvantages, the WOMAC questionnaire is the assumed standard method to obtain the WOMAC Index. In addition, it is an appropriate method to be used for the measurement of joint pain, considering the specific score related to it.

3.1.2.4 JOINT PAIN

Medical community agrees in defying joint pain such as an unpleasant sensation referred to discomfort and aches which has both physical and emotional components. The physical part of pain results from stimulation of peculiar nerve terminations, i.e. the nociceptors. By the fact that pain is a self-reported sensation, the emotional component greatly influences pain perception and tolerance, making the objective pain assessment difficult and occasionally misleading. In this regard, socio-economical and psychosocial factors have been seen to be strictly associated with pain severity perception and higher are life satisfactory and educational level, lower is the pain declared [38]. Age is the main risk factor for joint pain onset, which can be localized to a single joint or more diffuse,

562 generalized. Thus, even if joint pain is usually recorded among older people, children can also be
563 involved and, in this regard, musculoskeletal pain represents a frequent reason for children, mainly
564 from 3 to 14 years old, presentation to primary care[39]. Painful sensation is widely experienced by
565 active, sportive people due to joint injuries of various degree, from distortion to fractures and it is,
566 in that case, usually benign and self-resolving. Otherwise, joint pain can be among the symptoms of
567 several pathological conditions, most commonly osteoarthritis and rheumatoid arthritis [40] but also
568 fibromyalgia, bursitis, cancer, Systemic Lupus Erythematosus, rickets and sarcoidosis.
569 To evaluate the appropriateness of joint pain as OV of maintenance of joint mobility, the literature
570 deriving from database #6 was critically evaluated (Table 1).
571 The musculoskeletal pain affecting the healthy population, which is primarily due to joint stress,
572 injuries surgical outcome and trauma, should be a great point of interest for clinicians by the fact
573 that, correlating with its severity, it has been seen to be associated with physical and psychosocial
574 disability, leading to poor mobility, difficulty with daily-life activities, social isolation and also loss
575 of employment opportunity. Nevertheless, relatively to literature studies, joint pain is almost never
576 considered as a risk factor for the onset of osteoarticular diseases in healthy people, highlighting it
577 as a prognostic index. Rather, the medical community agree in considering the painful sensation
578 perceived at joints as a symptom and, as example, its assessment is reported in multiplicity of works
579 concerning knee or hip osteoarthritis, whose mean feature is indeed joint pain [37,38]. On the lights
580 of these evidences, pain severity is routinely measured with self-reporting, disease-specific
581 instruments, like the WOMAC questionnaire and the Visual Analogue Scale (VAS), with the
582 substantial limitation that the extrapolation of results obtained in patients with joint diseases, to the
583 target, healthy population is not possible. Thus, even if joint pain evaluation is a valid assessment
584 tool in the context of articular diseases, it is not appropriate for the scientific substantiation of health
585 claims related to the maintenance of joint function in healthy population. On the contrary, the pain

586 measurement, together with other parameters of Health Related Quality of Life[41], fulfills the need
587 to monitor joint-related conditions in patients.

588 #### 3.1.2.4.1 WOMAC QUESTIONNAIRE

589 See Section 3.1.2.3.1

590 #### 3.1.2.4.2 VISUAL ANALOGUE SCALE

591 The VAS is a method commonly used for the evaluation of severity of joint pain and relief, thanks
592 to its easiness to use, reproducibility and the variety of clinical practices it can be applied to[41]. In
593 general, VAS has been developed to measure a parameter (in this case, pain) that is believed to range
594 across a continuum of values and therefore not directly measurable. Operationally, a VAS is usually
595 a line both vertical and horizontal, 100 mm in length, flanked at each end by word descriptors. The
596 patient is asked to rate his current pain perception on a scale of 1-10. The rating of "1", on the left,
597 corresponds to a mild discomfort from time to time, while "10", on the right end of the scale, means
598 the worst possible pain. By the fact that the pain assessment with VAS is clearly highly subjective,
599 the VAS is useful when looking at changes in pain severity within individuals, whereas it is less of
600 value for correlating results across a group of individuals at onetime point. As such a subjective tool,
601 reliability of VAS has to be primarily assessed, thus several studies focused on the evaluation of
602 VAS reliability in measuring both acute and chronical joint paint, confirming its high reliability.
603 Practically, a few minutes (usually from one to ten) after the first VAS, the patient is asked to rate
604 his pain severity again on a fresh VAS without reference to the first measurement. Then, parametric
605 statistical tests are used to analyze the derived data, leading to the determination of the smallest
606 significant change in pain severity that is clinically important. It depends on the type of pain taken
607 into account and on the time occurring between the two measurement, but it usually ranges from 9
608 to 13 mm with a confidence index higher than 90%. The VAS is frequently used in combination
609 with other tools measuring pain intensity, such as the Faces Pain Scale-Revised, which has a high
610 degree of concurrent validity and includes six facial expressions covering the entire range of pain

levels in a hierarchical order[42]. Thus, the summed score obtained by the combination of the two previous techniques describes pain according to the facial expression of patient, leading to the translation of subjective pain into a quantitative numeric measure. Furthermore, literature data demonstrated the high correlation between WOMAC pain scale and VAS pain scale across several joint-related diseases, like osteoarthritis, rheumatoid arthritis and fibromyalgia.

In conclusion, the VAS is an appropriate method, better if used in association with another pain evaluation method, for the assessment of joint pain.

3.1.2.5 JOINT SPACE WIDTH

Articular cartilage separates two adjacent bones within a joint, like knee or hip, and the area between the consecutive bone extremities is known as joint space. Physiologically, the joint space width decreases with aging in a sex-specific manner, being older women more likely to joint space narrowing than men, probably due, at least in part, to an estrogen-based mechanism[43]. Joint tissue homeostasis is characterized by the equilibrium between breakdown and regeneration of joint structural components. This is a highly-regulated mechanism that is prone to be altered by trauma or pathological events, leading to the loss of articular function and micro- and macro- architectural changes within the joint structure. Articular, hyaline cartilage is therefore often interested by damage due to trauma or degeneration, and the joint space width, which is related to the amount of cartilage, undergoes critical changes in such conditions. In this regard, osteoarthritis is a disease of the whole joint that does not only affect the cartilage thickness but also its composition and the structural appearance of all the surrounding synovial tissues, with associated clinical manifestation of pain and loss of function. Indeed, in osteoarthritis, joint space narrowing, due to cartilage breakdown, is an early event preceding osteophytes development, subchondral sclerosis, cystis formation and bone deformities. The severity of osteoarthritis heavily relies on joint space narrowing and subchondral bone lesions, and the complete loss of joint space width, leading to an abnormal bone-to-bone contact is one of the main factors in deciding for surgical joint replacement[44]. Clinically, joint

636 space width, even better known as “minimal joint space width”, is a radiological parameter used to
637 define osteoarthritis severity and progression. The threshold value of 2.5 mm is usually used as
638 cutoff for osteoarthritis diagnosis, even if it is predominantly derived from studies in men and
639 variation is also related to individual factors such as sex and age[43].
640 To evaluate the appropriateness of joint space width as OV of maintenance of joint mobility, the
641 literature deriving from database #7 was critically evaluated (Table 1).
642 Changes in joint space width, leading to changes in joint structure, is one of the main feature of
643 osteoarthritis, whose pathological process include the breakdown of hyaline cartilage and damages
644 in the surrounding joint tissue, i.e. the subchondral bone, the articular capsulae, synovium,
645 meniscum and soft periarticular tissues. The joint space width is the most generally used and
646 accepted outcome variable for the assessment of osteoarthritis severity, by the fact that both a
647 reduction in cartilage thickness and meniscal damage are clinically inferred from a reduction of joint
648 space width[44]. Differently speaking, it is worldwide assumed that loss of joint space width is a
649 surrogate marker of cartilage damage in osteoarthritis. Moreover, the minimal joint space width is
650 commonly used to assess osteoarthritis progression because it has been seen to be very sensitive to
651 changes, even tiny, occurring over time[45]. In this regard, the radiographic joint space width
652 measurement is a powerful tool, on which clinicians heavily relay for taking decision about
653 treatment[44]. Due to the fact that modifying the structural progression has become a need for drug
654 development in osteoarthritis, the joint space width is considered the essential outcome used to
655 quantify the expected rate of structural progression in clinical trials regarding the, so called, disease-
656 modifying osteoarthritis drugs[29].
657 In conclusion, established the fundamental role of joint space width measurement in monitoring
658 osteoarthritis severity and progression, and in the evaluation of joint structure response to treatments,
659 joint space width represents a disease-specific outcome measure; therefore, it is not an appropriate
660 outcome variable for the scientific substantiation of health claims regarding the maintenance of joint

function in the healthy population. On the contrary, it is worth repeating that joint space width measurement fulfills the need to monitor joint-related conditions in osteoarthritis patients.

3.1.2.5.1 ARTHROGRAM

Radiographic techniques are routinely carried out to monitor progression of common and potentially disabling diseases, as rheumatoid arthritis and osteoarthritis. The evaluation of radiographic changes in joint space width is widely considered the 'gold standard' to assess the progression of such diseases and is a common outcome variable for clinical trials [29,34,44,46]. Indeed, despite the onset of other diagnostic technologies, such as magnetic resonance imaging (MRI), which provides semi-quantitative measures of cartilage volume, thickness and composition, bone size and shape, meniscus lesions, joint effusion, synovitis and ligament status, the joint space width assessed through radiographic measurement is still considered the most appropriate method to assess and monitor both joint disease onset and progression. In this context, joint space width is a surrogate measure of cartilage degeneration and loss, and can be appreciated on radiographs by a decrease in the distance between the projected margins of the considered joint[46]. Radiography, or X-ray-based technique, is the oldest and most common imaging technique used in diagnostic, and when referred to joint also known as arthrography or arthrogram. This type of clinical test, leading to X-ray images acquisition can show not only the joint bones, but also the soft tissues lining the joint, thus being more useful than a regular planar X-ray exam in the evaluation of the whole joint structure. The traditional scoring methods for radiographic assessment, such as the Sharp[47] and the Larson and Thoen ones [48], have shown to be subjective and based on a qualitative evaluation of joints, not providing a true measure of the size of radiographic structure, rather giving a score on an ordinal scale based on comparison to representative method [46]. Due to the necessity of reproducible and quantitative surrogate outcome variable, Image analysis software has been shown to be more responsive to change than semiquantitative scoring and can be used to provide quantification of articular structural changes on a continuous scale. Computerized methods also provide automated archiving of scores

which can be directly integrated with digital imaging modalities. Moreover, several computer-based methods for the evaluation of radiographic joint space width have been recently developed, providing an objective and continuous measure with enhanced reliability and sensitivity to change. Nevertheless, also these technologies are prone to errors, mainly due to not 100% reliability of software, which should be improved through quality assurance procedures using a correction software [46]. Measurement errors can also be due to patient repositioning between radiographic acquisitions; therefore, standardization of patient positioning procedures should be required [44]. Due to radiation exposure, some precaution must be taken; pregnant women should not undergo radiographies, unless the benefits of findings would outweigh the risks of radiation exposure. Computed Tomography Arthrography and Magnetic Resonance Arthrography have been increasingly utilized in the last ten years because they combine the images provided by the standard arthrogram with the high-resolution and sensitive outputs from CT scanning or MRI.

Finally, all these aspects considered, arthrogram is an appropriate method of measurement to assess joint space width.

3.1.3 COLLAGEN FORMATION

3.1.3.1 NET COLLAGEN FORMATION AND BREAKDOWN

Collagen is an insoluble fibrous protein which is, in particular, the most abundant protein in the animal kingdom making up from 25% to 35% of the whole-body protein content. Indeed, collagen is a structural component of the ECM in several connective tissues including bones, cartilage, gums, skin, tendons and blood vessels[49-51]. In the human body there are 28 types of collagen, all sharing the same basic structural unit, that is a right-handed triple helical molecule particularly rich in glycine, proline and hydroxyproline. The distinct properties of each type of collagen mainly depend on folding, which leads to peculiar 3D structures, and on the protein segments binding the triple helix. Furthermore, post-translation modifications also play a role in characterizing the properties of each type of collagen and essential cofactors, such ascorbic acid, are fundamental to successfully

711 carry out the process, leading to the synthesis of functional collagen fibers. Type I collagen is the
712 most abundant form of collagen in the human body, and because of its enormous tensile strength it
713 is the main component of the organic part of bones and tendons, helping these tissues to withstand
714 loading and stretching forces[51]. Differently, type II is the major collagen in articular cartilage,
715 where it forms rigid macromolecules, whose reciprocal orientation allows joint to bear mechanical
716 shocks.
717 To evaluate the appropriateness of net collagen formation and breakdown as OV of collagen
718 formation, the literature deriving from database #8 was critically evaluated (Table 1).
719 Even though the main cellular type secreting collagen in the connective tissue are fibroblasts, recent
720 evidences demonstrate that others, such as epithelial cells, make certain type of collagen. Collagen
721 synthesis is based on a very complex and high regulated biochemical pathway during which various
722 immature forms of collagen are sequentially produced, then shortened and modified until mature
723 collagen molecules are obtained. As it happens during synthesis, also collagen degradation involves
724 the release of protein fragments in the extracellular space. Therefore, peculiar collagen fragments
725 are considered surrogate markers of collagen synthesis or breakdown occurring in a specific organ
726 or tissue [49,52]. As example, PIINP, CPII and PICP, PINP are fragment of type II collagen the first
727 and of type I collagen the last and therefore considered markers of cartilage and bone synthesis,
728 respectively. Similarly, recognized markers of cartilage breakdown are CTXII and neoepitope C2C,
729 while collagen type I N- and C- terminal telopeptide are indicative of bone degradation. According
730 to literature, their level variation is frequently assessed in serum or urine samples in order to monitor
731 the effect of an intervention program on the collagen turnover in different organs [52]. However, it
732 must be highlighted that variation in levels of collagen formation and breakdown not always reflects
733 the variation of functionality of a specific organ or tissue. Indeed, changes of functionality relies on
734 several variables, as example the amount of collagen present in the structure of the tissue/organ and
735 the specific relation between structure and function. Therefore, although net cartilage formation and

breakdown is appropriate to be used alone as outcome variable for the scientific substantiation of health claims related to the normal collagen formation, differently its appropriateness must be assessed on a case-by case basis whether it is considered in relation with tissue or organ functionality. In this case, it can be used as supportive of a mechanism through which the food/constituent could exert the claimed effect, in addition to the evaluation of appropriate and specific outcome variables of organ or tissue functionality.

3.1.3.1.1 DIRECT COMPETITIVE ELISA

See Section 3.1.1.2.1

3.1.3.1.2 DIRECT NONCOMPETITIVE ELISA

See Section 3.1.1.2.2

3.2 RISK REDUCTION CLAIMS Art 14(a)

3.2.1 OSTEOPOROTIC BONE FRACTURES

Osteoporotic fractures, also known as fragility fractures or minimal/low trauma fractures, represent the hallmarks of a chronic and disabling disease characterized by low bone mass and micro-architectural deterioration of bone tissue[53] resulting in decreased mechanical strength. Osteoporosis grounds its roots in childhood but generally affects adults and especially elderly worldwide, with different age and sex distribution regarding the clinical presentation. It leads to bone fragility and an increment of susceptibility to fractures even due to minimal trauma, such as strain, bump or minor fall. An osteoporotic fracture is generally defined as a fracture due to a fall from no more than standing height or less, excluding those caused by road-traffic accidents. It may occur at vertebral and non-vertebral locations, without considering hands, feet, digits, face or skull [54].

Wordwilde, 200 million of women are estimated to be osteoporotic[55]. On the basis of statistical data, it is estimated that approximately more than 50% of postmenopausal women and 30% of men over the age of 60 years will suffer at least one osteoporotic fracture during their remaining life [56].

Although osteoporotic fractures can occur in many skeletal sites, their incidence at vertebral level is relatively higher, especially in patients with postmenopausal osteoporosis. Vertebral fractures are generally classified as wedge, biconcave or crush fractures according the shape of deformity, and further as grade 1, 2 or 3, by the degree of deformity[57]. Among non-vertebral osteoporotic fractures, they occur most frequently at the hip, humerus, and wrist. Together with spinal fractures, the formers are the most serious in terms of cost and morbidity. Fractures occurring at the hip, spine and wrist, listed by order of the related disability burden, are the best characterised. Furthermore, other peripheral fractures are related to low density or poor quality of bone mass, such as proximal humeral, pelvic, rib, proximal tibia or ankle fractures [58]. Hip fractures are associated with 20-25% mortality in the 12 months after the event. Approximately 50% of the patients do not return at their prior level of self-sufficiency, many lose their independence and require long-term care. On the other hand, vertebral fractures may affect the overall quality of life causing pain and limiting the spinal movement. One-fifth of patients require hospitalization and some will require subsequent long-term care[58]. Moreover, owing to the absence of significant pain, a large proportion of vertebral fractures, mainly at lumbar level, are asymptomatic and remain undiagnosed for long time. Comparing to hip and vertebral sites, forearm fractures tend to occur at earlier ages, with a peak incidence in 40-65 years old women[58].

Osteoporotic fractures have a complex aetiology composed by both non-modifiable risk factors, such as endocrine disorders, genetic predisposition, age, sex, ethnic origin and behavioural risk factors. The most critical are represented by bone mass, which progressively decrease with age, and increased frequency of falls. Family history of osteoporosis, mainly in case of first degree of kinship, plays a major predictive role into disease development. Among modifiable risk factors, lifestyle (e.g. cigarette smoking, inappropriate diets), low body weight, drugs (e.g. alcohol, anti-epilepsy medications, loop diuretics, aromatase inhibitors and steroids), physical inactivity and low calcium intake can exert a negative effect on bone health predisposing the subject to develop this pathology.

According to the definition given by the World Health Organization (WHO)[3], in absence of a defining fragility fracture, the diagnosis of osteoporosis can be applied when bone mineral density is 2.5 standard deviations or more below the mean peak bone mass (defined as the average value for young healthy adults) measured by DXA at lumbar spine, femoral neck, total hip or one-third radius sites. However, the ability of bone mineral density (BMD) measurements to predict osteoporotic fractures is only partial, although important. In fact, about two-thirds of individuals who suffer a fracture do not present osteoporosis as defined from WHO diagnostic criteria (DXA). Because of the limited sensitivity of BMD test, different clinical risk factors have been identified in order to enhance fractures risk prediction valid both in the presence and in the absence of the BMD measurements. However, the identification of additional biomarkers will improve the assessment of fracture risk.

3.2.1.1 OSTEOPOROTIC BONE FRACTURES

See Section 3.2.1

To evaluate the appropriateness of osteoporotic bone fractures as risk factor of osteoporotic bone fractures, the literature deriving from database #9 was critically evaluated (Table 1).

Osteoporosis-related fractures, not osteoporosis *per se*, are associated with significant morbidity, mortality and health care expenditure worldwide[58]. In fact, even if population-based studies have found a consistent relationship between low BMD at different sites and mortality[59,60], the classical way to measure the burden of osteoporosis in terms of mortality is to assess the death rates after osteoporotic fractures. Several conditions, independent from BMD, have been identified as risk factors for the occurrence of fragility fractures. These include non-modifiable risk factors, but also falls, previous fractures and smoking. It has been established that the occurrence of any osteoporotic fracture predisposes to significant morbidity and premature death, besides a two/four-fold increased risk of subsequent fractures. In detail, about 30% of women and 22% of men with a prior history of fracture experience a new fracture during the following 5 years[59]. BMD measures alone have

limited sensitivity and specificity in the prediction of an osteoporotic fracture, as demonstrated by the fact that a great proportion of the overall incident fractures occurs in subjects with osteopenia. Furthermore, fractures can occur also in subjects with normal BMD. It has been reported a progressive loss of the power of BMD at the femoral neck on predicting hip fracture risk with increasing age. This fact can be explained by the higher frequency of additional clinic risk factors leading to co-morbidity in the elderly groups, whereas in a young population low BMD might be a stronger predictor of overall fracture risk. In a randomized controlled trial (RCT), the risk of osteoporotic fracture can be expressed as relative risk (RR) comparing the control and the intervention group, on the basis of the number of fractures occurred. The occurrence of fractures can also be used to obtain fracture rates (number of fracture/person-years).

In conclusion, osteoporotic bone fractures, as a direct measure of the disease itself, are appropriate outcome variables to be used alone for the scientific substantiation of such risk reduction claims.

3.2.1.1.1 X-RAY RADIOGRAPHY

X-ray radiography is a conventional tool to diagnose fractures without use of contrast agents. It is considered the gold standard method to determine osteoporotic bone fractures not only in clinical setting, but also in intervention studies where it is preferred to questionnaire. It can be explained by the fact that some osteoporotic bone fractures are asymptomatic, mainly in case of those occurring at spinal level[61]. As a consequence, the use of questionnaire (both self- and non-self-administered) to record radiography-confirmed fractures may underestimate the real number of osteoporotic fractures occurred during the period of the intervention and follow up. During radiographic process an area of the body is penetrated with X-rays and visualized on suitable film or electronic sensors. X-rays are taken in various planes while standing, sitting or lying down, depending on the area to be examined, which have to be undressed and free of foreign bodies on the skin in order to guarantee optimum image assessment. The subject's area of examination is exposed to X-rays emitted by a generator for a few milliseconds. Although radiation is reduced to minimum, cells and tissue are

836 exposed to a low risk of radiation damage. For this reason, this approach is not suitable in case of
837 pregnancy. The procedure is able to detect simple or compound fractures. However, it provides little
838 information on the involvement of surrounding muscles, sinews, ligaments or joint. Additional
839 procedure, such as MRI, CT or ultrasound can be applied in case of joints and soft tissue
840 involvement.
841 In conclusion, X-ray radiography is generally considered an appropriate method of measurement of
842 osteoporotic bone fractures.

843 3.2.1.2 FALL(S)

844 The WHO and the Kellogg International Work Group on the Prevention of Falls in the elderly
845 defined a fall as “an event which results in a person coming to rest inadvertently on the ground or
846 other lower level and other than as a consequence of the falling: sustaining a violent blow; loss of
847 consciousness; sudden onset of paralysis as in stroke; an epileptic seizure”[62]. Broader definitions
848 are available and can be chosen depending on the focus of the study. Fall is a relatively common
849 event in older people. About 30% of individuals aged ≥ 65 fall at least once a year, and about half
850 of those subjects do so recurrently. Moreover, fall(s) is one of the most important determinants of
851 osteoporotic fractures, mainly, but not only, at the hip. In fact, about 90% of hip fractures in elderly
852 result from a fall. Because falls and related risk factors are a leading cause of adverse consequences
853 in older adults, ranging from partial loss of self-sufficiency to total disability and even to death, fall
854 prevention in older people represents a major healthcare priority.
855 To evaluate the appropriateness of falls as risk factor of osteoporotic bone fractures, the literature
856 deriving from database #10 was critically evaluated (Table 1).
857 The rate of falls and the likelihood of severe injury from a fall increase with age. Although most of
858 falls do not lead to serious injury, about 5% results in a fracture or require hospitalisation for
859 community-living elderly[63]. Moreover, falls and subsequent mobility alteration induce important
860 psychosocial effects, including fear of falling and social isolation leading to a faster functional

decline. The incidence of falls, when assessed in epidemiological studies, may vary deeply based on the population investigated. Lower rates occur among community-dwelling elderly (age ≥65), generally healthy people, whereas the higher rates are reported for people living in long-term care institutions, where 10-25% of falls tends to result in more serious complications, such as fractures and disability. Although falls in the elderly are often referred to as accidents, causal processes are involved. Falls are multifactorial events not randomly occurring[64]. Several factors influence the risk of falling: intrinsic or patient-related, extrinsic or environment-related and finally behavioural or activity-related. Among intrinsic risk factors, the most important are visual difficulties, impaired physical capacity and altered cognitive function, particularly crucial in recurrent falls[63]. Thus, an effective reduction of falls and of some fall risk factors is possible ameliorating physiological impairments. Comparing the BMD values in the proximal femur of women with hip fractures with those of control of similar age, a substantial overlapping is observed. The two groups generally differ on the basis of slightly higher values for the controls. Thus, factors, other than osteoporosis, are crucial in the pathogenesis of fractures, especially at the hip. Among these, fall(s) play an important role. Therefore, the risk of falling and the risk of falling at least once may be higher in older women with osteoporosis than counterparts without osteoporosis because of greater impairments in muscular strength and balance. In a RCT the risk of falls and the risk of falling at least once can be expressed as relative risks (RRs) comparing the control and the intervention group, on the basis of the number of falls occurred. A precise definition of fall needs to be provided by the investigators. The occurrence of falls can also be used to obtain incident fall rates (number of falls/person-years).

To conclude, fall(s) can be considered an appropriate risk factor for osteoporotic bone fractures to be used alone for the scientific substantiation of osteoporotic bone fracture risk reduction claims.

3.2.1.2.1 DIARY/CALENDAR

The methods of collecting fall data chosen in an intervention study and during the follow-up may affect the number of falls recorded and consequently the risk of falling calculated. The available

techniques can be principally distinguished into prospective and retrospective reporting systems. The former includes diary, post-card and calendar, whereas the latter use telephone/clinic visit interview or postal questionnaire. The incidence of falling assessed in longitudinal studies may result relatively more accurate using prospective systems that can help avoiding the drawback of the limited accuracy in remembering falls number during the time[65]. However, self-reporting, the only feasible mode of ascertain falls in community studies, may imply low accuracy in recording the number of falls. The situation is different in institutional settings, because the accuracy of falls reporting can be improved recurring to falls data recorded by nursing staff which provides an ancillary system able to reduce the cases of underreporting.

Calendar represents a validated method applied in longitudinal studies in which falls recall is controlled at different time points, most often monthly. Diary or calendar is generally considered the gold standard method, even if not declared as the most effective, to track falls[66]. When applied in research settings, this approach requires an expert staff to monitor calendars. It is considerably time-consuming because it needs to verify self-reported falls with phone calls to participants. The advantage is that for each day subjects are requested to indicate whether or not they have fallen. However, specific information about the details of any falls cannot be ascertained until the diary is returned to investigators. Return of the data generally happens at specific time points, ranging from one week to three months. Moreover, in community-dwelling elderly, the information about the circumstances of fall is sometimes incomplete or inaccurate due to the psychological effects of falling, such as the shock and distress. Furthermore, the tendency of the subjects to lay the blame on external factors for the fall is in part responsible of the phenomenon of underreporting. To conclude, diary/calendar seems to be an appropriate method of recording fall(s) in intervention studies. However, the limitations of this approach should be taken into account, mainly if community-dwelling elderly is investigated.

3.2.1.2.2 QUESTIONNAIRE

As described in Section 3.2.1.2.1, falls can be measured by using prospective and retrospective reporting systems. The latter include telephone/clinic visit interview or postal questionnaire. In a retrospective approach the participants are asked whether and/or how many times they fell in a past period (generally one week, two/four months or one year)[67]. The drawback associated to this system concerns the limited accuracy in remembering falls over a relatively long period[65]. Compared to collected prospective falls data, the recall of any fall could have high specificity but shows less sensitivity. Thus, the incidence of falling assessed in longitudinal studies may result relatively less accurate because of the possibility of underreporting [68]. Increasing the frequency of the submission of the questionnaire may partially reduce this drawback. Nevertheless, this phenomenon represents a relevant concern mainly in case of subjects with cognitive impairments.

The difference between telephone interview and mail-out questionnaire is that the former may require many calls to contact the subjects, resulting more time-consuming. Comparing to prospective systems, questionnaire has the advantage of obtaining all relevant details about the circumstances of falling. However, even with the most rigorous reporting technique, the number of falls is generally underreported and the information about fall event is sometimes incomplete or inaccurate due to psychological effects of falling (shock and distress). Moreover, the tendency to lay the blame on external factors lead the subjects to not count a fall as a “true” one. In conclusion, even if questionnaire is not considered the gold standard method or recording fall(s), it can be appropriately applied in intervention studies.

3.2.1.3 BMD

See Section 3.1.1.1

To evaluate the appropriateness of BMD as risk factor of osteoporotic bone fractures, the literature deriving from database #1 was critically evaluated (Table 1).

BMD has been already described in Section 3.1.1.1 as an appropriate OV for the scientific substantiation of health claims in the context of improvement/maintenance of bone mass. Although BMD is a worldwide approved measurement to evaluate bone strength and a well consolidated tool for fracture risk assessment and management[13], in recent years it is spreading out the certainty that BMD measures alone have limited sensitivity and specificity in the prediction of an osteoporotic fracture, as demonstrated by the fact that a great proportion of the overall incident fractures occurs in subjects with osteopenia. Furthermore, fractures can occur also in subjects with normal BMD. Indeed, about two-thirds of individuals who suffer a fracture do not present osteoporosis as defined from WHO diagnostic criteria (DXA). Because of the limited sensitivity of BMD test, different clinical risk factors have been identified in order to enhance fractures risk prediction valid both in the presence and in the absence of the BMD measurements. Nevertheless, sites where BMD is frequently measured are hip, lumbar spine and femoral neck[12]. Concerning human intervention studies, a reported decrease in BMD values is positively related with an augmented risk of osteoporotic fractures; on the contrary, high values of BMD do not necessary correlate with low or no risk of fractures. Indeed, recent studies demonstrated how a combination of BMD and VFA or, even better, a combination of BMD, VFA and FRAX significantly increases the efficacy in identifying individuals who need treatment[13]. Reduced BMD may be considered as a risk factor for osteoporotic fractures if an increase in (or reduced loss of) BMD following a particular nutritional intervention is accompanied by evidence of reduced bone fracture incidence in humans.

In conclusion, BMD is not an appropriate risk factor to be used alone for the scientific substantiation of health claims in the context of the reduction of the risk of osteoporotic fractures by reducing bone loss.

3.2.1.3.1 DXA

See Section 3.1.1.1.1

3.2.1.4 VITAMIN D STATUS

The term “vitamin D” encompasses different molecular forms. In humans, dietary ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) represent the two sources of vitamin D. The former derives from ergosterol in plants, whereas the latter has both exogenous and endogenous origin, being generated in the skin from 7-dehydrocholesterol by the action of ultraviolet irradiation. Biologically active compounds originate from vitamin D3. In the liver the two forms are respectively converted by oxidation into 25-hydroxyvitamin D3 ($25(OH)D_3$) and 25-hydroxyvitamin D2 ($25(OH)D_2$), which are further converted in the kidney into other metabolites of varying activity, the most of which is 1α25-dihydroxyvitamin D3[69]. Therefore, total serum level of 25(OH)D, reflecting the combined contribution of cutaneous synthesis and dietary intake, represents the best estimate of vitamin D status. As complementary measures, PTH levels or BMD can be considered.
To evaluate the appropriateness of vitamin D status as risk factor of osteoporotic bone fractures, the literature deriving from database #11 was critically evaluated (Table 1).
Vitamin D takes part in the modulation of immune respo nse and the maintenance of calcium homeostasis in the body, stimulating the intestinal absorption of this micronutrient. As shown by epidemiological studies, both BMD and muscle function positively correlate with 25(OH)D[70]. The concept of the optimal vitamin D status for bone health, associated with the maximization of bone mass and a low occurrence of osteoporosis, is based on the relation between serum 25(OH)D and serum PTH. Although the literature provides controversial data, there is a range of 25(OH)D level below of which PTH begins to rise promoting bone loss. This threshold is estimated to be a broad range that may be explained by:

1) the heterogeneity of populations studied;
2) the different dietary calcium consumption;
3) the possible influence of health disorders on PTH levels in the elderly;

4) the variability of quantitative assays for 25(OH)D caused by the lack of standardization.

Moreover, despite the similarity between the shapes of scatter plots of PTH and 25(OH)D reported in the literature, the ideal form of their mathematical relation is still not clear[71].

A recent systematic review of the results from randomized controlled trials (RCTs) indicates a significant decline of the osteoporotic fracture risk with 25(OH)D, showing the protective role of an adequate vitamin D status against the risk of falling. This effect has been particularly demonstrated in weak institutionalized elderly individuals[72]. Nevertheless, optimal serum concentration of 25(OH)D is still under debate and currently there is no consensus on a given range of values[73].

In conclusion, low vitamin D status is not an appropriate risk factor to be used alone to scientifically substantiate health claims regarding osteoporotic fractures risk reduction. However, it can be used as supportive of a mechanism through which the food/constituent could exert the claimed effect, in addition to direct measures of osteoporotic bone fractures.

3.2.1.4.1 CHROMATOGRAPHIC TECHNIQUES

A variety of methods is available to determine circulating concentration of 25(OH)D, from immunoassays, widely used in clinical laboratories, to chromatographic techniques. In comparison to the formers that may present unsatisfactory accuracy and precision, the latter show several advantages, such as the lack of immune interferences and the presence of a step that involves solvent extraction or protein precipitation. These procedures improve the analyte releasing from the vitamin D binding protein (the major carrier protein of 25(OH)D into the circulation), providing more accurate results. However, the methodological limitations associated to the various analytical procedures are responsible for the lack of their standardization resulting in both inter-assay and inter-laboratory variability. Moreover, in conjunction with these drawbacks, the lack of standard reference preparations and calibrating materials makes the assessment of vitamin D challenging. HPLC followed by ultraviolet detection has the ability to separately assay $25(OH)D_3$ and $25(OH)D_2$ and,

until recently, it has been regarded as the reference method for quantifying vitamin D[74]. In this context it is considered a reliable and robust methodology, but the requirement of an expensive equipment, of a large sample volume, and of specific technical expertise, limits its application in routine clinical analysis. Among available techniques for 25(OH)D measurement, liquid chromatography tandem mass spectrometry is presently considered the gold standard by many commentators, providing relatively higher selectivity, accuracy and sensitivity [75,76]. As well as HPLC, it is able to distinguish the two metabolites of 25(OH)D. It allows the separation of compounds on the base of their polarities, ionization behaviours and mass-to-charge ratios and offers very low limits of quantification. Moreover, it has the advantage of providing data not affected by the interference from dihydroxy metabolites of vitamin D during the quantification. However, in comparison with automated platforms, this technique presents some limitations, such as the requirement of expensive equipment and a lower throughput[73]. Currently, some research groups are making efforts in order to improve this last drawback. Moreover, the variability in sample preparation, chromatographic separation and finally ionisation/fragmentation should be considered. On the basis of current evidence, chromatographic techniques are appropriate methods of measurement of circulating levels of 25(OH)D.

3.2.1.5 BONE TURNOVER MARKERS

See Section 3.1.1.2.

To evaluate the appropriateness of bone turnover markers as risk factor of osteoporotic bone fractures, the literature deriving from database #2 was critically evaluated (Table 1).

As discussed in Section 3.1.1.2, biochemical markers of bone formation and resorption allow to evaluate the structural and functional conditions and the rate of metabolic processes undergoing in bone tissue [19]. Because the activity of osteoblast and osteoclast is intertwined during normal bone remodeling, bone formation or bone resorption markers must be used together to provide an indication of overall bone turnover. Regarding osteoporotic fractures, the increased bone loss due to

altered bone homeostasis, could be reflected well by the imbalance in favor of bone resorption markers. Nevertheless, the obtained results should always be evaluated taking into account the whole clinical background, as well as having a firm understanding of the biological sources of each marker. This is essential for a comprehensive interpretation. Indeed, they are not specific for the any bone conditions assessment therefore they are not, altogether, disease specific. Additionally, several of the available markers are non-specific, i.e. they are present in tissues other than bone and may therefore be influenced by non-skeletal processes. As matter of fact, bone turnover markers must be evaluated together with other markers of general health [18]. For an adequate diagnostics and monitoring of bone disease treatment efficacy special attention must be paid to the investigation of type I collagen fragments content in blood or urine as markers of bone formation and indirectly of resorption, because collagen type I is the major bone tissue protein[77,78]. The clinical determination of the potential bone metabolism markers allows investigating the rate of spontaneous bone loss, performing the bone diseases monitoring both in adults and in children and prognosis of the risk of fractures. Most bone turnover markers exhibit significant within-subject biological variability but also subject-indipendent variability, both from pre-analytical and analytical factors. In addition to standard factors of assay performance (e.g. choice of sample collection and storage), technical sources of variability are also present[77]. Therefore, knowledge of the sources of variability and the strategies used to minimize them are mandatory to obtain reliable and meaningful results. In conclusion, an impaired balance of bone turnover markers is not an appropriate risk factor to be used alone to scientifically substantiate osteoporotic bone fractures risk reduction claims. However, it can be used as supportive of a mechanism through which the food/constituent could exert the claimed effect, in addition to direct measures of osteoporotic bone fractures.

3.2.1.5.1 DIRECT COMPETITIVE ELISA

See Section 3.1.1.2.1

3.2.1.5.2 DIRECT NON COMPETITIVE ELISA

See Section 3.1.1.2.2

3.2.2 OSTEOARTHRITIS

Osteoarthritis (OA) is the most common type of arthritis affecting, worldwide, more than a half of the over 65 years old population and therefore one of the most significant causes of disability among the elderly. Despite OA has been commonly considered in the past as a wear and tear disease, leading to loss of joint structure and progressive deterioration of articular cartilage, the recent advancements in molecular biology have allowed to expand knowledge about the pathophysiology of OA. Indeed, OA is nowadays redefined as a very complex multifactorial and degenerative disease, complicated by inflammatory reactions, which mainly affects the joints of the knees, hands and hips, and further the surrounding tissues, i.e. ligaments, synovium and subchondral bone[79].

Two forms of OA have been recognized and even if they depend on different predisposing factors, the resulting pathological substrate is the same: a disabling, late-onset but progressive disease which starts from a low-grade inflammation of articular cartilage and synovium with related joint swelling, pain and stiffness; then, the narrowing of the joint space and lesions of the subchondral bone cause loss of articular mobility and, the late complete loss of joint space width leads to an abnormal bone-to-bone contact. This is one of the main factors in deciding for surgical joint replacement. Considering the two different form of OA, the pathogenesis of the primary, or idiopathic, OA relies on genetic and epigenetic predisposition, involving over than 80 recognized gene mutations and a multitude of heritable changes which occur in the phenotype, like DNA methylation and histone modification, as response to environmental changes. The secondary type of OA, also called post-traumatic OA, strictly depends on a traumatic insult like a joint injury or a surgery intervention. In particular, repetitive and delimited mechanical loading stress, like that experienced by athletes, plays a fundamental role in triggering inflammatory events characteristic of OA[79].

OA has a complex etiology composed by several risk factors, whose association influences the susceptibility and the individual predisposition to the disease. Some are considered as non-modifiable, also called "ordinary" risk factors: hereditary predisposition, sex, age, ethnicity, injuries and mechanical stress on weight-bearing joints. Considering sex and age, the percentage of women showing symptomatic OA is twofold respect to that of men, but this discrepancy only occurs after the fifth decade of life, likely due to hormonal changes in post-menopausal women. The disparities might also depend on differences in the structure of bones and ligaments, like alignment, strength and laxity of ligaments or just a reduced volume of cartilage in woman compared to that of men[79,80].

Among modifiable risk factors, wrong habits and behaviors of daily life can exert a negative effect on general body health predisposing the subject to develop OA. For example, the bad dietary habits may induce obesity, which is responsible for both metabolic destroying processes affecting the cartilage and overload of the joints, especially hips, knees and joints of the foot. Strong evidences are present in literature showing that type II diabetes and high glucose concentration are connected with the onset and progression of OA. Furthermore, the increased role of technology in our lives is potentially a risk factor for OA; in particular, the use of computers, smartphones and tablets involves an excessive stress of the joints of the hand with associated pain and site-specific disability. Nevertheless, the real link between technology and hand OA has not been proven yet.

3.2.2.1 NET CARTILAGE LOSS

Net cartilage loss is a distinctive event of OA, a disease of whole joints that, in fact, does not only affect the total amount of cartilage, but also its composition and the structural appearance of all the surrounding synovial tissues. The type of cartilage affected by OA is the articular, also named hyaline, cartilage, which is located in diarthrodial joints, is devoid of blood vessels, lymphatics, and nerves and therefore it has limited capacity for intrinsic healing and repair[81]. Even though the total amount of cartilage physiologically decreases in humans with ageing, diseases like OA accelerate

this process and, in addition, net cartilage loss is a significant factor that contributes to OA progression. Indeed, due to the loss of the highly-regulated mechanism necessary to maintain cartilage homeostasis, joint structure is altered: micro- and macro- architectural changes occurs and the equilibrium between breakdown and regeneration of joint articular components is shifted toward degradation, thus resulting in net cartilage loss. In severe OA, articular cartilage could be completely lost and the consequent loss of joint space width results in bone-to-bone contact, one of the main factor considered in deciding for surgical joint replacement. Net cartilage loss in OA is a multifactorial process which relies on several factors, including general ones (age, sex and weight) and mechanical factors like joint alignment and injuries. In this regard, knee is the articular joint most affected by OA and several studies here demonstrate the strong relation between net cartilage loss and meniscal position and damage. Indeed, the absence of functioning meniscus and the resulting lack of covering of the articular surface leads to the progressive deterioration of cartilage, up to its complete loss[82].

To evaluate the appropriateness of net cartilage loss as risk factors of, the literature deriving from database #12 was critically evaluated (Table 1).

The net degradation and loss of cartilage are the fundamental pathogenic events of OA, then often followed by osteophytes development, subchondral sclerosis, cystis formation and bone deformities[81]. Net cartilage loss is a structural outcome of cartilage volume and its changes assessed using MRI scans, commonly at tibial site, is a recognized method for quantifying disease severity in OA. Thus, considering net cartilage loss as a volume (cm^3) is a clinical and research approach whose validity and reproducibility have been ascertained by several studies[82,83]. Variables frequently evaluated in combination with net cartilage loss are, primarily, the WOMAC joint pain score and the joint space width, which in fact can be considered as a surrogate marker of net cartilage loss, and secondarily, other cartilage defects and bone marrow lesions[84]. According to literature statements, net cartilage loss is a variable of primary importance to be assessed in studies

aiming to clarify the effect of intervention like food supplementation on OA progression[84]. Moreover, monitoring the course of cartilage loss is a powerful tool fulfilling the need to early recognize OA onset. However, considering that the variation of the amount of cartilage loss does not affect the risk to OA onset because it is a peculiar early event of manifested OA, net cartilage loss cannot be considered an appropriate risk factor for the scientific substantiation of health claims regarding the reduction of the risk of OA in healthy subjects. On the contrary, it is worth repeating that the measurement of net cartilage loss has a fundamental role in monitoring OA progression and severity.

3.2.2.1.1 MAGNETIC RESONANCE IMAGING

MRI is a common procedure worldwide used in radiology to image the anatomy and physiological processes of the human body in both health and disease. MRI scans use magnetic field and pulses of radio wave energy to create images of tissues and organs. For imaging purposes, the hydrogen nucleus is used, due to its abundance in water and fats. When the body is placed in the strong magnetic field of a MRI scanner, all the protons axes line up and this uniform alignment creates a magnetic vector oriented along the axis of the MRI scanner. Then, energy, in form of radio waves, is added to the static magnetic field and the magnetic vector is deflected. The radio wave frequency causes the hydrogen nuclei to resonate in a manner which is dependent on the magnetic field strength and element sought. The strength of the magnetic field can be electronically altered using gradient electric coils and, through small increments, different slices of the body resonate as different frequencies are applied. After that, a signal is generated and emitted when the radiofrequency source is switched off because of the returning of the magnetic vector to its resting state. Finally, the intensity of the received signal is plotted on a grey scale and images are built up. In the context of joint state assessment, MRI is a powerful tool for the evaluation of the overall joint structure because it provides information based on different signal intensities between bone, cartilage, fibrous tissue, mineralized cartilage, hematopoietic and fatty marrow. It is therefore a much appreciated tool in OA

studies because it is the only imaging modality that can delineate articular cartilage in a direct and non-invasive way. It provides quantitative information about the amount of cartilage loss, being able to detect changes in cartilage volume as low as 40-60 mm^3 with a demonstrated confidence of 95%. Furthermore, several studies have established the accuracy, the reproducibility as well as the reliability of MRI technique in revealing the thickness and volume of articular cartilage both in moderate and in severe OA[82]. For these reason MRI is also widely used for effectively staging the net cartilage loss due to the progression of disease. Radiography is another imaging technique which can produce relatively accurate results, even if only in the medial and not in the lateral, femorotibial compartment of the joint. Despite the substantially higher cost, MRI shows a number of advantages respect to radiography: it is less prone to errors due to wrong joint positioning, and it provides more specific information on joint status because the images obtained with MRI differentiate between tibial and femoral cartilage loss and further show the distribution pattern of cartilage degradation throughout the articular surface[83]. Other advantages include the fact that there is no involvement of radiations, so it is preferred for people who can be vulnerable to the effect of radiations, such has pregnant women or children. Despite it is generally considered as safe, MRI implies some significant risks: gadolinium-based contrast material may cause nephrogenic fibrosing dermopathy in subjects with kidney failure, MRI is contraindicated in presenc e of metallic devices, such as cochlear implants, pacemakers and implantable cardioverter-defibrillators which may affect image quality, the expensiveness and the long-lasting procedure (20-40 minutes); other problems related to MRI exam is the loud noise and the enclosed space that can be unpleasant for those who are claustrophobic. Nevertheless, MRI is generally an appropriate method of measurement of net cartilage loss.

3.3 CLAIMS REFERRING TO CHILDREN'S DEVELOPMENT Art 14(b)

3.3.1 NORMAL GROWTH AND DEVELOPMENT OF BONE

3.3.1.1 BONE MINERAL CONTENT

BMC is a measurement of bone mineral found both in a specific area of the skeleton or in total skeleton system. Up to 50% by volume and 70% by weight of human bone is formed by hydroxyapatite, which is the mineral form of calcium apatite. BMC is expressed in grams (g) and it is used to obtain BMD, which is measured in grams per centimeter squared (g/cm^2), by dividing BMC by the area of the considered site [85]. Thus, due to the high association between BMD and BMC, it can be properly said that also BMC is characterized by a growing phase, depending on the availability of calcium and phosphate, during the childhood, with the following achievement of BMC peak during the early adulthood. BMC or areal BMD increase is due to the deposition of hydroxyapatite crystals into the preexistent bone matrix, but can also result from augmented bone size, thickening of bone cortex or trabeculae, or new synthesis of trabeculae. After reaching peak bone mass, the mineral deposition activity of osteoblasts and the resorption activity of osteoclasts are balanced, leading to a steady state of the total BMC. Then, during adulthood, a constant and progressive imbalance of neo-mineralization and bone resorption, with prevailing osteoclast activity, causes a loss of BMD, reflecting a diminished BMC with ageing. Progressive loss of BMC results in osteopenia and osteoporosis. Despite BMC, together with BMD and bone size, is an important risk determinant of osteoporotic fractures[86], it is also widely used in clinical practice for the assessment of the normal growth and development of bone in children. Additionally, by the fact that bone growth depends on hydroxyapatite deposition, BMC reflects calcium bioavailability in human body.

To evaluate the appropriateness of BMC as OV of normal growth and development of bone in children, the literature deriving from database #1 was critically evaluated (Table 1).

BMC measurement, with adjustments for changes in body mass and total bone size, is widely carried out in clinical practice for the assessment of bone health and mineralization in children and in adolescents[85,87]. BMC, expressed as grams of hydroxyapatite, depends on both the size and density of skeletal bone, and a difference in BMC may reflect a difference in either bone size or bone density. BMC is the preferred outcome variable over BMD because bone expansion and the increase in BMC occur at different rate during childhood. Consequently, BMD calculated as BMC/bone area is not an appropriate ratio to be used in growing children because it is influenced by bone size[85]. Instead, it is well-accepted that bone mineralization is assessed in three steps: height for age, bone area for height, and BMC for bone area. In comparative studies, it is important to adapt BMC measurement for age and sex, in order to adjust the heterogeneity in terms of the age- and sex- specific maturation[85]. Thus, to combine measurement results for children of different ages and to account for the growth-related changes in BMC, *z* scores for BMC-for-age and BMC-for-height were calculated based on the healthy reference sample. In addition, because hydroxyapatite is the main mineral component of the bone skeleton and it is primarily made of calcium, BMC evaluation is also a useful tool in calcium bioavailability studies, which also allows to analyze the association existing between dietary intake and bone development and metabolism[87].

In conclusion, BMC is an appropriate outcome variable to be used alone for the scientific substantiation of health claims in the context of normal growth and development of bone in children.

3.3.1.1.1 DXA

DXA, also known as bone densitometry or bone density scanning, can accurately analyze bone and non-bone tissue, providing a quantification of BMD, BMC, fat mass and soft lean mass; it has been validated across age groups, from premature infants to older adults, including both normal and overweight subjects. The use of DXA in infants and children is gradually increasing, with the aim to understand the impact of disease on bone health or nutritional impact on body composition.

Indeed, DXA has been demonstrated to measure skeletal maturity and body fat composition and has been used to evaluate the effects of pharmaceutical therapy. Even though the diagnosis of osteoporosis in children cannot be made using the basis of a densitometry criteria, DXA scans are routinely carried out on pediatric patients with conditions such as Systemic Lupus Erythematosus, Turner Syndrome, Osteogenesis imperfecta and nutritional rickets revealing DXA as a helpful tool for pediatricians in diagnosing and monitoring treatment of disorders of bone mass and BMC acquisition in childhood[85]. DXA is a peculiar imaging modality which differs from other X-ray systems because requires special beam filtering and near perfect spatial registration of two attenuations. Indeed, DXA system creates a two dimensional image that is the combination of low and high energy attenuations. Although density is typically given by mass per unit volume, DXA can only quantify the bone density as a mass per unit area, since it uses planar images and cannot measure the bone depth. By the fact that a two-dimensional output is given, DXA-based bone mass cannot distinguish between bone compartments, namely cortical and trabecular bone[10]. For this reasons DXA measurement can be integrated with additional 3D outputs from different technologies, as QCT. Nevertheless, regarded as a safe, with a minimal radiation exposure (0.1 μGy), fairly fast (6-7 min for total body assessment), accurate and non-invasive method[86], DXA is frequently used in many clinical settings. On the other hand, it is relatively more expensive than others and requires expert skills. Another limitation of DXA scanning is the need to remain perfectly still during the entire scan. Whole body DXA scans is primarily used for BMC measurements in children[87] and for body composition measurements in adults, while several common measurement sites, including the lumbar spine, the proximal hip and the forearm, are preferred when measuring BMD to diagnose osteoporosis or other bone loss-related malignancies, to follow-up osteoporosis treatment and to assess the risk of bone fractures, too. Importantly, as for the intervention studies, DXA measurement is made at baseline and then not earlier than 12 months, which is considered the most appropriate follow-up interval to detect (if any) significant changes in BMD and/or BMC.

In summary, DXA is generally an appropriate method to assess BMD, BMC and bone area in human intervention studies.

3.3.1.1.2 SINGLE PHOTON ABSORPTIOMETRY

In the early 1960s, a new method for bone densitometry, called single photon absorptiometry, was developed, which overcame the problems of previous radiographic photodensitometric techniques caused by polychromatic X-rays and non-uniform film sensitivity. Indeed, Single Photon Absorptiometry (SPA) technique uses a single energy gamma ray source (^{125}I) photon energy, and a scintillation detector to measure the single-energy photon beam passage through bone and soft tissue. The distal radius (wrist) is usually used as the site of measurement because the amount of soft tissue in this area is small. Changes in beam intensity are due to the attenuation by bone mineral and the integrated attenuation is proportional to the mass of mineral in the scan path, whose length is proportional to the width of the bone. Even if SPA has been widely used in the past for the assessment of bone mineral density and content[88], it is outdated and nowadays has been replaced by other densitometry techniques, such as Dual Photon Absorptiometry and DXA which have greater accuracy and are capable of measuring central skeletal sites. In fact, the radionuclide source (^{125}I) emitted an average energy of 27 keV, which is sufficient for the BMD measurement of appendicular bones but not for that of central skeletal sites. Other limitations are represented by the use of radionuclide source, which gradually decays and requires regular replacement, the scanning time, which is considerable (15-30 minutes) due to the low rate of photon flux. With the low scanning, undesirable incidents might occur, such as the patient moving during the scan and thus rendering poor quality of the scan image and limiting the reproducibility. Moreover, SPA method can compensate for variation in bone width but not for variation in bone thickness; the reproducibility of the measurement therefore depends upon the ability to reproduce exactly the location of the measurement. For this reason, it is necessary to control the stillness and the

pronation/supination of the bone site (generally the forearm), since rotation alters the photon beam path[88].

In summary, even if it was a widely used bone densitometric technique, SPA is generally an outdated method to assess BMC.

3.3.1.2 BMD

To evaluate the appropriateness of BMD as OV of normal growth and development of bone in children, the literature deriving from database #1 was critically evaluated (Table 1).

BMD has been already discussed as OV for improvement/maintenance of bone mass (Section 3.1.1.1) and as risk factor for osteoporotic bone fractures (Section 3.2.1.3). In addition to the use of BMD as important diagnostic tool in the assessment of the risk of osteoporotic fractures, BMD is widely employed for the evaluation of correct development of the skeleton system during childhood and adolescence; in this regard, BMD must be adjusted for changes in body mass and total bone size and it is therefore more used the Z score reference. Therefore, BMD is an appropriate outcome variable to be used alone for the scientific substantiation of health claims in the context of normal growth and development of bone in children.

3.3.1.2.1 DXA

See Section 3.3.1.1.1

3.3.1.3 CORTICAL BONE THICKNESS

Bone structure is made of two osseous tissues with different microstructures and functions. The cortical or compact bone is the most represented component of bone, forming about 80% of total skeleton weight. As its name implies, it forms the outer layer of most bones and is primarily found in the shaft of long bones, like femur or tibia. Microscopically, cortical bone is arranged in tightly packed osteons, concentric rings of matrix surrounding a central Haversian canal, giving rise to a dense, hard, strong and stiff structure. Childhood and adolescence are crucial moments for the correct development of the skeleton: the organization of cortical bone is regulated by mechanical

stimuli, which are thought to drive the orientation of Haversian lamellae along both stressing and loading directions, owing to cortical bone thickness and strength for supporting body weight and mechanical loading. Several factors have been shown to be implicated in the acquisition of thickness in cortical bone: physical activity, in particular exercises that involves impact and mechanic loads trigger the bone modeling and remodeling process[89], the nutritional intake, which must provide all the components needed for bone growth and mineral accrual (i.e. proteins, calcium, phosphate) and the anabolic agents, like GH which is a major regulator of postnatal bone growth, parathyroid hormone, and androgens, known to be fundamental regulators of bone expansion[90]. Physiologically, the cortex in women is thinner than in men, due to a lower bone mass acquisition during the puberty; therefore, in older age, the consequences of bone loss are more pronounced in women than in men, and the incidence of fractures is two to three times higher.

To evaluate the appropriateness of cortical bone thickness as OV of normal growth and development of bone in children, the literature deriving from database #13 was critically evaluated (Table 1).

Several studies demonstrated how an increase in body mass results in an increase in the thickness of cortex of long bones by the fact that larger is body mass larger is bone loading. At the same time, it can be stated that loading of bone in the form of increased activity, particularly high-impact activity, can also result in an increase in the cortical bone thickness. These evidences are therefore used for the assessment of bone quality, especially during childhood, when the increase of weight, mechanical loading and adequate physical activity support the correct development of the skeleton system[89]. Even though trabecular bone is the most affected compartment after the menopause, the parallel cortical bone loss occurring in elderly has a direct impact on the biomechanical properties of long bones and vertebrae, which is clinically associated with higher fracture risk[91]. Cortical thickness is a parameter of bone geometry and macroarchitecture which, together with trabecular and cortical bone area, periosteal and endosteal circumference, defines the bone structure, whose changes are even more taken into account instead of bone mass alone, in the evaluation of fracture

risk; in fact, it would be better to say that bone mass and bone structure are considered together for the prevention of the osteoporosis; thus maximizing bone mineral mass during childhood or adolescence may decrease the risk of osteoporotic fractures late in life, especially those occurred in cortical bones due to thinned cortex. Additionally, even if osteoporosis is worldwide considered as a disease affecting elderly subjects, it also occurs in children as primary osteoporosis, due to intrinsic skeletal defects of genetic or idiopathic origin, or secondary, caused by immobility, hematologic malignancies, inflammatory conditions, long lasting glucocorticoids therapy, hypogonadism or poor nutrition. In this context, low cortical thickness, together with low BMD, are used as radiological predictor of fractures.

In conclusion, cortical bone thickness represents an appropriate outcome variable, only if used in combination with the parallel measurement of other surrogate parameters of bone size and structure, like bone length, periosteal circumference and polar strength strain index of the radius, for the scientific substantiation of health claims regarding normal growth and development of bone in children.

3.3.1.3.1 QUANTITATIVE COMPUTER TOMOGRAPHY/ PERIPHERAL QUANTITATIVE COMPUTER TOMOGRAPHY

QCT and pQCT are established techniques for the measurement of BMD mainly in the lumbar spine and in peripheral skeleton (forearm and tibia). In fact, differently from DXA, it provides 3D non-projectional results: firstly, a true volumetric measurement of bone density in mg/cm^3 and secondly, a separate measurement of trabecular and cortical bone, providing information of bone geometry and trabecular structure[92]; indeed, it can also identify cortical thickness, which is the main bone variable affected by growth hormone deficiency. Moreover, by the fact that trabecular bone has higher turnover rate than cortical bone, QCT is a very useful and high sensitive technique to monitor bone turnover. QCT and pQCT are X-ray based techniques and the total linear X-ray absorption by

tissues is given by the coefficient μ. For clinical applications, the values of μ are calibrated to the X-ray attenuation of water (w), resulting in a number measured in Hounsfield Units. In an appropriately calibrated scanner the CT number of water is 0 and thus, in contrast to DXA, all CT scanners are calibrated equivalently. Then, the analysis of the outputs involves the use of dedicated software to extract quantitative parameters. Founded on the same basis, it is possible to distinguish pQCT from general QCT, because it defines the application of QCT to appendicular skeleton sites, such as the arms or legs and the term pQCT is frequently used to designate such dedicated peripheral scanners. pQCT measurement are performed on specially designed small gantry scanner using a translate- rotate movement with a multi detector head. In general, QCT is high reproducible and thus widely used for assessment of vertebral fracture risk, measurement of age-related bone loss, and follow-up of osteoporosis and other metabolic bone diseases[92]. Moreover, QCT allows spine BMD evaluation on patients with scoliosis, which cannot usually be measured using other techniques, as DXA; QCT can also avoid the artificial BMD measurements that often mislead results from DXA in arthritic patients, in overweight or obese patients, and in subjects suffering from disc space narrowing or spinal degenerative diseases, aortic calcification or osteophytes. Disadvantages include the exclusion of the following categories: patients who have recently had another radiological procedure that includes the use of high density contrast material or radio-opaque catheters and tubes, and pregnant women. pQCT is specifically useful for children, with spinal deformities, contractures or metallic implants even if reproducibility and positioning remain a problem both in children and in adults. A newest technique is the high-resolution pQCT, which has the spatial resolution to measure trabecular geometry and micro-architectural changes. However, it is limited to imaging extremities, is very expensive and for these reasons has been only used so far for research purposes.

In summary, QCT/pQCT is generally an appropriate method to assess cortical bone thickness, periosteal circumference and polar strength strain index of the radius in human intervention studies.

3.3.1.4 BONE LENGTH

Bone length is referred to long bones which, during the intrauterine and postnatal period, undergo longitudinal growth due to the action of chondrocytes in the proliferative and the hypertrophic zones of the growth plate in the metaphysis[93]. Other than the intertwined role of systemic and paracrine factors, the endochondral growth, leading to bone development in length is controlled by mechanical stimuli which ensure the alignment of bone axes with the predominant mechanical forces. Indeed, it is now extensively accepted that, starting from positional information for the basic outline of the skeleton provided by the genome, the key actors in bone length acquisition are growth factors and cytokines, hormones, intrinsic and extrinsic mechanical forces, environmental and nutritional factors. In detail, during childhood, the systemic control is ensured by growh hormone, insulin-like growth factor 1, thyroid hormones and glucocorticoids, whereas the sex steroids play the most significant role during puberty. At the beginning of fetal life, longitudinal bone development is characterized by a high rate of growth, with a rapid acceleration until the achievement of a peak and then, when the skeleton is approaching growth maturity, the growth rate decelerates up to puberty. Discrepancy in the growth rate has been seen at different anatomical sites which can be explained by differences in the degree of hypertrophy of local chondrocytes. Moreover, it must be remembered that longitudinal growth alone is detrimental to bone stability and thus is counteract by simultaneously bone growth in width[94].

To evaluate the appropriateness of bone length as OV of normal growth and development of bone in children, the literature deriving from database #14 was critically evaluated (Table 1).

Bone growth needs intense anabolic activity, mainly focused on protein synthesis and, in this regard, any disorder affecting cell replication and differentiation, collagen or any non collagenic bone protein synthesis may lead to disorders in bone growth, like Osteogenesis Imperfecta and other growth plate-related diseases. Lack in nutritional intake of Vitamin D, proteins, calcium and other ions has been seen to negatively affect bone quality, namely the acquisition of bone mass and mineral

content, increasing the risk of fracture during childhood. Mechanically appropriated loading must be well directed and balanced in order to escape limb discrepancy and angulation deformities. Bone length is directly related to leg length, an epidemiological marker used as indicator of the quality of the environment for growth during infancy, childhood and the juvenile years of development. Thus, the bone length assessment is a useful parameter to monitor the proceed of correct skeletal growth and, in case of deformities, it may help the surgeon to choose the best treatment. Therefore, a deep understanding of this process is not only fundamental for physicians treating pediatric bone disorders, but also for clinicians and researchers dealing with postmenopausal and senile osteoporosis[93]. Even if the proper accrual of bone length is a good predictor the correct skeletal growth, it cannot be considered alone to have an overall view on bone quality during skeletal development, and the information provided must be summed to those given from bone size, BMD and the grade of mineralization.

In conclusion, bone length is an appropriate outcome variable, only if considered in association with the parallel measurement of other surrogate parameters of bone size and structure, like cortical bone thickness, periosteal circumference and polar strength strain index of the radius, for the scientific substantiation of health claims regarding normal growth and development of bone in children.

3.3.1.4.1 RADIOGRAPHIC TECHNIQUES

Several different methods are available to clinicians for the assessment of bone length and the eventual discrepancies between the lower limbs[95]. There is general consensus that radiographic techniques, as orthoentgenogram, scanogram and teleoroentgenogram are more reliable and accurate than clinical exams consisting, for example, in the use of a standing block under the shorter leg to level the pelvis and measurement with tape. Orthoentgenogram is a plain radiographic technique, which has been developed in the early 1950s in order to minimize measurement errors due to magnification[96]. Specifically, it uses three distinct radiographic exposures centered over the hip, knee ankles joints. Orthoentgenogram differs from scanograms because a larger cassette, which is

placed under the laying patient is required for measurement, entailing an additional burden of costs, storage and special equipment, such as grids, filters, and processors. Scanogram technique, which is one of the most commonly used method for assessing bone length, is similar to teleoroentgenogram technique, except for the use of three different radiographic cassettes, respectively placed under the hip, knee and ankle joints, which are moved under the patient laying supine during the three consecutive exposures. The distance of the X-ray beam source from the patient is usually 101 cm and the beam is consecutively centered over the knee, hip and ankles. Scanograms reveals less magnification errors respect to teleoroentgenogram but entails a greater radiation exposure; additionally, this technique fails both in the visualization of the entire length of femur and tibia, and in the account for any shortening related to foot. Teleoroentgenogram is a full-length standing AP radiographic technique consisting of a single radiograph exposure of both lower limbs with the X-ray beam centered at the knee joints. While patient standing erect with both patellae pointed anteriorly, the X-ray beam source lies at distance of approximately 80 cm, and the cassette is placed behind. Several authors pointed out magnification errors related to the use of such instrument, whose magnitude depends on various factors, like the girth and the length of the limb, the divergence of the X-ray beam and the distance of the beam source from the cassette. Because of the magnification errors, teleoroentgenogram may not accurately measure the true bone length[97,98]. Despite of this limitation, its fair accuracy is commonly accepted in measuring the relative length of the two extremities at a single exam. Moreover, it provides low dose of radiation, proving to be valid tool for the detailed assessment of leg length discrepancy, for better underlie the etiology and deformities analysis. Although there is no single imaging method that can be considered ideal, the standing full-length AP teleoroentgenogram of both lower extremities with the pelvis level, along with use of a magnification marker, should be the primary modality for the initial evaluation of bone leg length. Indeed, this technique is not only an accurate and reliable imaging tool, but the measurements can be obtained with limited radiation exposure in a cost effective manner. In conclusion, it must be

taken into account that, although the previous techniques have been described referring to lower limbs, they can be also successfully applied for the assessment of bone length in upper limbs. Thus, it can be stated that, at present, radiographic techniques represent the goal standard in the assessment of bone length and therefore they are appropriate methods of measurement.

3.3.1.5 PERIOSTEAL CIRCUMFERENCE

The periosteum is the thin fibrous layer covering the entire surfaces of bones, except for the intra-articular surfaces, tendon insertions, and sesamoid bones; therefore, the periosteal circumference often corresponds to long bone circumference itself[99]. The periosteum consists of an outer fibrous layer containing fibroblasts, collagen along with a nerve and microvascular network. These components provide mechanical stability to the periosteum. The inner cambium layer contains adult mesenchymal skeletal progenitor cells and osteoblasts, cells that are responsible for bone growth, increasing bone width, and bone repair. The periosteal osteogenic capacity is greatest in children, whose cambium is thick and has considerable osteoblastic potential to ensure the correct bone size achievement. In adults, the periosteum is much less active under physiological conditions but it can be reactivated, for example, after a bone fracture. As the bone ages, the reduction in osteoblast number leads to a distinctive atrophy and thinning of the cambium layer and a corresponding decrease in the periosteal circumference.

To evaluate the appropriateness of periosteal circumference as OV of normal growth and development of bone in children, the literature deriving from database #15 was critically evaluated (Table 1).

The periosteal circumference is an aspect of bone size which is strictly related to other parameters assessing bone quality, such as BA, BMD and BMC. Increases in bone circumference, accomplished through periosteal, is widely studied during childhood, because its expansion is a part of the process of bone modeling which, when deregulated, lead to osteogenic diseases. Periosteal apposition during growth has been seen to be affected by a distinct set of environmental determinants, like gender and

ethnic identity, and intrinsic endocrine factors, i.e. estrogens in females and androgens in males during puberty[100]. Equally to other determinants of bone size, bone circumference grows faster in male than in females. Gender differences in periosteal expansion, like hip circumference, during puberty may help to explain the higher prevalence of hip fractures in women compared with men in later life. Periosteal expansion is also studied to identify the prevailing risks of fractures with ageing. Indeed, it is thought to continue after longitudinal growth has ceased, although this subsequently declines in later life, limiting its ability to compensate for the higher resorption and endocortical expansion that characterizes bone loss in the elderly. In the light of evidences demonstrating that physical activity, mechanical loading and a proper nutritional intake have a positive impact of bone health, periosteal circumference is a widely used to monitor the correct mineral accrual in the developing skeletal system[101].

In conclusion, periosteal circumference represents an appropriate outcome variable, only if used in combination with the parallel measurements of other surrogate parameters of bone size and structure, like bone length, cortical bone thickness and polar strength strain index of the radius, for the scientific substantiation of health claims regarding normal growth and development of bone in children.

3.3.1.5.1 QCT/pQCT

See Section 3.3.1.3.1

3.3.1.6 POLAR STRENGTH STRAIN INDEX OF THE RADIUS

The polar strength strain index, which has also been termed the density-weighted polar moment of resistance (R; mm^3) is a surrogate measure of bone strength and bone stability. It is determined from a cross-sectional scan by QCT/pQCT imaging and it is used to compare the structural bone parameters, assessed by QCT/pQCT analysis, to the results of three points bending test, which is a physical test providing values for the modulus of elasticity in bending, flexural strain, flexural stress and the flexural stress-strain response of a given material. The polar strength strain index of the

radius is usually measured together with physical parameters, such as the polar moment of inertia, the minimal and maximal bending moment of inertia in order to determine the geometric and mechanical properties of the outer cortical shell of bones and to estimate the torsional and bending strength of bone structures, with the final purpose to early diagnose bone strength loss for risk assessment and treatment of osteoporosis and other diseases inducing bone fragility, like type 2 diabetes or Turner syndrome[102,103]. Moreover, it is considered with BMD, BMC, BA, thickness, endosteal and periosteal circumference, all parameters of bone quality, for the evaluation of the correct skeletal development in children and adolescents.

To evaluate the appropriateness of polar strength strain index of the radius as OV of normal growth and development of bone in children, the literature deriving from database #16 was critically evaluated (Table 1).

Scientific literature studies almost never include indices of bone strength per se, but rather measure age-related changes in bone size parameters, like bone area, volume and BMD. Even if these measures are directly correlated with bone stability, a better estimation of bone strength can be obtained using engineering theory through calculation of polar strength strain index and other mechanical indices, such as moments of inertia or section moduli. Hence, polar strength strain index of the radius is evaluated in growth studies to investigate the relationship existing between body size, muscle size, and bone structural development[102,103]. Moreover, a decrease in polar strength strain index of the distal radius, measured by pQCT, can be considered a parameter of loss of strength in bones, thus allowing the detection of individuals at risk of osteoporotic fractures late in life. In this regard, the measurement of the polar strength strain index of the radius is an indispensable evaluation when it is necessary to obtain a clear overview on the strength of the total skeleton. Indeed, conventional QCT scans allow only measurement of the backbone, which usually not correlate highly with the parameters of the long peripheral bones, such as the distal radius, often leading to misleading or uncompleted data. Due to these considerations, polar strength strain index

of the radius, given by peripheral QCT scans, is often requested, in order to provide a fully-understanding on the health condition of the whole skeleton in humans, both in childhood and in elderly.

In conclusion, polar strength strain index of the radius can be used as appropriate variable, only if used in combination with other parameters of bone size and structure, like cortical bone thickness, bone length and periosteal circumference, for the scientific substantiation of health claims regarding normal bone growth and development in children.

3.3.1.6.1 QCT/pQCT

See Section 3.3.1.3.1

3.3.1.7 BONE AREA

Human skeleton is made of two types of bone tissues, classified on the basis of their characteristics of porosity and unit microstructure: cortical bone, that is primary found along the axis of long bones and forms the outer shell around trabecular bone at the end of joints and the vertebrae, is dense and little porous, whereas trabecular or cancellous bone has a higher degree of porosity (ranging everywhere from 50% to 90%) and it is located at the end of long bones, in flat bones like the pelvis, and in vertebrae. Bone area is a quantitative measure of bone surface, meaning either total skeleton or a single bone area. Specifically, considering bone area as the outer bone surface, clinicians commonly refers to cortical bone area, while trabecular bone area is assessed by summing trabecule total surfaces. Thus, trabecular bone area is bigger than cortical bone area for an equal unit volume considered, thus resulting on lower bone density[104]. Bone area, currently expresses as squared centimeters (cm^2), is known to be affected by bone size and increases during skeletal development in childhood and adolescence, when other factors like physical exercise, dietary intake and many hormones (e.g. PTH, calcitriol, GH, testosterone and estrogens)[105] play a fundamental role in bone accrual. Differently, bone area remains substantially unchanged among adult life and may have pathological change in the elderly.

To evaluate the appropriateness of bone area as OV of normal growth and development of bone in children, the literature deriving from database #17 was critically evaluated (Table 1).

Bone area is considered a measure of the bone size and it is especially evaluated in children and adolescents, together with other skeletal parameters, such as bone mass, BMC and areal BMD for the assessment of the correct development and growth of bones during the earlier stages of life. In a similar fashion, bone area is a supplementary tool for diagnosis and follow-up of diseases characterized by bone loss, affecting both children and adults (Turner syndrome, osteogenesis imperfecta, sickle cell disease, bone cancers and osteoporosis)[106]. Although the majority of the studies founded in literature report osteoporosis as an elderly disease, it has to be taken into consideration that early life events are equally important in its pathogenesis and, finally, it can be viewed as pediatric disorder that manifest itself late in life[105]. Because BMD is the recognized best parameter for osteoporosis clinical assessment and management, bone area is not considered for itself, but is studied in order to understand the relevance of bone size on BMD[105]. Other than whole-body bone area, sites where bone area is usually measured are hip, femoral neck, lumbar spine and wrist. In particular, total hip bone area is considered a measure of skeletal size by which fracture prediction can be assessed[107]. It is very difficult to find studies reporting bone area considered by itself, because it is almost always evaluated in order to primarily obtain derived measures like BMD, BMC and bone mass and consequently correlate these parameters to genetic, environmental and behavioral determinants affecting bone health during the lifespan. Anyway, bone area eq[106]ually to the other bone parameters, should be size-adjusted when evaluated in children, other than adjustments that are to be made for confounding effects on bone area given by sex, ethnicity and pubertal age. Moreover, bone area quartiles are frequently obtained through statistical analysis and then used as reference tool for subjects' categorization in population studies, allowing to study how other variables (e.g. BMD, fracture risk) changes among quartiles.

In conclusion, bone area is not an appropriate outcome variable to be used alone to substantiate health claims regarding normal growth and development of bone in children.

3.3.1.7.1 DXA

See Section 3.3.1.1.1

3.3.1.8 VITAMIN D STATUS

See Section 3.2.1.4.

To evaluate the appropriateness of vitamin D status OV of normal growth and development of bone in children, the literature deriving from database #11 was critically evaluated (Table 1).

Vitamin D as risk factor for osteoporotic bone fractures has been already discussed in Section 3.2.1.4.

Optimal serum concentration of 25(OH)D in children and in adults has been widely debated in the recent years. Recently, the consensus on the cut-off that defines the lower limit of adequacy or sufficiency specifically in infant and children was obtained in 2014. On the basis of the recommendations of the experts regarding the prevention of nutritional rickets, 25(OH)D levels >50 nmol/l are considered sufficient, whereas values <30 nmol/l are considered to be deficient [108]. If prolonged severe vitamin D deficiency leads to clinical disorders, skeletal abnormalities and short stature, also subclinical vitamin D deficiency may have a detrimental effect on bone mineralization, leading bones to become unnaturally curved and misshapen. Thus, low serum concentrations of vitamin D in children and adolescent is an important public health issue across different latitudes.

In conclusion, vitamin D status is not an appropriate outcome variable to be used alone for the scientific substantiation of health claims regarding normal bone growth and development in children. However, it can be used as supportive of a mechanism through which the food/constituent could exert the claimed effect, in addition to appropriate outcome variables, such as BMD or BMC.

3.3.1.8.1 CHROMATOGRAPHIC TECHNIQUES

See Section 3.2.1.2.1

3.3.1.9 BONE TURNOVER MARKERS

See Section 3.1.1.2

3.3.1.9.1 DIRECT COMPETITIVE ELISA

See Section 3.1.1.2.1

3.3.1.9.2 DIRECT NONCOMPETITIVE ELISA

See Section 3.1.1.2.2

4 CONCLUSION

To date, owing to the important contribution of the diet to bone function and health, several foods or food components have been proposed as subject of application for authorization of health claims in this context, pursuant to Regulation EC 1924/2006. However, for most of them, EFSA has issued negative opinions for reasons pertaining to an insufficient characterization of the food/food component, the choice of a not appropriate claimed effect, as well as an insufficient substantiation of the claim. The selection of adequate OVs and the related MMs used in the RCTs is a basic requirement for obtaining the authorization to associate a certain health claim to a food or a food component. It is crucial that OVs and MMs are chosen according to the specific claimed effect, taking into account that the target population must be healthy. The results provided by the present manuscript are relevant to drive the applicants towards a suitable choice of OVs and MMs in RCTs. However, it is wortly repeating that an effective substantiation of a claimed effect is provided considering also all the parameters which affect the quality of a RCT, such as an adequate choice of placebo/control, a proper sample size, and an adequate statistical analysis. Beyond the qualitative improving of the applications, the present results could serve to EFSA to update the guidance for the scientific requirements to bear health claims in the framework of bone health.

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Table 1 Strategies used for retrieving the literature pertinent with outcome variables and methods of measurement in the area of bone health.

Legend: BMC: Bone Mineral Content; BMD: Bone Mineral Density; WOMAC: Western Ontario and McMaster Universities.

DB Number	Syntax	Total articles	Narrative reviews	Systematic reviews / metanalyses	Validation studies	Outcome variables
1	"bone density"[mesh] AND "english"[language] AND "humans"[mesh]	32462	4542	894	197	BMD, BMC
2	"bone and bones"[mesh] AND ("turnover"[title/abstract] OR "metabolism"[mesh] OR "biomarkers"[mesh] OR "alkaline phosphatase"[mesh] OR "osteocalcin"[mesh] OR "deoxypyridinoline"[supplementary concept] OR "pyridinoline"[supplementary concept] OR "pyridinium crosslinks"[title/abstract] OR "procollagen type i carboxy terminal peptide"[supplementary concept] OR "tartrate-resistant acid phosphatase"[supplementary concept]) AND "english"[language] AND "humans"[mesh]	12859	1540	77	22	bone turnover markers
3	"range of motion, articular"[mesh] OR ("joints"[mesh] AND ("mobility"[title/abstract] OR "motility"[title/abstract] OR "flexibility"[title/abstract])) AND "english"[language] AND "humans"[mesh]	35004	2711	768	545	joint mobility

4	"cartilage"[mesh] AND ("turnover"[title/abstract] OR "metabolism"[mesh] OR "biomarkers"[mesh] OR "collagen type i"[mesh] OR "collagen type ii"[mesh] OR "c-terminal cross-linking telopeptide of type ii collagen, human"[supplementary concept] OR "collagen ii c-telopeptide"[supplementary concept] OR "procollagen type ii carboxy-terminal peptide" [supplementary concept]) AND "english"[language] AND "humans"[mesh]	2888	356	20	4	cartilage metabolism markers
5	("womac"[title/abstract] OR "western ontario) AND mcmaster universities arthritis index"[title/abstract] AND "english"[language] AND "humans"[mesh]	2000	63	73	90	WOMAC index
6	"musculoskeletal pain"[mesh] OR ("joints"[mesh] AND ("discomfort"[title/abstract] OR "pain"[title/abstract])) AND "english"[language] AND "humans"[mesh]	23091	2750	653	187	joint pain
7	"joints"[mesh] AND "space"[title/abstract] AND "english"[language] AND "humans"[mesh]	3675	292	43	50	joint space width
8	"collagen"[mesh] AND ("metabolism"[mesh] OR "turnover"[title/abstract] OR "synthesis"[title/abstract] OR "breakdown"[title/abstract]) AND "english"[language] AND "humans"[mesh]	12006	915	26	14	net collagen formation and breakdown
9	"osteoporotic fractures"[mesh] AND "english"[language] AND "humans"[mesh]	2194	432	186	25	osteoporotic bone fractures
10	"accidental falls"[mesh] AND "english"[language] AND "humans"[mesh]	15091	1573	604	163	fall(s)
11	"vitamin d"[mesh] AND "osteoporosis"[mesh] AND "english"[language] AND "humans"[mesh]	2932	797	134	2	vitamin D status

12	"cartilage"[mesh] AND ("loss"[title/abstract] OR "impairment"[title/abstract] OR "deterioration"[title/abstract]) AND "english"[language] AND "humans"[mesh]	2395	354	36	11	net cartilage loss
13	(("bone AND bones"[mesh] AND "thickness"[title/abstract]) OR "bone density"[mesh]) AND "english"[language] AND "humans"[mesh]	37829	4178	928	257	cortical bone thickness
14	"bone and bones"[mesh] AND ("length"[title/abstract] OR "dimension"[title/abstract]) AND "english"[language] AND "humans"[mesh]	14815	622	181	129	bone length
15	"periosteum"[mesh] OR ("periosteal"[title/abstract] AND "circumference"[title/abstract]) AND "english"[language] AND "humans"[mesh]	2220	165	14	2	periosteal circumference
16	"strain index"[title/abstract] OR ("bone AND bones"[mesh] AND "stability"[title/abstract]) AND "english"[language] AND "humans"[mesh]	9204	747	148	76	polar strength strain index of the radius
17	"bone and bones"[mesh] AND ("area"[title/abstract] OR "volume"[title/abstract]) AND "english"[language] AND "humans"[mesh]	23081	1389	218	219	bone area

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