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Titolo della tesi

Micronutrient deficiency and bone metabolism in inflammatory bowel diseases and celiac disease

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ABSTRACT

Inadequate dietary intake of Vitamin D, Vitamin K and calcium as well as inadequate sunlight exposure favor bone loss in the general population, more so in inflammatory bowel disease (IBD) and celiac disease. In these diseases other risk factors are present, including inflammation, but dietary factors, if present, are easily corrected. While the relation between calcium and Vitamin D, and bone metabolism are well known, less data is available for Vitamin K both in healthy individuals and patients.

Aim of the study was to evaluate a wide set of bone metabolism markers, bone specific alkaline phosphatase (BALP), carboxylated and undercarboxylated osteocalcin (ucOC), procollagen type I N-terminal propeptide (PINP), tartrate resistant acid phosphatase (TRAcP), serum type I collagen cross-linked C-telopeptide (CTX), in IBD-, celiac patients and controls, correlating these findings with blood levels of vitamin D and K, and their nutritional intake.

A 28-item quantitative food frequency questionnaire and a 12-item sunlight exposure questionnaire were administered to 111 IBD patients (58 Crohn's disease and 53 ulcerative colitis), 61 patients with celiac disease and 112 controls. Patients' demographics, clinical, endoscopic and laboratory findings were analyzed in relation to recommended daily allowances (RDA). Bone mineral density was assessed by densitometry.

PINP, a marker of osteoblastic activity, was significantly higher in IBD patients compared to controls (9.16±8.84 vs. 7.60±8.84 ng/ml, p=0.0005). No difference was detected for BALP, TRAcP, and CTX.

Age influenced the levels of BALP and TRAcP in females and values were significantly higher in older subjects, both in IBD and controls compared to younger age groups. High levels of CTX were present In IBD patients with active disease vs. inactive disease. No other marker was influenced by disease activity.

With regard to vitamin K intake markers, ucOC levels were non-significantly lower in IBD patients versus controls (p=0.07). A trend toward higher concentrations in Crohn's disease compared to ulcerative colitis was also observed. Significantly higher concentrations of ucOC were present in male (p= 0.02) both in IBD and controls, likely due to a lower intake of green leafy vegetables compared to women.

The mean Vitamin K intake calculated on the base of questionnaires was less than adequate in IBD and normal in controls (IBD 59.56% RDA vs. controls 107.15% RDA, $p < 0.0001$). A significant difference in vitamin K levels was detected also between celiac patients and controls, but was less pronounced than in IBD (celiac disease 98.78% vs. controls 107.15%; $p = 0.0001$; celiac disease 98.78% vs. IBD 59.56%, $p < 0.0001$).

The vitamin D intake was inadequate in IBD, celiac and control patients (55.29% RDA, 60.47% RDA, and 65.34% RDA, respectively), resulting in the absence of statistically significant differences between controls and patient groups.

No significant correlation was found between blood vitamin D levels and sunlight exposure In IBD and celiac patients, as well as in relation to the season of blood sampling. Conversely, as expected, the levels of vitamin D in controls were significantly lower in winter than in summertime (IBD 17.41 vs. 17.15 mg/dl, p= 0.81; controls 21.35 vs 26.93 mg/dl, $p< 0.05$).

No difference in vitamin D concentration was reported In IBD in relation to disease activity indexes as c-reactive protein, fecal calprotectin, clinical or endoscopic disease activity.

Calcium intake was non-significantly lower in IBD and celiac disease patients compared to controls (IBD 100.17% RDA, celiac disease 102.45%, controls 107.15%).

Bone densitometry showed osteopenia in 27/63 IBD patients (42.86%) and 14/23 celiac patients (60.87%), and osteoporosis in 13/63 IBD (20.64%) and 4/23 celiacs (17.39%).

These data confirm that factors affecting bone health, namely diet and sunlight exposure, are abnormal in IBD and celiac patients and provide new interesting information on vitamin K intake and their effects on bone-metabolism markers. Some of these deficiencies are also observed in controls. Thus, the present study prompts further investigation on the impact of dietary factors on the increased risk of osteoporosis and osteopenia in IBD and celiac patients, including larger series of IBD patients and celiac patients not on gluten-free diet.

1. INTRODUCTION

Inflammatory bowel diseases (IBD) and celiac disease (CD), reducing absorption or increasing losses of micronutrients, often result in deficit of vitamins and minerals, exerting negative effects on metabolic processes, immune-response, and the quality of life (QoL) of these patients [1,2].

Vitamin B12, folate and iron deficit are actively sought and treated since decades [3]. Similarly, the increased risk of osteoporosis prompts the measurement of vitamin D levels and calcium, and results in active therapeutic intervention [4]. Less attention is paid to deficit of other vitamins and micronutrients, despite their importance in a wide range of metabolic processes.

IBD and CD patients indeed are at increased risk for osteoporosis due to different mechanisms. High levels of pro-inflammatory cytokines and need for drugs, such as steroids, negatively affect bone homeostasis [5,6]. Inadequate nutritional intake of vitamin D and calcium also impairs bone homeostasis but, when present, can be easily corrected representing a viable target for intervention. More recently, the primary role of vitamin K for bone health has been recognized, and a significant inverse association exists between vitamin K dietary intake and risk of fractures (RR=0.78, 95% CI: 0.56-0.99; I2=59.2%, P = 0.04) [7].

Vitamin K is required for the synthesis of coagulation factors but also acts as a cofactor in the carboxylation of several bone proteins, including osteocalcin [8]. The carboxylation of glutamic acid in gamma-carboxyglutamic promotes the binding of calcium to these proteins and partially counteracts the risk for osteoporosis [8]. Vitamin K is preferentially used for the synthesis of coagulation factors than bone metabolism [9]. Thus, the harmful effect of vitamin K deficit on bone metabolism takes place at blood concentrations that do not affect normal clotting.

The measurement of vitamin K levels is cumbersome and concentrations are indirectly evaluated by dosing vitamin K-dependent carboxylation products, such as the protein induced by vitamin K absence-II (PIVKA-II) and undercarboxylated osteocalcin (ucOC) [10]. However, these indirect markers are not routinely dosed in clinical laboratories, further limiting high-quality data on this topic. The dosage of prothrombin time (PT) is widely used in clinical settings, and abnormally low values associated with normal thromboplastin time

suggests severe vitamin K deficiency. However, the approach is inaccurate and underestimates the prevalence of vitamin K deficit [10].

A recent meta-analysis on fat-soluble vitamin deficits reported high prevalence of vitamin K deficiency in patients affected by Crohn's disease [11]. The study, however, did not evaluate vitamin K levels in ulcerative colitis patients. Similarly, data on vitamin K in adult celiac disease patients are surprisingly lacking, whereas two studies in pediatric series led to contrasting results [12,13]. Thus, prevalence and role of prolonged vitamin K deficiency on bone homeostasis in adult patients with IBD and celiac disease remain an unsolved question.

Vitamin D and bone metabolism

Bone is a highly dynamic tissue, which undergoes continuous remodeling, a physiological process that guarantees bone renewal. Indeed, a correct bone remodeling relies on a perfect balance between bone resorption, performed by osteoclasts and the subsequent deposition of new bone matrix by osteoblasts, which also take care of its mineralization. Bone remodeling is regulated by bone-derived factors, including cytokines, growth factors, chemokines, and endocrine factors. The seco-steroid 1α,25-dihydroxyvitamin D3 (1,25-OH_2D3), influences bone remodeling both through direct and indirect mechanisms [14,15].

The indirect mechanisms consist in the control of calcium reabsorption in the kidney, and intestinal absorption, as well as modulation of parathyroid hormone (PTH) production.

Vitamin D stimulates transcellular and paracellular calcium absorption from the intestinal epithelium. Transcellular transport mechanism is predominant in the proximal small intestine, while paracellular transport occurs in the jejunum and ileum. When the dietary intake is high, paracellular transport provides adequate amounts of calcium [16]. When this is not the case, vitamin D upregulates transcellular transport, enhancing the expression of several proteins, including the epithelial calcium receptor (TRPV6), calbindin and calcium binding protein (CaBP). Interacting with parathormone, 1,25-OH_2D also stimulates calcium and phosphate reabsorption in the kidney [17].

PTH, secreted in response to low plasma calcium- or increased inorganic phosphate levels, binds to parathyroid hormone-1 receptor (PTH1R) in the proximal tubules of the kidney, inducing 1,25-OH_2D production through 1α-hydroxylase (Cyp27B1) stimulation, and 24-hydroxylase (Cyp24A1) suppression. The latter represents the key enzyme of the inactivation pathway of vitamin D. By feedback inhibition, 1,25-OH_2D downregulates its own production stimulating the activity of 24-hydroxylase, inhibiting the activity of 1α-hydroxylase, as well as PTH transcription and secretion [18].

However, the suppressive effect of vitamin D signaling on PTH transcription is less relevant in vivo than in vitro, as the main regulation mechanism is represented by ionized serum calcium levels [19].

The renal effects of vitamin D are primarily mediated by FGF23, belonging to the fibroblast growth factors (FGFs) family, mainly produced by bone osteoblasts and osteocytes [20,21]. It induces increased phosphate urinary excretion and is bidirectionally linked to vitamin D levels [22].

FGF23 binds to a receptor complex consisting of FGF receptor-1c (FGFR1c) and of the co-receptor αKlotho in the kidney. In proximal renal tubules it reduces the number of membrane sodium-phosphate cotransporters, lowering transcellular phosphate uptake. Transcription of 1α-hydroxylase (CYP27B1) is inhibited while the transcription of 24-hydroxylase (CYP24A1) is stimulated [23,24].

In the distal renal tubules, it enhances reabsorption of calcium and sodium, upregulating the number of membrane calcium channel TRPV5 and of the sodium-chloride cotransporter NCC [25].

As inhibitory feedback, the secretion of FGF23 is regulated by several factors including 1,25-OH_2D, PTH, phosphate, and pro-inflammatory cytokines. Vitamin D is the most relevant one [26].

Direct effects are primarily mediated by the presence of vitamin D receptor (VDR) on osteoblasts. Conversely, the effects of VDR expression on osteoclasts are debated [27].

During development, bone is formed according to two different mechanisms: intramembranous (i.e. flat bones of the skull) and endochondral (long bones) ossification. In the former, bone arises directly from a condensation of the mesenchymal tissue, where osteoprogenitor cells proliferate, produce osteoid, a type I collagen-rich matrix, and subsequently differentiate into osteoblasts. In turn, they trigger the deposition of calcium phosphate crystals to produce bone, which is than remodeled into lamellar bone [14].

Endochondral ossification requires the formation of a hyalin cartilage template. Indeed, it starts following the differentiation of mesenchymal stem cells into chondroblasts, first producing type II collagen matrix, and subsequently type X collagen. These cells also produce degradative enzymes (metalloproteinases and phospholipases, alkaline phosphatase). Mature chondrocytes produce osteocalcin, osteopontin, and type I collagen, then they become hypertrophic and promote cartilage matrix calcification. Under these conditions, chondrocytes can no longer receive nutrients from the matrix and undergo apoptosis; at the same time, they produce factors that favors vascularization, which in turn allows the arrival of osteoclast precursors and of osteoprogenitor cells. The former will degrade the calcified cartilage, while the latter will differentiate in osteoblasts, and will lay down bone, eventually leading to primary ossification center in the diaphysis. Later on, secondary ossification centers also form in the epiphysis of long bones. The chondrocytes that remain between primary and secondary ossification centers form the growth plate, where linear bone growth occurs [14].

Data on the effects of 1α,25-OH_2D3 on osteoblast proliferation are conflicting. Both inhibition [28] and stimulation [29] have been reported. Similarly, the effects on cell survival and apoptosis are conflicting [30,31].

Some effects of 1α,25-OH_2D3 on differentiation from immature mesenchymal stromal cells into osteoblasts have also been reported [32-34].

It should be pointed out that most studies evaluating the effect of vitamin D on osteoblasts have been carried out in vitro. Thus, results do not entirely reflect what takes place in vivo. Indeed, outcome is influenced by the experimental micro-environment, and minor changes in the extracellular- (growth factors, cytokines, matrix proteins, ions) or intracellular milieu affect the response to 1α,25-OH_2D3 [35,36].

1,25-OH_2D stimulates osteoclastogenesis in vitro, and at high doses binds the VDR in osteoblasts increasing the expression of RANKL from osteoblasts [37,38]. RANKL binds its receptor on osteoclasts, and increases osteoclast formation and activity [39]. High 1,25-OH_2D in the presence of hypocalcemia increases osteoclastogenesis to restore normal calcemic levels [40].

Animal studies in VDR-deficient mice reported that skeletal abnormalities are reversed by dietary calcium supplementation alone, suggesting minor direct effects of vitamin D [41,42]. However, osteoblast-specific VDR deletion results in minimal increase in trabecular bone volume [43], while osteoblast VDR overexpression results in increased bone mass, due to increased osteoblastic bone formation and reduced osteoclastic resorption [44,45].

These data strongly indicate that vitamin D, besides playing a primary role in the regulation of calcium absorption, has direct effects on bone cells.

Vitamin D, immune system modulation and gut

Vitamin D, besides the effects on bone metabolism, has been linked to a wide range of biological activities, including modulation of gut mucosal immunity and integrity of the intestinal barrier [46,47].

Moreover, vitamin D deficiency has been associated in differing conditions with increased chronic inflammation, and immune system deregulation; a role in IBD has also been suggested [48].

The effects of vitamin D on the immune system are related to the expression of VDR on several types of immune cells, including macrophages, dendritic cells, B- and T cells [49,50]. Inhibition of IL-12 and toll-like receptors expression in dendritic cells and macrophages, as well as the activation of T cells induced by dendritic cells [51,52] have been reported. In the acquired immune system, vitamin D inhibits the proliferation of B- and T-cells, as well as the T-cell production of pro-inflammatory cytokines, IL-2, interferon (IFN)-γ, IL-17, and TNF-α included [53].

Conversely, vitamin D induces the production of IL-10 and other anti-inflammatory cytokines by regulatory T-cells and IL-4 by Th2 cells [53,54].

The effects of vitamin D, inhibiting T cells activity, reducing IFN-γ and IL-17 levels, as well as inducing regulatory cells (T regs, CD8αα, and T), have been documented in animal models of colitis. CD4 T cells from VDR KO and Cyp27B1 KO mice overproduce IFN-γ and IL-17 cells compared to wild-type CD4 cells [55,56].

Conversely, VDR induces FoxP3+ T reg cells, which reduce inflammation through the production of IL-10 and TGF-β in animal models of experimental colitis [57,58].

All these data support some role of vitamin D in chronic inflammatory disorders, in man, but the clinical relevance is still to be defined.

The effects of Vitamin D on gut epithelium are in keeping with this hypothesis in IBD patients.

VDR is highly expressed in the gut epithelium, with a surface-to-crypt gradient [59,60], and plays a central role in regulating the expression of tight junctions (TJ) and adherens junctions (AJ) [61]. The VDR-related control of epithelial permeability also resides in the release of antimicrobial peptides and mucins [61].

Despite the lack of VDR in goblet cells [62], a thinning of the mucus layer is present in CYP27B1−/− mice [63].

The role of vitamin D on intestinal wellbeing is further supported by studies carried out in animals. The VDR/IL-10 double knockout mice develop experimental colitis 8 weeks following stimulus whereas single IL-10- or VDR-knockout animals remain relatively healthy at the same timepoint [64]. Moreover, the induction of epithelial VDR reduces disease activity [59].

Thus, animal studies strongly support the pivotal role of vitamin D/VDR for mucosal barrier function, but results are more controversial in human studies.

VDR expression was significantly lower in IBD versus non-IBD controls in two studies [59,65], but differences were not significant in two other studies, despite inverse correlation between VDR expression and inflammation [66,67]. Lower VDR staining in inflamed samples despite similar VDR gene expression and protein immunohistochemical staining intensity in different intestinal segments, have been reported. The same difference has been observed between IBD patients and controls [66] but, again, the clinical relevance of these findings is still to be determined.

Sunlight exposure, vitamin D synthesis and immune-system modulation

Sunlight exposure induces cutaneous synthesis of vitamin D. It represents a central determinant of bone health, as only minor amounts of the vitamin derive from dietary intake.

UVB photons are absorbed by epidermal chromophores, such as 7-dehydrocholesterol (7-DHC) of the plasmatic membrane of epidermal cells, which is converted into pre-vitamin D [68].

The molecule subsequently undergoes thermal isomerization to vitamin D3 (cholecalciferol), which enters the blood flow. Vitamin D3 is still biologically inactive, and requires activation by two hydroxylation reactions occurring in liver and kidney. Hydroxylation in position 25 takes place in the liver, forming 25-hydroxyvitamin D (25-OHD). This represents the main circulating form of the vitamin and the best indicator of vitamin D status. The active hormone, 1,25-dihydroxyvitamin D (1,25OH_2D), is produced by an additional hydroxylation in the proximal tubules of the kidney [68]. Minor amounts of 25-OHD are also converted into 1,25-OH_2D by other cells expressing 1α-hydroxylase [69,70].

As the major determinant of 25-OHD levels is sun exposure, this form of vitamin D represents the best marker of recent sun exposure [71]. However, only few studies investigated the direct effect of UVB-exposure on the markers of bone metabolism. Despite the lack of adequate data, a modest benefit on bone ALP, as well as on bone formation, has been reported [72].

Sun exposure, through ultraviolet radiation (UVR), affects the immune system upregulating innate response, and modulating adaptive response [73].

UV photons induce the formation of pyrimidine photoproducts, cis-urocanic acid, and oxidized membrane lipids (platelet activating factor and platelet activating factor-like lipids), which induce migration of Langerhans cells to the lymph nodes. The efficiency of these cells in presenting antigens within the germinal center is modified, resulting in increased production and activation of regulatory T-cells (T-regs) [74,75].

Similarly, UV-irradiation of the skin favors the migration of dermal mast cells to lymph nodes, which in turn increase the production of regulatory B cells (B-regs) under the influence of several factors, platelet activating factor and cis-urocanic acid included [74].

Indirect evidence links low sun exposure to increased risk of developing IBD. Living at high latitude minimizes sun exposure [76], and is also associated with high incidence of IBD [77,78]. A latitude-related gradient in eastern Countries has been claimed in some studies [79], but not by others, reporting similar incidence and prevalence of IBD, irrespective of latitude [80].

Although some series suggested increased relapse rates and hospitalization in relation to higher latitude [81,82], the hypothesis that sunlight exposure reduces incidence and severity of IBD lacks hard data.

Interestingly a small clinical study carried out in 21 patients reported that repeated UVB irradiation significantly increased the diversity of the intestinal microbiome [83], and an association between sunlight exposure, microbiota modifications and IBD has also been advocated.

However, at present, no direct evidence suggests that UVR exposure, directly or indirectly through vitamin D-dependent pathways, affects the risk of IBD or disease relapse.

A north-south gradient was also reported in celiac disease, with more celiac patients living at latitudes of 35◦ north or higher, independently of ethnicity [84].

A Swedish study associated summer birth and increased risk of CD. It was hypothesized that low maternal 25(OH)D levels, resulting from less sun exposure during pregnancy represents an adjunctive risk factor for celiac disease, besides timing of gluten introduction and viral infections [85].

The role of UV light exposure in relation to inflammation and bone health is unclear. Long-lasting subclinical intestinal inflammation leads to persistent osteoclast activity stimulation and prevents reversal of bone damage [86]. The importance of this mechanism, as well as the interplay with sunlight exposure, needs further validation. Strict adherence to gluten-free diet (GFD) significantly improves bone mineral density (BMD) [87] but no differences in overall UV exposure were reported in accordance with the presence or absence of symptoms [88]. Finally, as GFD rarely reverts bone losses to normal in adults [89,90], additional mechanisms are likely present.

Vitamin K and bone metabolism

Vitamin K represents an additional factor involved in bone metabolism [91], but its role is less well documented than that of vitamin D. More so the clinical relevance of deficiency.

Vitamin K is a fat-soluble vitamin characterized by a 2-methyl-1,4-naphthoquinone ring. The vitamin K family consists in two sub-groups: vitamin K1 (phylloquinone) and vitamin K2 (menaquinones). Vitamin K1 contains a phytyl chain in C3 position, while vitamin K2 includes several compounds characterized by a polyprenyl chain in the same position. Distinct forms of menaquinones differ in the length of the side chain [92].

Dietary vitamin K1 in vegetables represents the main dietary source of vitamin K in humans [93,94]. Vitamin K2 is present in eggs, meat and dairy products [93], but is primarily synthesized by gut bacteria. The process mainly occurs in the ileum, irrespective of dietary intake [95,96].

Dietary Vitamin K-1 is absorbed in the jejunum by active transport and Vitamin K-2 is absorbed in the small intestine by diffusion. Bile salts and pancreatic enzymes are required for assimilation [97], and the length of the side chain influences absorption rates [98].

The vitamin K family retains its biological activity in the presence of the naphthoquinone ring, which is a cofactor for enzymes involved in a number of biological processes such as coagulation, prevention of vascular calcification, bone metabolism and modulation of cell proliferation [99].

The active form of vitamin K is hydroquinone, produced by quinone reductase or vitamin K epoxide reductase. Hydroquinone is an electron donor for γ-glutamylcarboxylase. The process involves oxidation to 2,3-epoxide, which is converted back to quinone by vitamin K epoxide reductase, allowing multiple use of the molecule [100].

Within the bone metabolism vitamin K is involved in the carboxylation of bone proteins, including osteocalcin (also termed bone Gla-protein) and matrix Gla-protein (MGP) [8]. Osteocalcin is specifically expressed by osteoblasts, while MGP shows a broader expression pattern (chondrocytes, vascular smooth muscle cells and epithelial cells) [101]. Osteocalcin is the main non-collagenous protein in the bone matrix. Upon being released by osteoblasts, it binds hydroxyapatite crystals in the extracellular matrix and promotes mineralization. The osteocalcin affinity for calcium depends on the carboxylation of three glutamic acid residues in gamma-carboxyglutamic [8]. Several other bone proteins require vitamin K-dependent carboxylation, including growth arrest specific protein 6 (Gas6), periostin, periostin-like factor and Upper Zone of Growth Plate and Cartilage Matrix Associated Protein [102].

Osteocalcin has been suggested to be a negative regulator of bone formation as osteocalcin-deficient mice exhibit higher bone mass than wild-type [103]. However, hydroxyapatite crystals are less organized in vitamin K deficiency, possibly favoring frailty [104]. This suggests that osteocalcin plays some role in bone remodeling, rather than being a crucial constituent of bone.

The role of this vitamin K is not limited to the carboxylation of bone-related proteins. Vitamin K2 promoted in vitro mesenchymal stem cell differentiation in osteoblasts, enhancing the expression of cartilage-associated growth differentiation factor 15 (Gdf15) and stanniocalcin 2 (Stc2) [105,106]. Vitamin K also prevents osteoblast apoptosis by inhibiting Fas and Bax genes [105].

NF-κB downregulation induced by Vitamin K also regulates osteoclasts, inhibiting osteoclastogenesis [107], inducing osteoclast apoptosis [108], and reducing the RANKL/OPG mRNA ratio, leading to decreased cell activation [109].

In humans low blood concentrations of vitamin K in IBD patients correlate with decreased bone mineral density [110,111], and inadequate intake favors hip fractures [7,112]. The harmful effect of vitamin K deficiency on bone metabolism is present at blood concentrations that do not affect clotting, as vitamin K is preferentially used in the synthesis of coagulation factors than for bone metabolism [9].

Vitamin K immune-system modulation and gut

Anti-inflammatory and immunosuppressive activities of Vitamin K in the intestine have recently been hypothesized.

Vitamin K indeed reduces the levels of IL-6 in human macrophagic THP-1 cells in vitro [113] and Vitamin K2 MK-4 represses IKKa/b phosphorylation, inhibiting NFkB [114]. Synthetic Vitamin K3 and K4, but not naturally occurring vitamin K1 and K2, inhibit the activation of NLRP3 inflammasome [115]. These results, however, have not been replicated in vivo.

Vitamin K acts in vitro as a scavenger for free radicals as MK-4 suppressed the upregulation of iNOS, COX-2, p38, NF-kB expression, and caspase-1 activation in intestinal cell lines [116,117]. Dietary supplementation of vitamin K1 reduces the lipopolysaccharide-induced inflammatory response in animals [113]. Similarly, low levels of Vitamin K are associated with worse inflammation in murine DSS colitis, whereas Mk-4 supplementation reduces the levels of IL-6 and IL-10 [118].

Bidirectional effects suggest a relation between vitamin K levels and gut microbiota. Lower diversity, with marked reduction in Lachnospiraceae and Ruminococcaceae, has been reported in Vitamin K-deficient

subjects compared with controls [119]. MK-4 and MK-9 supplement reduce the number of caecal Bacteroides and increase Lactobacillus [120]. Vitamin K supplementation increases Proteobacteria counts, such as C. lanceolatus, P. phenylpyruvicus, and P. excrementihominis, while reducing inflammation- and cancer-related strains, such as H. mesocricetorum and H. apodemus [121].

In turn, microbial changes influence the production of bacterial metabolites, short chain fatty acids (SCFA) included. Microbial MK-7 boosts the levels of caecal acetic acid and butyric acid [121] and high concentrations of vitamin K1 in the diet increase butyrate concentration and diminish those of propionate, isobutyrate, and isovalerate [122].

Bone metabolism markers

Some bone metabolism markers are useful in predicting fracture risk in different clinical settings, gastrointestinal diseases included [123]. Bone alkaline phosphatase (BALP), tartrate resistant acid phosphatase (TRAcP), serum type I collagen cross-linked C-telopeptide (CTX), and osteocalcin (OC) are most consistently associated with bone fractures [123,124]. In gastroenterological patients the association is less well documented for elevated procollagen type I N-terminal propeptide (PINP) and beta-CTX levels [125]. However, according to the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine, PINP represents the marker of choice of bone formation, and beta CTX of bone resorption [123,126]. Unfortunately, despite this, widespread clinical application is limited by high cost and large biologic variability [127].

In IBD low bone mineral density was linked to suppression of bone formation rather than enhanced bone resorption in a small cohort of 26 patients affected by Crohn's disease [128]. Low levels of OC and high levels of CTX, representing risk factors for osteoporosis, were documented in a cohort of IBD patients [129]. High levels of TRAcP, more so in those with highest disease activity, were reported in a single series of IBD patients [130]. Noteworthy, despite the high prevalence of low bone density in IBD, bone metabolic alterations were evaluated in only small series. As a consequence, in some instances surprisingly low prevalence of altered levels of bone biomarkers were reported (4.1% elevated OC, 14.4% raised CTX) [131].

Data on bone turnover in Crohn's disease and ulcerative colitis are not entirely concordant, higher levels of bone resorption in Crohn's disease were reported in some studies [131], and the opposite in other [132].

The evaluation of bone metabolic markers in IBD is further complicated by the opposite influence of drugs as steroids are detrimental, and biologics exert favorable effects. The use of anti-TNFα is associated with favorable effects on bone resorption and apposition, as documented by the concentrations of specific markers as PINP, CTX, ALP and OC [133,134]. The effect of other biologics and small molecules on bone metabolism has not been clearly assessed but a favorable impact may be anticipated, resulting from modulation of cytokines involved in bone remodeling.

Even less clear is the behavior of bone biomarkers in newly diagnosed celiac disease. At diagnosis some cohort studies reported significantly higher levels of ALP, OC, and CTX versus controls [135]. Conversely, other studies reported low CTX concentrations in 50% of patients, associated with normal OC levels. PINP levels were instead higher than normal in celiac patients on free diet [136,137]. Overall, in most series the levels bone markers did not significantly differ from those in the controls [138,139].

Contrasting results have been published also in celiac patients following gluten-free diet.

As expected, the levels of BALP, PINP and N-telopeptide of type I collagen (NTX) of celiac patients on long-term gluten free diet did not differ from those of controls [140].

This contrasts with results from the same study group, reporting that serum BALP concentrations of patients with celiac disease were significantly lower than those of controls upon diagnosis ($p=0.006$), but gradually increased on gluten-free diet ($p=0.024$). Conversely, patients with untreated disease had significantly higher urinary concentrations of NTX than controls ($p<0.0001$), but the concentrations were not significantly affected by diet ($p=0.37$) [141].

Few studies, small number of patients and conflicting results highlight the existing knowledge gap in this area.

2. AIMS OF THE STUDY

The study was aimed at providing information on the relationship between Vitamin D and K, and bone metabolism in two population of patients characterized by increased risk of osteoporosis, inflammatory bowel disease and celiac disease, comparing data with those of a normal control population.

As primary endpoints, four main areas were investigated:

- **prevalence of vitamin D and vitamin K deficiency**

  direct dosage of 1-25-OH-vitamin D.

  indirect evaluation of Vitamin K through the dosage of the vitamin K-dependent enzyme ucOC.

- **assessment of the nutritional intake of vitamin D, vitamin K and calcium.**

  using a 22-item, validated food frequency checklist evaluating the 7-days intake of Ca^{2+} and Vitamin D

  using a 26-item, validated food frequency checklist evaluating the 7-days intake of Vitamin K

- **assessment of sunlight exposure**, to correlate the nutritional intake of vitamin D and its metabolically active form in blood, using a validated questionnaire for UV light exposure.

- **direct dosage of bone remodeling markers**

  bone specific alkaline phosphatase (BALP), carboxylated osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP) for evaluating **number and function of osteoblasts**

  tartrate resistant acid phosphatase (TRAP) and serum type I collagen cross-linked C-telopeptide (CTX) for evaluating **number and function of osteoclasts**

  sclerostin (SOST) and DKK1 as **negative bone mass regulators**

As secondary endpoints, correlations were sought between:

- Vitamin K and vitamin D deficiency and **bone mineral density** in patients with IBD and celiac disease by bone densitometry (**DEXA**)

- prevalence and severity of vitamin D and K deficits with **disease activity**, location and extent of the lesions in Crohn's disease and ulcerative colitis

- prevalence and severity of vitamin D and K deficits with

  severity of histological lesions (Marsh-Oberhuber classification) in celiac patients

  the effect of **one-year gluten-free diet**

-vitamin D and K deficits and the lack of **other micronutrients**:

  iron, vitamin B12 and folate in IBD and celiac disease.

3. MATERIALS AND METHODS

The present observational cohort study prospectively enrolled patients affected by IBD and CD, visited in the Gastroenterology Unit, San Salvatore Hospital, L'Aquila.

Inclusion criteria were former diagnosis of IBD or CD, age between 18-65 years, absence of concomitant diseases listed as exclusion criteria. All patients were asked to sign a written informed consent to participate in the study.

Age under 18 or over 65 years, previous diagnosis of cancer, previous diagnosis of HIV infection and pregnancy represented exclusion criteria, as well as treatment with bisphosphonates, history of spontaneous bone fractures, presence of diseases that affect vitamin K levels (acute and chronic hepatitis, pancreatic insufficiency, short bowel syndrome, hematological disorders), presence of diseases affecting bone metabolism (type I diabetes, hyperparathyroidism, chronic kidney or liver disease). Patients taking drugs that interfere with vitamin K levels (warfarin) or bone metabolism (phenytoin, NSAIDs, oral contraceptives, corticosteroids) were also excluded from the study.

The control population consisted in subjects aged 18-65 years undergoing screening colonoscopy or esophagogastroscopy at the UO of Gastroenterology, Hepatology and Nutrition of the PO San Salvatore of L'Aquila, which were enrolled after signing written informed consent. Neoplasia or inflammatory colorectal pathologies detected by endoscopy were considered exclusion criteria, as well as those listed in the exclusion criteria for patients with IBD and CD.

Demographics (age and gender), were recorded in all participants. Clinical data including duration and clinical activity of the disease were investigated in all patients, as well as site and extent of lesions in IBD.

The nutritional intake of vitamin D, vitamin K and calcium and the levels of sunlight exposure, essential for the transformation of vitamin D into its metabolically active form, were assessed in patients and controls using specific validated questionnaires [142-144].

Laboratory parameters including blood cell count, serum iron, serum ferritin, vitamin D, ucOC, vitamin B12, folate, parathormone were dosed as well as anti-endomysium antibodies and anti-transglutaminase antibodies in patients with CD, and C-reactive protein and fecal calprotectin in IBD.

The following bone remodeling markers were also investigated in patients and controls: bone specific alkaline phosphatase (BALP), carboxylated osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP) for evaluating number and function of osteoblasts. Tartrate resistant acid phosphatase (TRAP) and serum type I collagen cross-linked C-telopeptide (CTX) were dosed for the evaluation of number and function of osteoclasts [145,146]. Negative bone mass regulators included Sclerostin (SOST) and DKK1 [147,148].

Blood samples were obtained in the morning, after an overnight fast. The blood was left 30 minutes at room temperature to induce clotting. Serum was separated by centrifugation for 10 minutes at 1000 rpm, at 4°C. Using a clean pipette, 210µl of serum were collected into labeled cryovials and immediately frozen in a –80 °C freezer.

All the bone remodeling markers were dosed in the research laboratory of Morphology and Function of Skeletal System, PO San Salvatore, L'Aquila using the following ELISA kits: Octeia Ostase BAP (Pantec S.r.l., Torino, Itay), b Cross-Laps Siero (Pantec S.r.l., Torino, Itay), Human Trap 5b, PINP human Procollagen I N-terminal (Pantec S.r.l., Torino, Itay), Human Undercarboxylated Osteocalcin (ucOC) (Pantec S.r.l., Torino, Itay). Bone mineral density was assessed by densitometry (DEXA) in patients and controls.

Severity and extent of disease activity was assessed in IBD by ileocolonoscopy (CS) with multiple biopsies in ulcerative colitis and Crohn's disease, and by magnetic resonance imaging in Crohn. The severity of histological lesions of the duodenal mucosa according to the Marsh-Oberhuber classification, was evaluated in six duodenal biopsies collected during esophagogastroduodenoscopy (EGDS) in celiac disease patients.

Evaluation of food intake

Information on the diet during the week prior to observation was acquired by completing a food frequency checklist. The questionnaire was adapted from a 22-item quantitative FFQ, previously validated for calcium [143] and Vitamin D intake, integrated with 6 specific questions focused on foods with the highest phylloquinone concentration (100–400 µg VitK/100 g). Green leafy vegetables, including spinach, iceberg lettuce, chicory, beets, turnip tops and rocket salad, as well as eggs, are the main contributors to Vitamin K intake. In accordance with previous studies, using validated FFQ, the food list included country-specific items [149,150] to minimize the risk of over- and underestimating Vitamin D and Vitamin K intake.

The interview assessed and recorded how often each item was consumed. The usual serving size of foods was evaluated using a photographic atlas of food portions [151]. The daily phylloquinone and Vitamin D intake from the diet was calculated by multiplying the frequency and serving size for each portion by the nutrient content of the food.

As the available reference data for vitamin content show marked differences, in the present study we used the mean value from three different sources (EFSA, United States Department of Agriculture and Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione). The food composition values we used are shown in Appendix A.

Data recorded from patients and controls were expressed as a percent of the recommended daily allowances (RDA) for Vitamin D, using the reference values of 15–20 µg/d [152]. An inadequate intake of Vitamin D was defined as <66% of RDA.

Conversely, the Average Daily Requirements (AR) and Population Reference Intakes (PRI) of Vitamin K differ according to Country and local eating habits [153,154]. Thus, we chose as reference AR values 140 µg/d for females and males up to 59 years old, and 170 µg/d for subjects >60 years, as proposed by the Società Italiana di Nutrizione Umana (SINU) [154]. These values are higher than those proposed by NIH and EFSA [152,153] and reflect the Mediterranean diet in our Country. Vitamin K data were thus expressed as a percent of the reference intake levels. Daily intakes of Vitamin K <66% AR were defined as inadequate.

Evaluation of sunlight exposure

The dedicated questionnaire included an evaluation of skin phototype and questions about sport and outdoor activities, work included, time of the year, location and duration of holidays, time spent outdoors differentiating between summer and winter, use of sunscreen, sunglasses, and hats.

The score for calculating sun exposure was developed by giving different weights (ranging between 0 and 1), according to all sun exposure domains listed in the questionnaire (1 if most of the body was uncovered, 0 if the body was covered or 0.5 if partially covered). The untanned skin characteristics of patients were evaluated as follows: skin phototype I (very light complexion, blond or red hair, blue eyes, no tan, 80% UVB penetration) received a score of 0.8. Type II (fair complexion, light brown or dark blond hair, blue eyes or brown, light tan, 67.5% UVB penetration), received a score of 0.675. Type III (clear brown complexion, brown hair, blue or brown eyes, dark tan, 55% UVB penetration) a score of 0.55; Type IV (olive complexion and dark hair, dark brown or black, dark eyes, intense tan, 42.5% UVB penetration) a score of 0.425 and Type V (olive-brown complexion, hair blacks, dark eyes, 30% of UVB penetration) a score of 0.3; type VI phototype (black complexion, black hair, dark eyes, always tanned, <30% of UVB penetration). The final scoring algorithm was created by multiplying the time spent in the sun by the proportions of the different domains. On the base of the scores <10, 11-20 and >20, the study population was divided into three groups: low, moderate, and high sunlight exposure, respectively. The original questionnaire [142] was then integrated with two more questions examining the use of tanning lamps and the frequency of outdoor activity at high altitude, like skiing, to evaluate the contribution of these factors to overall sunlight exposure.

Sample size calculation and statistical analysis

The sample size was calculated based on published data for vitamin D and K, as no adequate information is available on bone activity markers.

Considering the occurrence of adequate intake of vitamin D in 75% of control subjects and 50% of patients with IBD and CD, with alpha error of 0.05 and power of 95%, 95 patients per group were needed.

Using the same levels of alpha error and power for vitamin K, considering adequate intake in 85% of healthy subjects and 60% of patients with IBD and CD, the sample required is 80 patients per group.

Therefore, enrolling at least 95 patients with IBD, 95 patients affected by CD and 95 healthy control subjects, was considered adequately powered.

The statistical analysis was carried out using the SAS statistical package (version 9.4, 2002-2012 by SAS Institute Inc., Cary, NC, USA). Data were compared using Wilcoxon Rank Sum Test for continuous variables and Chi-Squared Test or Fisher's Exact Test, as appropriate, for dichotomous variables. Spearman's nonparametric correlation was used to evaluate the degree of relationship between variables. $P<0.05$ has been considered statistically significant.

4. RESULTS

Patient population

The patient population consisted of 111 IBD patients, 61 patients affected by celiac disease and 112 controls observed in our Institution between March 2021 and September 2023 (Table 1a and Table 1b).

Fifty-one (45.94%) IBD patients were male and 60 (54.06%) females. The mean age was 48.86 ± 15.84 years (range 18–65 years).

IBD patients were affected by Crohn's disease in 58 cases and ulcerative colitis in 53 cases. Diagnosis of ulcerative colitis and Crohn's disease was based on clinical, endoscopic, and histological findings.

Disease localization and behavior were classified according to the Montreal Classification [155].

Ulcerative colitis patients presented with extensive colitis in 29 cases (E3), left-sided colitis (E2) in 19 and proctitis (E1) in 5 cases.

Crohn's disease patients presented with ileal disease in 24 cases (L1), colonic disease in 8 cases (L2) and 26 had ileo-colonic disease (L3). Thirty patients had inflammatory disease, 19 stenosing disease and 9 fistulizing disease.

Most patients were treated with biologics or small molecules (49 Crohn's disease, 38 ulcerative colitis). Only one patient in each IBD group was treated with azathioprine and steroids. The remaining patients were treated with mesalamine only (5 Crohn's disease, 13 ulcerative colitis). Two patients affected by Crohn's disease and one with ulcerative colitis did not take any medication.

Disease activity was assessed using the Harvey–Bradshaw index (HBI) in CD, and the Partial Mayo score in UC [156,157]. Remission was defined as HBI ≤ 4 for CD and as a partial Mayo score ≤ 1 for UC patients.

Patients affected by Crohn's disease were in clinical remission in 25 cases and presented with active disease in 33 cases (mild 26 patients, moderate 7 patients).

Twenty-six patients with ulcerative colitis were in clinical remission, and 27 had clinically active disease (15 mild-, 11 moderate- and 1 severe disease).

Endoscopic disease activity was recorded using the Simple Endoscopic Score for Crohn's Disease (SES-CD) and the Mayo endoscopic score for ulcerative colitis [158,159].

Patients with Crohn's disease showed endoscopic activity in 37 cases (19 mild, 17 moderate, 1 severe disease), while 15 were in remission. Six patients did not undergo endoscopic examination in the 12 months preceding enrollment.

Thirty-one patients affected by ulcerative colitis had endoscopically active disease (12 mild, 13 moderate, 6 severe), while 15 were in remission and 7 did not undergo endoscopy in the 12 months preceding enrollment.

Sixty-one celiac patients participated in the study: 16 male and 45 female. Mean age of the enrolled patients was 40.13±15.54. The majority of patients had already been diagnosed as affected by celiac disease and were on a gluten-free diet. Five patients were enrolled at the time of diagnosis.

The histological grade of CD was assessed by the Marsh classification. Upon diagnosis patients were classified as follows: Marsh-1 in 3 patients (4.93%), a Marsh-2 in 12 patients (19.67%), and a Marsh-3 grade in 46 patients (75.4%). All but one patient on gluten-free diet, showed reversal of histologic damage at the last follow-up endoscopy. Adherence to gluten-free diet was evaluated with serum anti-transglutaminase IgA and anti-endomysial antibodies. The same patient showing histologic persistence of mucosal damage, was the only one showing positive antibodies during follow-up, likely reflecting non-adherence to the gluten-free diet.

Control patients were 49 male and 63 female. Mean age was 48.01±15.69. Patients underwent upper or lower endoscopy for the following reasons: screening (43), heartburn or dyspepsia (38), stypsis (19), recurrent abdominal pain (12).

Sera for bone metabolism markers and the indirect evaluation of vitamin K were collected in 91 IBD (47 Crohn's disease, 44 ulcerative colitis), 55 celiac patients, 99 controls.

Vitamin D levels

Vitamin D levels were 17.32±7.14 ng/mL in the 49 IBD patients not taking supplements, and 31.63±8.84 ng/mL in patients taking supplements. Patients affected by celiac disease that did not supplement vitamin D (24) had mean vitamin D levels of 22.26±6.06 ng/mL, in those taking supplements was 36.69±14.87 ng/mL. Healthy controls did not take supplements and showed mean vitamin D concentration of 24.26±5.58 ng/mL (Table 2).

Statistical analysis showed a significant difference in both IBD patients' groups versus controls ($p<0.05$), as well as in both celiac disease groups versus controls ($p<0.05$). In the subgroup of patients not taking supplements, significantly lower levels of vitamin D were detected in IBD compared to celiacs ($p<0.05$).

The same was observed in the IBD and celiac patients supplementing vitamin D ($p<0.05$).

No sex-related difference in the mean vitamin D levels was detected in patients not taking supplements (IBD: males 16.94± 6.95 ng/mL vs. females 17.79± 7.51 ng/mL, $p=0.85$; Celiac disease: males 20.35± 4.24 ng/mL vs. females 20.44± 7.25 ng/mL, $p=0.87$; controls: 26.05± 4.64 ng/mL vs. females 23.57± 6.03 ng/mL, $p=0.05$). However, controls showed significantly higher levels compared to celiac and IBD patients in males ($p<0.0001$) and in females ($p=0.0014$). No significant sex related difference was instead observed between IBD vs. celiac disease patients.

Patients taking supplements did not show significant gender-related difference in vitamin D levels (IBD: males 32.02± 12.53 ng/mL vs. females 31.45± 6.87 ng/mL, $p=0.56$; Celiac disease: males 30.42± 4.64 ng/mL vs. females 38.17± 16.65 ng/mL, $p=0.43$). Similarly, no difference was detected in patients affected by IBD vs. celiac disease (males: $p=0.86$; females: $p=0.26$).

In the present study we used the median age of 47 years as a cut-off to evaluate age-related differences. In IBD patients not taking supplements younger individuals showed non-significantly lower hematic concentrations of vitamin D (15.53± 6.50 ng/mL vs. 19.04± 7.43 ng/mL, p=0.10). The trend was not confirmed in celiac patients not supplementing vitamin D (≤47: 20.63±7.01 ng/mL; >47: 19.70±5.66 ng/mL, p= 0.91). Conversely in controls significantly higher hematic vitamin D concentrations were observed in younger patients (≤47: 26.04± 5.64 ng/mL; >47: 23.45±5.27 ng/mL, p= 0.009).

In patients taking supplements, no-age related difference was detected (IBD: 31.18±11.49 ng/mL vs. 31.96±6.46 ng/mL, p=0.19; Celiac disease: 35.67±12.29 ng/mL vs. 36.44±17.37 ng/mL, p=0.69).

In Crohn's disease patients not taking supplements, disease localization did not influence vitamin D levels (L1: 17.38±5.71 ng/mL; L2: 19.65± 11.87 ng/mL; L3: 14.84± 4.54 ng/mL; p= 0.85). Similarly, no difference was detected in patients taking supplements (L1: 32.76± 6.01 ng/mL; L2: 31.50 ng/mL; L3: 28.38± 5.91 ng/mL; p= 0.17) (Table 3).

Conversely, in patients affected by ulcerative colitis not taking supplements, individuals affected by extensive disease showed significantly higher vitamin D concentrations (E1: 16.00 ng/mL; E2: 14.06± 7.77ng/mL; E3: 20.94± 5.59 ng/mL, p= 0.03). In patients supplementing vitamin D no difference was instead observed (32.62±9.87 ng/mL for E1, 35.41±15.41 ng/mL for E2, 29.21± 8.07 ng/mL for E3, p=0.45) (Table 4).

Vitamin D dietary intake and RDA

In IBD patients that did not supplement the vitamin D, the mean intake assessed on the basis of food questionnaires was 8.10±4.99 µg/day (50.88±35.12% RDA). In IBD patients taking vitamin D supplements was 9.09±5.10 µg/day (61.21±34.19% RDA). In celiac patients not taking supplements dietary intake of vitamin D was 8.58±3.58 µg/day (68.45±55.11% RDA). Celiac patients supplementing vitamin D had a mean intake of 9.00±6.05 µg/day (57.17±23.86% RDA). Controls did not supplement vitamin D and showed mean intake of 9.84±5.18 (65.34±34.65% RDA). Intake of controls significantly exceeded those of the IBD patients not taking supplements (p=0.04). No significant difference was observed in vitamin D intake in the other groups (p = 0.21) (Table 5).

In the control group significantly higher vitamin D intake was observed in males (males: 11.15±5.44 μg/day, 74.19±36.46% RDA; females: 8.80±4.76 μg/day, 58.35±31.73% RDA, p=0.014). In IBD patients that did not take supplements no significant sex-related difference was detected, with mean vitamin D intake of 8.54±3.85 μg/day (51.75±30.12% RDA) in males and 7.58±6.14 μg/day (49.85±40.97% RDA) in females (p=0.35). No difference was observed also in celiac patients not taking supplements (males: 8.17±6.33 μg/day, 54.43±42.19% RDA; females 8.63±3.43 μg/day, 57.53±22.86% of RDA; p =0.94).

In this sub-group, significantly lower RDA was reported in IBD males versus controls (p=0.04), but not in IBD versus celiacs, or celiacs versus controls. A trend was observed in female IBD patients, showing non-significantly lower RDA versus controls (p=0.07). No significant difference was detected in IBD versus celiacs, or celiacs versus controls.

A trend of higher dietary intake of vitamin D in males, was observed also in IBD patients taking supplements (males: 11.56±7.21 μg/day, 77.03±48.05% RDA; females 7.81±3.01 μg/day, 52.68±20.18% RDA; p=0.05). This was not the case in celiac patients taking supplements (males: 10.83±5.13 μg/day, 72.20± 34.19% RDA; females: 8.29± 6.41 μg/day, 54.76± 43.16 RDA; p=0.27).

No significant sex-related difference in RDA was recorded between IBD and celiac patients, taking supplements.

Considering the median age of 47 years as cut-off, in IBD patients not taking supplements, the mean vitamin D intake was higher in younger individuals (≤47: 9.79± 5.89 μg/day, 60.17± 43.17% RDA; >47: 6.41± 3.22 μg/day, 41.58± 21.83% of RDA, p= 0.04). In IBD patients taking supplements the difference was not observed (10.02± 6.73 μg/day, 66.76± 44.88% of RDA vs. 8.29± 3.03, 56.18± 20.26 of RDA; p=0.84).

In celiac disease, no age-related difference was found in patients not taking supplements, both considering the mean intake value (8.80±3.78 μg/day vs. 7.85± 3.22 μg/day, p= 0.73) and RDA (58.66±25.18% vs. 52.30± 21.44%, p= 0.73).

The same was observed in celiac patients taking supplements both for mean intake value (8.04± 5.58 μg/day vs. 9.76± 6.59 μg/day, p= 0.32) and RDA (53.60±37.19% vs. 64.41±44.63%, p=0.46)

Controls showed inadequate mean values for Vitamin D consumption in all age groups, with no significant age-related difference (≤47: 10.17±4.64 μg/day, 67.83± 30.97% RDA vs. >47: 9.54±5.63, 63.15±37.73% RDA, p=0.18).

In patients affected by Crohn's disease not taking supplements, disease localization markedly influenced vitamin D the daily intake (L1: 10.91± 3.18 μg/day; L2: 4.36±1.75 μg/day; L3: 10.15± 4.17μg/day, p= 0.03) and RDA (L1: 72.73± 21.21%; L2: 27.58± 12.83%; L3: 67.64± 27.82%, p= 0.03).

Instead, in patients taking supplements median daily vitamin D consumption was not influenced by disease localization (L1: 9.15± 2.46 μg/day; L2: 7.05 μg/day; L3: 11.06±4.88 μg/day, p= 0.48). The same was subsequently observed for RDA (L1: 63.44± 14.96%; L2: 47.00%; L3: 73.75± 32.53%, p= 0.44) (Table 3).

In ulcerative colitis, disease localization did not influence vitamin D intake (E1: 8.66±2.54 μg/day; E2: 7.39± 2.73 μg/day; E3: 6.46± 2.77 μg/day, p=0.19) and RDA (E1: 57.73±16.93%; E2: 46.94± 19.44%; E3: 36.64± 21.12%, p=0.15) in patients not taking supplements.

In patients supplementing vitamin D no difference in dietary intake (E1: 7.43±5.63 μg/day; E2: 6.93± 2.82 μg/day; E3: 8.82± 9.22 μg/day, p=0.56), and RDA was observed (49.53±37.53% for E1, 46.19± 18.82% E2, 58.78± 61.47% E3, p=0.56) (Table 4).

Vitamin K dietary intake and RDA

The mean Vitamin K intake was 88.04±75.62 μg/day in the IBD group, corresponding to 59.56±49.71% of the RDA, and 197.33±131.52 μg/day in the controls (135±91.68% RDA). The difference between the healthy controls and IBD patients was statistically significant ($p < 0.001$). The intake of vitamin K in celiac patients was 140.23±80.59 μg/day (98.78±56.86% of RDA), and was significantly higher than in IBD patients, but significantly lower than in controls ($p < 0.001$) (Table 6).

Within the IBD group, the total daily Vitamin K intake was non-significantly lower in Crohn's disease (80.75± 60.41 μg/day, 55.14±40.64 % of RDA) than in ulcerative colitis (95.77± 88.94 μg/day, 64.15±57.72 % of RDA) (p=0.65) (Table 7).

No sex-related difference in vitamin K intake was detected in the three enrolled groups.

In the IBD group vitamin K intake was 73.83± 56.42 µg/day (50.10± 39.03% of RDA) and 100.44± 87.71 µg/day (67.96±56.61% of RDA) in males and females, respectively (p=0.13). The intake of this vitamin in celiac patients was 131.87±79.65 µg/day (90.58±54.32% of RDA) in males and 142.69±81.88 µg/day (101.19±58.16% of RDA) in females (p=0.72). Vitamin K intake in the control males was 187.86±111.56 µg/day (130.40±80.26% of RDA) and 204.82±145.84 µg/day (139.84±100.38% of RDA) in females (p =0.97).

The IBD group showed lower levels of vitamin K intake compared to celiac patients and controls in both genders (p<0.000001). No significant difference in daily intake was instead observed in celiacs compared to controls in both genders.

The mean daily intake of vitamin K was low in IBD patients aged ≤47 (78.18±62.17 µg/day, 55.83± 44.40% of RDA, respectively) as well as in those aged >47 (97.71±86.34 µg/day, 63.28± 54.69% of RDA) (p=0.25).

In celiac patients aged ≤47 the levels were 124.09± 78.64 µg/day (88.63± 56.17% RDA) and in those aged >47 163.55± 79.74 (113.44±56.16% RDA) (p=0.10).

Controls showed adequate mean values for Vitamin K assumption in all age groups (≤47 188.40±128.57 µg/day, 134.51±91.77% RDA; >47 205.21±134.66 µg/day, 136.64± 92.39%) (p=0.56).

Young IBD patients showed lower levels of vitamin K intake compared to celiac patients and controls (p=0.000001). The same was observed in celiacs aged less than 47 years, versus controls (p=0.000001).

In patients with more than 47 years a significant reduction in vitamin K intake was observed in IBD versus celiac patients and controls (p=0.000001), but no significant difference was present between celiacs and controls.

Disease localization in Crohn's disease did not influence the daily intake (L1: 79.05± 67.66 µg/day, 54.63± 45.37% RDA; L2: 57.79± 40.59 µg/day, 37.48± 23.38% RDA; L3: 85.40± 55.94 µg/day, 58.28± 37.68 RDA; p=0.67) (Table 8). The same applied to ulcerative colitis (E1: 97.47± 119.92 µg/day, 69.62±85.65% RDA; E2: 100.96 ± 111.86 µg/day, 64.92 ±68.08 % RDA; E3: 93.96 ± 73.23 µg/day, 64.40 ±51.41 % RDA; p= 0.96) (Table 9).

Calcium dietary intake and RDA

The mean calcium intake was 1084.01±524.64µg/day in the IBD group, corresponding to 100.17±49.16% of RDA, and 1142.42±645.94 µg/day in controls (107.15±60.99% RDA). The difference between controls and IBD patients was statistically significant ($p<0.05$). The intake of calcium in celiac patients was 1127.29±513.16µg/day (102.45±47.67% of RDA), and was significantly higher than in IBD patients, but significantly lower than in controls ($p<0.05$) (Table 10).

Within the IBD group, the total daily calcium intake did not show differences in Crohn's disease (1118.44±525.85 µg/day, 103.70±48.08 % of RDA) versus ulcerative colitis (1047.51±526.20 µg/day, 96.43± 50.50% of RDA) ($p=0.50$) (Table 7).

No sex-related difference in calcium intake was detected. It was 1038.95±497.43 µg/day (99.36±47.81% of RDA) in males and 1123.33±548.80 µg/day (100.88±50.74% of RDA) in females, respectively ($p=0.39$), within the IBD group. The intake of calcium in celiac patients was 1121.93±475.61 µg/day (108.13±50.17% of RDA) in males and 1128.87±530.50 µg/day (100.72±47.55% of RDA) in females ($p =0.98$). Calcium intake in control males was 1185.54±596.06 µg/day (114.39±59.88% of RDA) and 1108.35±685.69 µg/day (101.44±61.73% of RDA) in females ($p=0.17$). No sex-related difference between the three groups was observed.

The mean daily intake of calcium was normal in both in IBD patients aging ≤47 and >47 (1029.08±488.96 µg/day, 101.62± 47.53% of RDA; and 1137.89± 556.90 µg/day, 98.75± 51.13% of RDA, respectively) ($p=0.33$). Normal calcium intake was observed also in celiac patients, irrespective of age (≤47: 1071.76± 503.73 µg/day, 104.85±51.31% RDA; >47 1207.49±530.48 µg/day, 98.76±42.75%) ($p=0.37$).

Similarly, in controls the mean dietary calcium was adequate in both age groups (≤47 1119.98±564.14 µg/day, 111.99± 56.41% RDA; >47 1162.20± 714.58 µg/day, 102.89± 64.93% RDA) ($p=0.93$).

No difference was observed between IBD, celiac patients and controls in the same age-subgroup.

The disease localization in Crohn's disease did not influence the daily intake of calcium (L1: 1065.42± 518.24µg/day, 98.22± 46.04% RDA; L2: 1384.55± 734.55µg/day, 125.16± 62.91% RDA; L3: 1117.01± 520.79µg/day, 105.54± 50.15 RDA; $p=0.54$) (Table 8). The same was true in ulcerative colitis (E1 882.31 ± 235.84 µg/day, 82.26 ± 32.02 % RDA; E2 1027.28 ± 539.11 µg/day, 92.83 ± 45.83 % RDA; E3 1071.33 ± 551.09 µg/day, 99.42 ± 56.12 % RDA; $p= 0.93$) (Table 9).

IBD activity and vitamin D, K, and calcium

Blood vitamin D did not significantly differ in relation to disease activity (Table 11).

In the absence of vitamin D supplements, the levels of the vitamin did not differ when disease was in clinical remission or active (17.45±6.27 ng/mL vs. 17.23±7.80 17.23 7.80, p= 0.53). No difference was detected comparing mild, moderate, and severe disease (17.77±6.27 ng/mL, 16.80±8.22 ng/mL, and 18.33±7.59 ng/mL, respectively) (p=0.65). Despite non-significant difference between inactive vs. active disease (33.06±9.68 ng/mL vs. 29.51±7.21 ng/mL, p= 0.14) the same was true in patients supplementing vitamin D. Similarly, no difference was detected in patients with clinically inactive disease (p=0.31) compared to those with mild 32.72±9.65 ng/mL, moderate 29.66±6.47 ng/mL and severe disease 28.80±11.95 ng/mL.

Endoscopic activity was not related to significant differences in the mean levels of vitamin D of patients who did not take supplements (remission: 19.63±7.78 ng/mL vs. active disease: 16.91± 6.96 ng/mL, p=0.42). No statistical difference was identified between patients in remission (p = 0.87) versus those with mild (17.18±6.46 ng/mL), moderate (18.22±8.04 ng/mL), and severe (16.50±7.38 ng/mL) endoscopic lesions.

Comparable levels of vitamin D were also observed in IBD patients taking supplements, regardless of endoscopic disease activity (remission: 32.03±11.80 ng/mL; active: 30.17± 7.19 ng/mL, p=0.88) or severity of endoscopic lesions (mild- 30.88±11.80 ng/mL, moderate- 29.14±7.73 ng/mL, and severe disease 22 ng/mL) (p=0.30).

Patients were classified on the base of biomarkers, and using a cut-off value of 250 μg/g for fecal calprotectin and of 0.5 mg/dl for C-reactive protein were selected to differentiate active from inactive disease.

The blood levels of vitamin D in patients not taking supplements did not differ in relation to fecal calprotectin levels (inactive- 16.75±6.92 ng/mL versus active mucosal inflammation 16.75±15.60 ng/mL, p=0.35). The same was observed in patients on vitamin D supplementation (inactive: 31.79±8.92 ng/mL; active: 29.35±8.58 ng/mL, p=0.52).

The blood levels of vitamin D were significantly lower in patients with high C-reactive protein levels (17.13± 7.98 ng/mL versus those with normal values of CRP 23.05± 6.40 ng/mL, p= 0.0002). This was not the case in

those supplementing vitamin D (33.67± 12.26 and 30.89± 5.07 ng/mL, for inactive and active inflammation, respectively, p= 0.91).

Vitamin D dietary intake was not influenced by clinical disease activity in patients not taking supplements (inactive: 8.44± 6.50 µg/day, 53.15± 45.56% RDA; active: 7.85± 3.68 µg/day, 49.25± 26.07% RDA; p= 0.94) (Table 12). Analysis by disease severity confirmed the lack of significant differences (inactive: 8.44± 6.50 µg/day, 53.15± 45.56% RDA; mild 7.14± 3.55 µg/day, 42.28± 25.23% RDA; moderate: 9.16± 3.74 µg/day, 60.93± 24.99% RDA, p= 0.15).

Conversely, in patients taking supplements, vitamin D RDA was significantly higher in case of disease activity (inactive: 7.77± 4.32 µg/day, 52.47± 29.24% RDA; active: 10.96± 5.64 µg/day, 73.03± 37.62% RDA, p=0.02). Patients with moderately active disease showed significantly higher RDA compared to those with mild or inactive disease (inactive: 7.77± 4.32 µg/day, 52.47± 29.24% RDA; mild 10.17± 2.84 µg/day, 67.82± 18.92% RDA; moderate 14.61± 13.35 µg/day, 97.37± 88.99% RDA, p=0.05).

Vitamin D intake was analyzed in relation to endoscopic activity. No difference in this respect was observed in patients not taking supplements (inactive- 9.47± 4.42 µg/day, 53.60± 36.00% RDA vs active disease 7.78± 5.12 µg/day, 50.25± 35.36% RDA, p=0.19). Considering separately different levels of endoscopic severity (mild: 8.68± 7.77 µg/day, 53.84± 54.33% RDA; moderate: 6.98± 2.77 µg/day, 46.55± 18.44% RDA; severe: 8.75± 1.71 µg/day, 58.05± 11.85% RDA; p=0.44) no significant difference versus patients in remission was detected.

Within patients taking supplements, higher vitamin D RDA were observed in active disease 10.12± 5.60 µg/day, 68.62± 37.53% RDA, vs those with inactive disease 6.87± 2.87 µg/day, 45.81± 19.16% RDA; p=0.04). Patients with moderate disease showed non-significantly higher levels of vitamin D intake compared to other sub-groups, as well as vs patients in remission (mild: 8.63±3.01 µg/day, 57.54± 20.07 % RDA; moderate: 13.31±8.24 µg/day, 88.70±54.94% RDA; severe: 7.39 µg/day, 49.26% RDA; p=0.12).

Clinical activity did not influence the daily intake of vitamin K (inactive: 97.45±88.31 μg/day, 66.57± 57.82% RDA vs active disease: 79.51± 61.52 μg/day, 53.32± 40.74% RDA, p=0.32) (Table 13). Considering separately different levels of clinical activity no difference was detected in mild- 87.17± 67.47 μg/day, 57.67± 44.08% RDA; moderate- 63.43± 44.83 μg/day, 42.94± 30.30% RDA and severe disease 186.51 μg/day, 133.22% RDA versus patients in remission (p=0.28).

Patients with endoscopically active disease showed lower levels of vitamin K intake compared to controls, but without attaining statistical significance (inactive: 114.46±103.63 μg/day, 75.56± 65.94% RDA vs 79.12± 61.82 μg/day, 54.08± 41.94% RDA in active disease; p=0.12). No difference was observed between patients in remission and different subgroups of patients with active disease subdivided on the base of endoscopic severity (mild 87.17± 67.47 μg/day, 57.67± 44.08% RDA; moderate: 63.43± 44.83 μg/day, 42.94± 30.30% RDA; severe 186.51 μg/day, 133.22% RDA; p=0.28).

The same, calcium intake was not affected by clinical disease activity (inactive: 1000.76± 390.21μg/day, 92.26± 37.55% RDA; active: 1159.55± 616.12μg/day, 107.35± 57.13% RDA, p=0.33) (Table 13). Different levels of clinical severity showed were not associated with significantly different calcium intake compared to inactive disease (92.26± 37.55% RDA; mild 1149.96± 566.03μg/day, 104.99±52.33% RDA; moderate: 1231.10±775.853 μg/day, 116.25±71.95% RDA; severe 805.36 μg/day, 80.53% RDA; p=0.86).

The same was observed in relation to endoscopic activity, as no difference was found between inactive- (965.00± 445.69μg/day, 88.64± 43.53% RDA) and active disease (1124.19± 545.52 μg/day, 104.06± 50.59% RDA, p=0.14). Considering separately different levels of severity of endoscopic lesions, daily calcium assumption was similar in different groups (mild- 1149.96± 566.03μg/day, 104.99±52.33% RDA; moderate- 1231.10±775.853 μg/day, 116.25±71.95% RDA and severe endoscopic activity 805.36 μg/day, 80.53% RDA; p=0.23).

Interestingly significant correlation was observed between calcium and vitamin D intake (r=0.24, p=0.0001).

Sunlight exposure and vitamin D levels

Fifty-one (45.94%) of the 111 IBD patients had low-, 46 (41.45%) moderate and 14 (12.61%) high-levels of sunlight exposure. Roughly similar distribution was present in the 112 controls: 55 (49.11%) had low-, 51 (45.54%) moderate- and 6 (5.35%) high-degree sunlight exposure. In the 61 patients with celiac disease, 29 (47.54%) had low-, 24 (39.35%) moderate- and 8 (13.11%) high- degree sunlight exposure. The difference between IBD and controls was non-significant ($p>0.05$). The same applies for celiacs vs. controls, and IBD vs. celiacs ($p>0.05$ and $p>0.05$).

Within 58 Crohn's disease patients, 27 (46.55%) had low-, 24 (41.38%) moderate- and 7 (12.07%) high-degree sunlight exposure, compared to 24 (45.29%) low-, 22 (41.51%) moderate- and 7 (13.20%) high-degree sunlight exposure in the 53 ulcerative colitis patients ($p>0.05$).

Within the IBD group, males showed higher median sunlight exposure, as 34 IBD females had low- (56.6%), 20 moderate- (33.3%) and 6 high-degree sunlight exposure (10.0%); compared to 13 IBD males with low- (25.4%), 27 moderate- (52.9%) and 11 high-degree sunlight exposure (21.5%) ($p = 0.002$).

No sex-related difference was instead observed in celiac disease as 5.5% males had low-, 22.2% intermediate- and 22.2% high-exposure and 51.8% females had low- 37.0% intermediate- and 11.1% high exposure $p=0.53$). The same applied to controls (males: 44.9% low- 48.9% intermediate- and 6.1% high exposure versus females: 53.2% low- 41.9% intermediate- and 4.8% high exposure; $p=0.38$).

IBD male patients showed significantly higher levels of sunlight exposition compared to celiac patients and controls ($p = 0.04$). No difference was detected between female patients ($p=0.26$).

No age-related difference in sunlight exposition was observed between patients younger and older than 47 years in all disease groups (IBD $p = 0.55$, celiacs $p = 0.83$, controls $p = 0.18$). No difference was observed between the different groups of patients belonging to the same age group (≤47 $p=0.63$; >47: $p=0.16$).

No correlation between sunlight exposure and vitamin D levels was observed in IBD patients that did not take supplements (r value 0.28, p = ns). The same applied to celiac patients not taking supplements (r value of 0.21, p = ns), whereas low positive correlation between sunlight exposure and vitamin D levels was present in controls (r value 0.34, $p=0.04$)

Seasonal differences in vitamin D levels were also evaluated comparing vitamin D levels observed in summer time (July-September) versus late winter/early spring (February-April) (Table 14). No significant difference was detected in IBD patients not taking supplements (winter: 17.41±7.52 ng/mL, summer 21.80±9.86 ng/mL, p=0.59). A trend to higher vitamin D levels during summer-time was conversely observed in celiac patients (winter: 17.97 ±5.76ng/mL, summer 24.11±5.72 ng/mL, p=0.07), while statistical significance was reached in controls (winter: 19.29±6.26 ng/mL vs. 28.27±4.03 ng/mL, p=0.001).

Sub-group analysis showed significantly lower vitamin D levels during winter in celiac patients versus controls, while statistical significance was not reached in the other groups. In summer both IB and celiac patients showed lower vitamin D levels versus controls.

Bone densitometry

Sixty-three IBD patients underwent bone dual-energy x-ray absorptiometry. Osteoporosis was observed in 13, osteopenia in 27, and 23 had normal densitometric values (20.64%, 42.86% and 36.50%, respectively).

Twenty-three celiac patients underwent bone densitometry. Four patients presented with osteoporosis, 14 with osteopenia, and five with normal density values (17.39%, 60.87% and 21.74%, respectively).

In patients with IBD PTH levels were within the range of normality in 97/111 patients (57.77±34.53 pg/ml; n.v. 3-73 pg/ml). In the 14 patients showing abnormal levels, parathyroid adenoma was excluded.

PTH levels always were within the normal range in celiac patients (34.18±10.93 pg/ml).

No alteration of hematic concentrations of calcium and phosphorus were detected in IBD (calcium: 9.30± 0.87 mg/dl; n.v. 8.1-10.4 mg/dl; phosphorus: 3.43± 0.68 mg/dl; n.v. 2.5-4.5 mg/dl) nor in celiac patients (calcium: 9.56± 0.39 mg/dl; phosphorus: 3.34± 0.39 mg/dl).

Bone metabolism biomarkers

Sera for investigating bone metabolism biomarkers were collected in 91 IBD (47 Crohn's disease, 44 ulcerative colitis), 55 celiac patients, 99 controls.

The biomarkers under investigation were bone specific alkaline phosphatase (BALP), carboxylated osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP) for evaluating number and function of osteoblasts. Tartrate resistant acid phosphatase (TRAcP) and serum type I collagen cross-linked C-telopeptide (CTX) were dosed for the evaluation of number and function of osteoclasts. Negative bone mass regulators included Sclerostin (SOST) and DKK1.

The evaluation of the markers of bone metabolism was delayed due to problems in the acquisition of specific kits. Laboratory material was eventually delivered in the second half of December 2023, analysis is under course and results will be available in short time.

Only sera from 61 male patients have been so-far analyzed (41 IBD, 15 controls, and 5 celiac patients).

With the limitations related to the small sample size, consisting of male patients, reduced osteoblastic activity, with normal osteoclastic activity was present in IBD patients. No difference was in fact detected in BALP levels, that expresses the number of osteoblasts, between IBD, celiacs and controls. The same was observed for TRAcP, a marker evaluating the number of osteoclasts, and CTx, assessing osteoclastic activity.

Interestingly PINP, a marker of osteoblastic activity, was significantly reduced compared to controls and celiac patients ($p<0.05$). Preliminary sub-group analysis showed that this difference was true both for Crohn's disease versus controls ($p<0.0001$) and ulcerative colitis versus controls ($p=0.016$). No difference was at present observed between celiacs and controls ($p=0.74$).

PINP was evaluated also in female patients showing opposite results. Analysis to detect differences in pre- and postmenopausal women are underway.

This outcome is particularly interesting as it suggests possible sex-related differences, that need to be confirmed when the evaluation of the other bone remodeling markers will be finalized.

Vitamin K-dependent biomarkers

For indirect evaluation of vitamin K deficiencies coagulation INR was evaluated in 75/111 IBD (39 Crohn's disease, 36 ulcerative colitis) and 26/61 celiac patients. No patient showed abnormal INR values (IBD 1.02± 0.10; n.v. 0.9-1.2 and celiac patients 0.98± 0.07). The measurement of partial prothrombin time PTT also led

to normal values in IBD (29.28± 4.03 sec, n.v. 25-35 sec.) and celiac patients (32.48± 4.14 sec), excluding the presence of severe vitamin-K-dependent coagulation defects.

For more precise investigation of vitamin K levels, ucOC was dosed in our patient series. Sera for investigating ucOC were collected in 91 IBD (47 Crohn's disease, 44 ulcerative colitis), 55 celiac patients, 99 controls.

The evaluation of ucOC was delayed due to problems in the acquisition of specific kits. Laboratory material was eventually delivered in the second half of December 2023, analysis is under course and results will be available in short time.

UcOC was so far evaluated in 61 male patients only. The levels of this marker were significantly higher in patients with Crohn's disease versus controls (p=0.047). Similar concentrations were observed between controls, ulcerative colitis patients and celiacs. A non-significant trend toward higher levels of ucOC was detected in Crohn's disease versus ulcerative colitis and celiacs, but data will be thoroughly re-evaluated upon completion of laboratory procedures.

5. DISCUSSION

IBD and CD patients are at increased risk for osteoporosis due to dietary deficiencies, high levels of pro-inflammatory cytokines and need for drugs, such as steroids, that negatively affect bone homeostasis [5,6].

The prevalence of osteoporosis ranges between 4-9% in most adult IBD cohorts [160], and osteopenia affects a significantly higher proportion of patients (up to 39.4%) [161]. This issue has significant impact also in pediatric cohorts in which osteoporosis is detected in 27.1%- and osteopenia 33.3% of patients, respectively [162].

The pooled prevalence of bone mineral reduction in celiac patients upon diagnosis is higher (14.4% osteoporosis, and 39.6%osteopenia) [163], but improves following the institution of gluten-free diet [164].

The prevalence of osteoporosis in the general population is much lower, around 3%, indicating that the study of bone metabolism in patients with chronic gastrointestinal diseases may prove clinically relevant [160,165]. More so in our IBD cohort in which the prevalence of osteoporosis and osteopenia was more frequent than reported in other studies, 20.64% and 42.86%, respectively. The discrepancy is likely related to the

characteristics of our population. Most IBD patients indeed were under biologic (78.3%) suggesting aggressive disease course. Besides direct detrimental effects of long-lasting chronic inflammation, more steroids' courses were likely needed than in patients with mild, less aggressive disease.

The prevalence of osteoporosis (17.39%) and osteopenia (60.87%) slightly exceed those reported in literature also in our series of celiac disease patients, but are consistent with those reported by other Authors. Indeed, regardless the young age of most patients, deficit of bone mineral density to some extent persists despite long-term gluten-free diet [90,166,167].

Despite this, with the exception of vitamin D and to some extension, chronic inflammation, data are surprisingly lacking. More so for vitamin K, highlighting the need for further research on the causal mechanisms underlying osteoporosis and bone repair processes in patients with chronic gastrointestinal pathologies.

The main aim of our work was to evaluate a wide set of bone metabolism markers in IBD-, celiac patients and controls, correlating these findings with the blood levels of vitamin D and K, and their nutritional intake. Unfortunately, due to delay in the availability laboratory kits, the measurement of bone metabolism markers in sera of a large proportion of our patients is currently still underway. The preliminary results, however, suggest that at least in IBD males, osteopenia and osteoporosis are related to reduced osteoblastic activity, in the presence of normal osteoclastic activity. Thus, reduced bone formation represents the primary mechanism of damage, and not increased bone resorption.

No difference was detected in BALP levels, that express the number of osteoblasts, between IBD, celiacs and controls. The same was observed for TRAcP, a marker evaluating the number of osteoclasts, and CTx, assessing osteoclastic activity.

Interestingly PINP, a marker of osteoblastic activity, was significantly reduced compared to controls and celiac patients ($p<0.05$). Sub-group analysis showed that the difference is highly significant for Crohn's disease versus controls and to a lesser extent for ulcerative colitis versus controls, $p<0.0001$ and $p=0.016$, respectively. No difference was instead observed between celiacs and controls ($p=0.74$).

Our data in IBD patients are in line with those of most of the studies reporting lower levels of bone formation markers. BALP represents a surrogate marker to estimate the number of osteoblasts. Most studies reported normal concentrations of BALP [132,168] while other found lower levels of this marker compared to controls [128,130]. However, in most studies carried out in GI disease patients, BALP measurement was not associated to that of other, more specific markers, such as PINP. In present study normal concentrations of BALP were observed in presence of reduced levels of PINP.

Conversely, we found normal levels of CTX, reflecting the number of osteoclasts, that have been previously reported to be high, by some authors [129-131]. In these preliminary results, no difference of TRAcP was present between patients and control. This is in line with what observed in few, small studies [130]. Moreover, the levels of these marker correlated with disease activity, in a single study [130]. Upon completing dosage in our entire series of patients we will confirm, or not, this interesting hypothesis.

In celiac patients the role of altered bone metabolism biomarkers is unclear. At diagnosis some cohorts reported significantly higher levels of ALP, OC, and CTX versus controls [135], while other series documented low CTX concentrations, and normal OC levels [136,137]. PINP levels were higher than normal in patients on normal diet [136,137]. These markers, however, were only slightly different in most series from those recorded in controls [138,139].

Following gluten-free diet, results are contrasting, although BALP and PINP levels were similar to those of controls in most cohorts [140]. Moreover, the levels of BALP increased gradually following gluten-free diet in patients with low concentrations this marker at diagnosis ($p=0.024$) [141]. A reduction in BALP was instead observed after 4 weeks of gluten challenge [169].

In our patients no difference was observed in the concentrations of BALP, TRAcP, CTx, and PINP compared to controls. This likely depends on the characteristics of our cohort, mostly consisting of young adults, on gluten-free diet.

Besides inflammation and the need for drugs negatively affecting bone metabolism, several diet-associated risk factors are involved in the genesis of osteoporosis in IBD and celiac disease. Vitamin K deficiency is the less well investigated one, despite representing a relevant cause of low bone density [11,170,171]. This is not

unexpected considering that vitamin K is involved in the carboxylation of bone proteins, including osteocalcin (also termed bone Gla-protein) and matrix Gla-protein (MGP) [8].

Unlike vitamin D, the direct measurement of vitamin K levels is cumbersome, and not widely available. Moreover, vitamin K is preferentially used in the synthesis of coagulation factors than for bone metabolism [9]. Thus, the harmful effects of vitamin K deficit on bone metabolism take place at blood concentrations that do not affect normal clotting. Vitamin K-dependent clotting factors cannot be considered reliable indicators of vitamin K deficit. Thus, its concentrations are indirectly evaluated by dosing vitamin K-dependent carboxylation products, such as protein induced by vitamin K absence-II (PIVKA-II) and undercarboxylated osteocalcin (ucOC) [10].

The results of ucOC levels in our cohort were significantly higher in patients affected with Crohn's disease compared to controls ($p=0.047$). Despite lower levels compared to controls were detected also in ulcerative colitis statistical significance was not reached. No difference was observed in Crohn's disease versus ulcerative colitis. Our data are in keeping with the results of other cohorts suggesting higher levels of ucOC in Crohn's disease patients [110,111,171,172].

Green leafy plants are the primary dietary source of phylloquinone (Vitamin K1), providing over 90% of requirements [173]. Thus, detailed information on the consumption of these vegetables have been sought in the present study, adding four additional questions to a validated questionnaire used for calculating the intake of vitamin D and calcium.

A significantly lower intake of vitamin K compared to controls (59.56±49.71% RDA vs. 135±91.68% RDA, $p < 0.001$), was present in our cohort of IBD patients. Lower vitamin K RDA are in line with the available literature, suggesting a reduced intake of vegetables in IBD patients [11,110,111,171,174]. No vitamin K sex- or age-related difference was observed within groups.

Some authors reported lower RDA in patients affected by Crohn's disease, possibly reflecting diet changes due to the presence of stenosing disease, while other factors such as bile salt malabsorption and gut microbiota dysregulation could further reduce the absorption of this vitamin [11,172,174]. In our cohort

vitamin K intake was non-significantly lower in Crohn's disease (55.14±40.64 % of RDA) than in ulcerative colitis (64.15±57.72 % of RDA).

Disease localization did not influence the daily intake of vitamin K in our series, both in Crohn's disease (p=0.67) and ulcerative colitis (p=0.96). This is in line with the observations of other cohorts [119,171]. Nonetheless, a non-significant trend toward lower vitamin K levels was documented in ileocolonic Crohn's disease and extensive ulcerative colitis [174].

In our cohort patients with active disease showed non-significantly lower levels of vitamin K intake compared to patients with inactive disease, considering both clinical (66.57± 57.82% RDA vs. 53.32± 40.74% RDA, p=0.32) and endoscopic activity (75.56± 65.94% RDA vs. 54.08± 41.94% RDA, p=0.12). Despite the lack of statistical significance these trends are in line with part of [171,174,175], but not all studies [110,119].

The results of ucOC levels confirm the data regarding dietary intake showing significantly higher levels in patients affected with Crohn's disease compared to controls.

The intake of vitamin K in our series of celiac patients was within the normal range (140.23±80.59 μg/day; 98.78±56.86% of RDA). Nonetheless, it proved significantly higher than in IBD and significantly lower than in controls ($p < 0.001$). In our cohort no sex or age-related difference was detected.

Only few studies report the daily intake of vitamin K offer contrasting results. Some of them report lower intake compared to controls [13] and other do not [12,176]. The results of these studies, carried out in newly diagnosed children, are not comparable to our findings in a cohort consisting of young adult patients following gluten-free diet.

No difference in the levels of ucOC was reported between celiac patients and controls. To our knowledge there are no other data on this bone marker reported in literature in celiac patients.

Vitamin D deficiency is common in patients affected by IBD, ranging from 16 to 95% according to different composition of patient series, and the definition of deficiency [177].

Vitamin D concentration in controls significantly exceeded in our cohort that of IBD patients not taking supplements (24.26±5.58 ng/mL vs. 17.32±7.14 ng/mL, $p<0.05$). The finding is similar to that reported in

previous metanalysis [178]. However, no sex- or age-related differences were observed between patients and controls. Although Kabbani reported significantly higher prevalence of vitamin D deficiency in young males [179], most authors documented higher prevalence of vitamin D deficiency in females and aged patients [180-182].

In our Crohn's disease patients, no significant difference of vitamin D concentrations was observed in relation to disease localization. Lower levels of vitamin D where instead detected in left-sided ulcerative colitis patients not taking supplements (p=0.03), but this observation was not confirmed in those supplementing vitamin D. The importance of the involved intestinal segment is unsettled, as extensive ulcerative colitis and ileo-colonic Crohn's disease were associated with low vitamin D concentrations in some studies [144], but not by others [183].

Conversely, a recent metanalysis reported an association between disease activity and low vitamin D levels [11,184]. This association is however often weak and burdened by high heterogeneity among studies [11,184].

A significant association between C-reactive protein ≥ 0.5 mg/dl and lower vitamin D levels was observed in our cohort (p=0.0002), but not with fecal calprotectin ≥250 μg/g, and clinical- and endoscopic activity.

Celiac disease patients not taking vitamin D supplements had significantly lower vitamin D levels compared to controls (22.26±6.06 vs. 24.26±5.58 ng/mL, p<0.05), despite gluten-free diet. Our results are in line with data from pediatric cohorts [176,185,186]. Data in adults on gluten free diet are scarce and partially contrasting. Most studies, however, report the persistence of hypovitaminosis despite diet [12,135,186].

Our data showed that celiac males, but not females, had significantly lower vitamin D levels compared to controls (p<0.0001). This is not in keeping with some studies who did not document sex-related differences [12,176,185]. The difference may depend upon the characteristics of our population, consisting of adult patients following long-term gluten-free diet, while most published data refer to recently diagnosed pediatric patients. In our cohort no age-related difference was observed.

Interestingly, the vitamin D levels in celiac patients have intermediate values between IBD and controls, despite long-term gluten-free diet. This suggests that despite complete regression of histologic damage, additional mechanisms of hypovitaminosis are present.

Several factors contribute to vitamin D deficiency in patients affected by gastrointestinal diseases, including inadequate dietary intake, impaired absorption, inadequate exposure to sunlight, or increased catabolism [177].

The present data confirm that vitamin D intake was abnormally low in IBD and celiac patients, but also in healthy controls. Fifty-three (47.74%) IBD patients (18 with Crohn's disease and 35 with ulcerative colitis) showed vitamin D dietary intake lower than 50% of RDA.

In our cohort, IBD patients showed lower vitamin D dietary intake compared to controls. (50.88±35.12% RDA vs. 65.34±34.65% RDA, p=0.04). This was particularly significant for males (p=0.04). No significant difference was observed between all other groups.

Our results are in line with what has been reported in other cohorts [174,187-189] and suggest that lower blood levels of this vitamin in IBD to some extent derive from dietary restrictions.

In our patients, the dietary intake was similar in IBD-, and celiac patients, regardless of vitamin D supplementation. This implies that higher hematic concentrations are directly related to supplements, and this approach proves effective.

Unlike previous cohorts [174], young patients not taking supplements, have higher mean vitamin D intake than older patients (p= 0.04). The strict correlation between vitamin D and calcium intake (r=0.24, p=0.0001), can be attributed to lower intake of dairy products in older patients.

Some studies indicate that the vitamin D intake does not differ in Crohn's disease and ulcerative colitis [172,174,188]. This was also the case in our cohort.

Interestingly, however, the disease localization markedly influenced the daily consumption of vitamin D in Crohn's disease (p= 0.03), but not in ulcerative colitis (p=0.19). The intake was particularly low in patients with colonic Crohn's disease, and despite the lack of statistical significance, in extensive colitis. The fear that dairy products may trigger diarrhea likely accounts for the finding.

Unexpectedly, no difference in vitamin D intake was observed according to disease activity. This result is consistent with previous data [174], and in our series may reflect the high proportion of patients with ileal/ileo-colonic- versus colonic disease in Crohn and proctitis/left-sided lesions versus extensive disease in ulcerative colitis.

Available data on the dietary intake of vitamin D in celiac disease mostly derive from children or newly diagnosed adults, while information on adult patients following gluten-free diet is scarce.

In our cohort, despite overall low levels of vitamin D intake, no difference between patients and controls was observed (not taking supplements: 68.45±55.11% RDA; taking supplements: 57.17±23.86% RDA; controls: 65.34±34.65% RDA, p = 0.21), nor in relation to age and gender. Our findings are in line with the literature [190-192].

Sunlight exposure is a major determinant of vitamin D concentrations and sunlight exposure in IBD patients is lower compared to controls, more so in Crohn's disease [193,194].

Some evidence suggests that is also associated to higher rates of hospitalization and surgery in IBD [81]. However, a metanalysis did not find significant correlations between vitamin D and latitude, or vitamin D and annual sunshine exposure, in Crohn's disease (r = 0.069, P = 0.856; r = -0.439, P = 0.265, respectively) [178]. No difference in reported sunlight exposure was observed in our cohort between patients and controls, nor correlations with vitamin D levels.

The seasonal concentrations of serum vitamin D levels in IBD are significantly higher in summer compared to winter/early spring [193,195,196]. In IBD patients not taking supplements higher hematic levels of this vitamin were detected in summertime, but statistical significance was not reached (winter: 17.41±7.52 ng/mL, summer 21.80±9.86 ng/mL, p=0.59). This can be explained by the relatively small size of our series and the high proportion of patients working outdoor or, being unemployed/retired (18/49), spend much time in outdoor even in winter.

No difference in sunlight exposure was detected versus controls or IBD patients in our cohort of celiac patients. Published data are surprisingly scarce and mostly obtained in untreated patients, but sunlight

exposure does not differ in symptomatic and asymptomatic patients [88]. The levels of vitamin D, as expected, are higher in summer versus winter/spring [185,197,198], but were obtained in pediatric patients, with recent diagnosis.

A trend toward higher vitamin D levels during summer-time was observed in our celiac patients (winter: 17.97 ±5.76ng/mL, summer 24.11±5.72 ng/mL, p=0.07), while statistically significant seasonal differences were present in controls (winter: 19.29±6.26 ng/mL vs. 28.27±4.03 ng/mL, p=0.001).

6. CONCLUSIONS

The prevalence of osteopenia and osteoporosis in IBD and celiac patients is well known, and results from differing factors including chronic inflammation, the use of steroids, reduced intake/absorption of critical micronutrients, including vitamin D, vitamin K, and calcium. Our results are in keeping with published data and confirm that young adult patients are not spared by the problem and require strict monitoring and intervention.

The importance of proactive strategies on modifiable risk factors is of prime importance to minimize harmful effects on bone metabolism. Besides confirming the central role of vitamin D, our data highlight the need for considering also vitamin K.

Low vitamin D concentrations are actively sought and routinely treated in IBD and celiac disease patients. In addition to vitamin D pharmacological supplements our data stress the importance of vitamin D nutritional intake and the primary role of adequate sunlight exposure to increase the production of metabolically active vitamin D. Information on the subject is surprisingly low and is an under-represented area of intervention both in IBD and adult celiac patients.

The same applies to vitamin K which has hardly been studied in IBD and celiac disease. The present data indicate that low concentrations of vitamin K are present in a large proportion of patients, but unlike vitamin D, only scarce attention is paid to deficiency. It can be anticipated that vitamin K supplementation represents a further tool for reducing the risk of osteoporosis. More so considering that concentrations of vitamin K that negatively affect the bone well-being are present also in the absence of alterations of vitamin K-dependent

coagulation factors. The measurement of PTT is at present the only tool for assessing vitamin K deficiency in the clinical setting but underestimates extent and severity of deficit. Further investigation is needed on vitamin K-dependent bone factors as ucOC, to favor the knowledge of the mechanisms involved in the genesis of osteopenia and osteoporosis in IBD and adult celiac patients. The same applies to other bone remodeling markers, which were addressed in the present study, including BALP, CTx, PINP, and TRAcP. Our preliminary data suggest that a deficit of bone apposition seems more relevant in this setting than bone resorption casting doubt on the role of steroids in the genesis of osteoporosis, at least in our series of patients.

This study strongly supports the need for an integrate approach to osteoporosis and osteopenia in IBD and adult celiac disease, to assess relative weight, and interactions between highly different factors. A comprehensive evaluation of different mechanisms is crucial for designing the preferable therapeutic intervention to prevent osteopenia and osteoporosis in differing subgroups of GI-patients.

7. ADDENDUM

BONE METABOLISM BIOMARKERS

Sera for investigating bone metabolism biomarkers were collected in 99/115 IBD, 54/61 celiac patients, 99/112 controls.

The biomarkers under investigation were bone-specific alkaline phosphatase (BALP), carboxylated osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP) for evaluating number and function of osteoblasts. Tartrate resistant acid phosphatase (TRAcP) and serum type I collagen cross-linked C-telopeptide (CTX) were used for evaluating number and function of osteoclasts.

At present, sera from 213/252 patients have been analyzed (95/99 IBD: 50 Crohn's disease- and 45 ulcerative colitis patients; 94/99 controls; 24/54 celiac patients), and results were partially different from those observed in the preliminary analysis on a smaller series. Definitive conclusions will be drawn upon completion of laboratory procedures.

Bone markers in IBD and controls. PINP, a marker of osteoblastic activity, was significantly higher in IBD patients compared to controls (9.16±8.84 vs. 7.60±8.84 ng/ml, p=0.0005). No difference was instead detected in BALP levels, that reflect the number of osteoblasts, TRAcP, that evaluates the number of osteoclasts, and CTX, assessing osteoclastic activity (Table A).

Sub-group analysis did not detect any difference in bone remodeling markers concentrations in Crohn's disease versus ulcerative colitis, as well as in the two IBD groups versus controls. This finding likely reflects insufficient statistic power of the study in relation to the sample size (Table B).

Bone markers in different genders and age groups. Sex-related differences were observed between genders (Table C), as PINP was higher in females vs. males, both in IBD patients and controls (p=0.0006 and p=0.0002, respectively). CTX was significantly higher in IBD males vs females (p=0.04).

Further analyzing PINP concentrations it appears that the significant difference between genders in IBD, but not in controls, was directly related to data from patients older than 47 years (Table D). Age did not affect the different CTX levels observed in males vs. females (Table D).

Age-related subgroup-analysis of bone metabolism markers was performed also within the same gender.

Age did not affect the concentration of bone metabolism biomarkers in IBD males. Significantly higher concentrations of PINP were present in older in controls (3.88±6.10 vs. 1.48±3.87 ng/ml, p= 0.01) (Table E). Conversely, aged IBD females showed significantly higher levels of BALP (12.72±3.81 vs. 10.54± 6.95 µg/l, p= 0.04) as well as a trend toward higher concentrations for TRAcP (2.91±1.15 vs. 2.46± 0.88 U/L, p= 0.05). The same was observed also in control females (BALP: 14.64±5.62 vs. 11.08±3.91 µg/l, p= 0.03; TRAcP: 3.43±1.27 vs. 2.49±0.83 U/L, p= 0.01).

Bone markers and inflammation. Data from IBD patients were evaluated also in relation to clinical and endoscopic activity in IBD patients, as systemic inflammation markedly affects bone metabolism and serum bone biomarkers.

As expected, significantly higher levels of CTX were reported in clinically active disease (0.50±0.30 vs. 0.36±0.21 ng/ml, p=0.02). The finding is in keeping with what has been reported in other inflammatory conditions [199, 200]. The correlation between CTX levels and clinical activity was statistically significant (R=0.22; p=0.03). All other markers did not differ in active versus quiescent disease (Table F).

A non-significant trend toward higher CTX, was documented in endoscopically active IBD (0.47±0.29 vs. 0.34±0.18 ng/ml, p=0.07), and correlation was significant (R=0.28; p=0.01). It can be hypothesized the level of statistical significance may result from larger patient series. No difference and no correlation with endoscopic activity were observed for PINP, BALP, and TRAcP (Table F).

As expected, sub-group analysis of disease activity (defined as low, moderate and severe) did not identify any difference in PINP, BALP, and TRAcP (Table G and Table H). Inadequate power related to the sample size prevented significant differences of CTX, in relation to endoscopic activity (Table H).

Clinical activity did not significantly influence the bone remodeling biomarkers in the present study in relation to gender (Table I).

PINP was invariably higher in female vs. males irrespective of clinical activity (Remission: 12.92± 10.75 vs.4.07± 2.88 ng/ml, p= 0.04; Active: 14.54± 9.04 vs. 4.72± 4.43 XX, p<0.0001). CTX was higher instead only in males with clinically active disease (0.52± 0.22 vs. 0.48± 0.38 ng/ml, p= 0.01) (Table I).

The same proved true for the studied biomarkers in relation to endoscopic activity (Table L).

PINP was significantly higher in females with endoscopically active disease (14.30±9.82 vs. 4.42±4.20 ng/ml, p<0.0001), but not in remission (11.87±10.33 vs. 4.42±2.55 ng/ml, p=0.27). Again, the statistical power was inadequate power in relation to the series size. CTX was higher in males with endoscopic disease activity (0.50±0.23 vs. 0.44±0.33 ng/ml, p= 0.01) (Table L).

Bone markers and C-reactive protein and fecal calprotectin. No difference was observed for any of the studied biomarkers in active/inactive inflammation using the 0.5 mg/dl threshold for CRP (Table M). Patients with FC >250 µg/g, a threshold level used for defining markedly increased inflammation, showed significantly higher CTX (0.64±0.25 vs. 0.40±0.26 ng/ml, p=0.001). This supports a relationship between inflammation and reduced bone density (Table M).

Bone markers and therapy. It is widely accepted that anti-TNFα treatment, reducing inflammation, promotes bone wellbeing [134]. Our data do not support this view (Table N). However, the study was not designed to investigate this endpoint, and more data are needed to define the issue.

Bone markers and computerized bone densitometry. As expected, an inverse correlation was detected between TRAcP and femoral and vertebral T-score (-0.352, p=0.01; -0.350, p=0.01 respectively). Unexpectedly, the same was observed for BALP (-0.325, p=0.01; and -0.281, p=0.04 respectively) (Table O).

Bone markers and Vitamin D levels. No significant difference between patients taking supplements and those not supplementing vitamin D (Table P). No correlation was also detected between vitamin D levels and bone biomarkers (Table Q).

Celiac disease.

The dosage of bone markers has not been completed in a proportion of celiac patients. Thus, data should be considered as preliminary, and conclusions require confirmation in the entire series of patients.

PINP, a marker of osteoblastic activity, was significantly higher compared to IBD and controls (celiac: 90.92± 244.01 ng/ml; IBD: 9.16±8.84 ng/ml, controls: 7.60±8.84 ng/ml, p=0.0005). Noteworthy, the difference is due to unexpectedly high values in two individual patients. A double-check of these values is under way. No difference was instead detected in BALP, TRAcP, and CTX levels from IBD or controls (Table A).

Some sex-related differences were instead observed between genders, with CTX being higher in celiac males vs females (p=0.04) (Table C).

No difference for any considered marker was observed between genders in patients in the same age subgroup (Table D), nor in aged vs. young patients (Table E).

No significant difference between patients taking supplements and those not supplementing vitamin D (Table P) nor in relation to vitamin D levels (Table Q).

VITAMIN K-DEPENDENT BIOMARKERS

The evaluation of ucOC was delayed due to problems in the acquisition of specific kits. Laboratory material was delivered with delay and analysis has not been completed. Thus, under-carboxylated osteocalcin was dosed in 74/99 IBD, 24/54 celiac patients 76/99 controls.

IBD patients. A trend toward lower ucOC levels was observed in IBD patients versus controls (IBD: 0.70±0.88 ng/ml, controls: 1.08± 1.44 ng/ml, p=0.007) (Table A). The trend was confirmed also analyzing the levels of this protein in Crohn's disease and ulcerative colitis versus controls (Crohn: 0.75±0.76 ng/ml; UC: 0.63± 1.03 ng/ml; Controls: 1.08±1.44 ng/ml; p=0.05) (Table B). These data are unexpected. They suggest that vitamin K intake is lower in controls than in patients and are not in keeping with what is suggested by dietary intake questionnaires. Patients affected by Crohn's disease showed lower ucOC than those affected by ulcerative colitis (p=0.05) (Table B). This, again, is in contrast with questionnaires.

Higher levels of ucOC were present in male versus female IBD patients (0.73±0.72 vs. 0.65±1.06 ng/ml, p=0.02), and the opposite in controls (males, 0.79±1.55 vs. 1.33±1.30 ng/ml in females, p= 0.02) (Table C).

No difference in was found in IBD patients in relation to age and gender (Table D and E). In young controls (age <47), significantly higher ucOC levels were observed in females (M: 0.57±0.33 vs. 1.57±1.37 ng/ml, p= 0.04). Numerically higher values of ucOC were reported in female controls in the other sub-analysis (Table D and E).

As disease activity may influence dietary patterns in IBD, favoring reduced intake of vegetables resulting in less vitamin K intake, serum ucOC was evaluated in respect to clinical and endoscopic activity.

No difference was however detected for clinical- (remission: 0.68± 0.89 ng/ml; active: 0.71±0.89 ng/ml; p=0.42), or endoscopic activity (remission: 0.75±1.02 ng/ml; active: 0.67±0.83 ng/ml; p=0.97) (Table F).

The same was observed in sub-group analysis grading the disease activity as low, moderate, and severe (Table G and H).

Male patients in clinical remission showed higher levels of ucOC than females (M: 0.90± 0.86 ng/ml; F: 0.47± 0.88 ng/ml; p= 0.001), while no difference was observed in active disease. Within genders, significantly increased levels of ucOC were present in females with active- versus inactive disease (0.87± 1.24 ng/ml vs. 0.47± 0.88 ng/ml, p=0.02) (Table I), but not in males. No difference was instead reported for endoscopic activity (Table H).

No difference was observed between patients with active versus absent inflammation using a CRP threshold of 0.5 mg/dl (Table M). Interestingly, patients with FC >250 µg/g showed a trend toward higher ucOC values (<250: 0.40±0.26 ng/ml; >250: 0.64±0.25 ng/ml, p=0.001) (Table M).

No significant difference in undercarboxylated osteocalcin levels was observed in relation to therapy (p=0.57) (Table N).

Computerized bone mineralometry values did not correlate with ucOC (Table O).

Surprisingly, no correlation of ucOC with vitamin K RDA was observed (0.132, p=0.29), but this needs to be confirmed after completing analysis in all samples (Table R).

As expected, no correlation emerged between ucOC and vitamin D levels (Table Q). Higher levels of ucOC were detected in patients not taking supplements, than in those supplementing vitamin D (0.78±0.77 vs. 0.51±0.85 ng/ml, p=0.03). The biological significance of this result is unclear (Table P).

Celiac disease. In celiac patients a trend toward lower ucOC levels was observed versus controls (celiac disease: 0.64± 0.68 ng/ml, controls: 1.08± 1.44 ng/ml, p=0.007) (Table A).

No difference in sex- (Table C) or age-related concentrations was detected (Table D and Table E).

No significant correlation occurred between ucOC and computerized bone mineralometry data in celiac patients (Table O).

Surprisingly, no correlation of ucOC with vitamin K RDA was observed (0.223 p=0.40), but this result needs to be confirmed in the entire series of patients (Table R).

As expected, no correlation was found between ucOC and vitamin D levels (Table Q) and between patients taking or not taking vitamin D supplements (Table P).

DISCUSSION

Aim of the work was to evaluate a wide set of bone metabolism markers in IBD-, celiac patients and controls, correlating these findings with the blood levels of vitamin D and K, and their nutritional intake. Due to delay in the availability of laboratory kits, the measurement of bone metabolism markers in a proportion of celiac patients is still incomplete.

PINP, a marker of osteoblastic activity, was higher in IBD patients than in controls. This finding, in the presence of normal values of BALP, which reflect the number of osteoblasts, rules out the hypothesis that osteopenia and osteoporosis in IBD are related to reduced osteoblastic activity. More so considering the normal osteoclastic activity documented by normal TRAcP and CTX, which evaluate the number of osteoclasts and osteoclastic activity.

Sub-group analysis did not show any difference between Crohn's disease, ulcerative colitis, and controls.

PINP, represents a marker of type-I collagen remodeling. An increase in PINP values has been observed in several fibrotic conditions [201]. It can be hypothesized that higher values in IBD, more so in Crohn's disease, could result from sources other than the bone. Further analysis on osteocalcin, which is secreted only by osteoblast, will be carried out to confirm or rule out the role of intestinal fibrosis in this unexpected high value.

BALP represents a surrogate marker to estimate the number of osteoblasts. Similarly to our findings, most studies reported normal concentrations of BALP [132,168] while in some instances lower levels of this marker compared to controls were reported [128,130]. Noteworthy, in most published studies, carried out in GI disease patients, BALP measurement was not associated to that of other, more specific markers, such as PINP. In present study normal concentrations of BALP were observed in presence of higher levels of PINP.

Conversely, we found normal levels of CTX, reflecting the number of osteoclasts, that have been previously reported to be high, by some authors [129-131]. CTX reflects the number of these cells but does not quantify their osteoclast activity. This is more precisely by TRAcP levels, which was normal in present study. This confirms what has been previously reported in some studies carried out in small series [130].

Sub-group analysis was also carried out, as some markers are to some extent influenced by age and sex. Indeed, higher than normal levels of PINP were present in females vs males. The reason of this finding is unclear.

Moreover, slightly higher concentrations of CTX were reported in males. The explanation may reside in the different behavior of the marker in the two genders. CTX levels are lower in young females than in males. The decrease reaches its lower value at the age of 35–45, whereas in males the lowest level is reached at 70 years [202].

In females age influenced the levels of BALP and TRAcP, that were significantly higher in older subjects, both in IBD and controls. Age-related osteoporosis may be responsible for the TRAcP increase in old patients [203]. BALP levels were also higher than normal. The finding is unexpected but is consistent with other data reported in the literature, associating increased BALP levels to more severe osteoporosis in females [204, 205]. Further studies are needed to clarify the inconsistency.

As expected, high levels of CTX in patients with active disease vs. inactive disease. No other marker was influenced by disease activity. Our findings in this respect are new and interesting, as data analyzing changes of bone markers in relation to intestinal inflammation are surprisingly scarce. A single study reported that the levels of TRAcP correlated with disease activity, but (despite overall higher levels vs. controls) no difference in CTX concentrations was reported according to disease status [130]. Another study did not confirm any change in bone marker levels in relation to disease activity [131].

In celiac patients the role of altered of bone metabolism biomarkers unclear. At diagnosis some cohorts reported significantly higher levels of ALP, OC, and CTX versus controls [135], while other series documented low CTX concentrations, and normal OC levels [136,137]. PINP levels were higher than normal in patients on

normal diet [136,137]. These markers, however, were only slightly different in most series from those reported in controls [138,139].

Results are contrasting in patients on gluten-free diet, nonetheless, in most cohorts BALP and PINP levels did not differ from those of controls [140]. Moreover, the levels of BALP increased gradually following gluten-free diet in patients with low concentrations this marker at diagnosis (p=0.024) [141]. A reduction in BALP was instead observed after 4 weeks of gluten challenge [169].

Increased concentrations of PINP were found in celiac patients compared to IBD patients and controls. Mean levels are surprisingly high and mostly derive from very high levels in a small number of patients, whereas results in most instances are in line with those of controls. These sera will be reprocessed to confirm the observed results. In our patients no difference was instead observed in the concentrations of BALP, TRAcP, and CTx compared to controls. This likely depends from the characteristics of our cohort, largely consisting of young adults, on gluten-free diet. Definitive results, following the analysis of sera in the entire series of patientsis needed to confirm this hypothesis.

With regard to the markers of vitamin K intake ucOC levels were non-significantly lower in IBD patients versus controls (p=0.07). A trend toward higher concentrations in Crohn's disease compared to ulcerative colitis was observed. This is in keeping with the results of other studies reporting higher levels of ucOC in Crohn's disease patients [110,111,171,172]. Analysis has not yet been carried out in all samples and results will be re-evaluated.

At present, significantly higher concentrations of ucOC were observed in men (p= 0.02) both in IBD and controls, indicating lower intake of green leafy vegetables compared to women. The difference was particularly marked in clinical remission. As significant elevation of ucOC was reported in women with active disease, but not in remission, it is suggested that the intake of green leafy vegetables reverts to normal when the disease is not active. This is not the case in males.

No difference in the levels of ucOC was reported between celiac patients on gluten-free diet and controls, nor between genders, or in relation to age. This finding is new as no data on this bone marker have so-far

been reported in the literature. Data need to be confirmed upon completion of analysis in the entire series of patients.

No correlation ucOC levels and the vitamin K intake questionnaire, suggesting the inadequacy of self-administered questionnaires, despite validation, to accurately assess micronutrients intake. This is possibly due to the patient's tendency to overestimate the intake of food which is widely considered as healthy. Our findings support the importance of direct measurement of ucOC for a reliable evaluation of the dietary intake of vitamin K.

CONCLUSIONS

Our data provide a simultaneous dosage of PINP, BALP, CTX and TRAcP. This information is hardly present in literature and helps understanding the complex relationship between bone wellbeing and inflammation in IBD.

The most interesting finding in this study is represented by the high PINP values in IBD, which contrast to what is expected in osteoporosis. A possible explanation resides in an origin of the marker other than the bone. Intestinal fibrosis represents a putative source but further investigations are needed to clarify the issue.

A new and interesting finding resides in the relationship between BALP and TRAcP. The presence of high BALP, which expresses the number of osteoblasts, was associated with high values of TRAcP, expressing the number of osteoclasts. This is expected in pediatric patients and teenagers, but not in adult population in which bone remodeling usually favors bone resorption. The same finding has been reported also by other Authors [204, 205], indicating that complex and still undefined factors affecting bone remodeling are present in IBD. This prompts further research.

As far as the vitamin K marker ucOC is concerned, data are not in line with the information available in literature. As the dosage has not been carried out half patients this observation needs to be confirmed. It appears nonetheless that poor correlation exists between vitamin K dietary intake and ucOC. This casts doubt on the reliability of dietary information deriving from self-administered questionnaires.

8. TABLES

Table 1a. IBD patients' characteristics

Gender	
Males	51
Females	60
Crohn's disease	
L1	24
L2	8
L3	26
Ulcerative colitis	
E1	5
E2	19
E3	29
Age	
≤47	52
>47	59
Clinical activity	
Active	60
Inactive	51
Endoscopic activity	
Active	68
Inactive	30
No endoscopy	13

Table 1b. Celiac patients' and controls' characteristics

Celiac disease	
Gender	
Males	16
Females	45
Age	
≤47	38
>47	23
Diet	
Gluten-free	56
Normal	5
Controls	
Gender	
Males	49
Females	63
Age	
≤47	53
>47	59

Table 2. Vitamin D levels expressed in ng/mL.

	a. IBD (no supplements)	b. IBD (supplements)	c. Celiacs (no supplements)	d. Celiacs (supplements)	e. Controls (no supplements)	
Total	17.32±7.14	31.63±8.84	22.26±6.06	36.69±14.87	24.26±5.58	p_{a-b} <0.05 p_{c-d} <0.05 p_{a-c} <0.05 p_{b-d} <0.05 p_{a-e} <0.05 p_{b-e} <0.05 p_{c-e} <0.05 p_{d-e} <0.05
Males	16.94± 6.95	32.02± 12.53	20.35± 4.24	30.42± 4.64	26.05± 4.64	p_{a-e} <0.0001 p_{c-e} <0.0001 p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} <0.0001 p_{d-e} <0.0001
Females	17.79± 7.51	31.45± 6.87	20.44± 7.25	38.17± 16.65	23.57± 6.03	p_{a-e} <0.001 p_{c-e} <0.001 p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} <0.001 p_{d-e} <0.001
	p=0.85	p=0.56	p=0.87	p=0.43	p=0.05	
Age ≤47	15.53± 6.50	31.18±11.49	20.63±7.01	35.67±12.29	26.04± 5.64	p_{a-e} = n.s. p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
Age >47	19.04± 7.43	31.96±6.46	19.70±5.66	36.44±17.3	23.45±5.27	p_{a-e} = n.s. p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
	p=0.10	p=0.19	p= 0.91	p=0.69	p= 0.009	

n.s.= non-significant.

Table 3. Vitamin D levels and intake in Crohn's disease according to disease localization.

Crohn's disease	Vitamin D*	p	Vitamin D‡	p	VitD Intake* (RDA%)	p	VitD Intake‡ (RDA%)	p
L1	17.38± 5.71	p= 0.85	32.76± 6.01	p= 0.17	10.91± 3.18 (72.73± 21.21%)	p= 0.03	9.15± 2.46 (63.44± 14.96%)	p= 0.48
L2	19.65± 11.87		31.50		4.36±1.75 (27.58± 12.83%)		7.05 (47.00%)	
L3	14.84± 4.54		28.38± 5.91		10.15± 4.17 (67.64± 27.82%)		11.06±4.88 (73.75± 32.53%)	

*: patients not supplementing vitamin D; ‡: patients supplementing vitamin D.

Table 4. Vitamin D levels and intake in ulcerative colitis according to disease localization.

UC	Vitamin D*	p	Vitamin D‡	p	VitD Intake* (RDA%)	p	VitD Intake‡ (RDA%)	p
E1	16.00	p= 0.03	32.62±9.87	p=0.45	8.66±2.54 (57.73±16.93%)	p=0.15	7.43±5.63 (49.53±37.53%)	p=0.56
E2	14.06± 7.77		35.41±15.41		7.39± 2.73 (46.94± 19.44%)		6.93± 2.82 (46.19± 18.82%)	
E3	20.94± 5.59		29.21± 8.07		6.46± 2.77 (36.64± 21.12%)		8.82± 9.22 (58.78± 61.47%)	

*: patients not supplementing vitamin D; ‡: patients supplementing vitamin D.

Table 5. Vitamin D daily intake expressed in µg/day.

	a. IBD (no supplements)	b. IBD (supplements)	c. Celiacs (no supplements)	d. Celiacs (supplements)	e. Controls (no supplements)	
Total (RDA%)	8.10±4.99 (50.88±35.12 %)	9.09±5.10 (61.21±34.19%)	8.58±3.58 (68.45±55.11%)	9.00±6.05 (57.17±23.86 %)	9.84±5.18 (65.34±34.65%)	p_{a-b} = n.s. p_{c-d} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{a-e} =0.04 p_{b-e} = n.s. p_{c-e} = n.s. p_{d-e} = n.s.
Males (RDA%)	8.54±3.85 (51.75±30.12 %)	11.56±7.21 (77.03±48.05%)	8.17±6.33 (54.43±42.19%)	10.83±5.13 (72.20±34.19 %)	11.15±5.44 (74.19±36.46%)	p_{a-e} = 0.04 p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
Females (RDA%)	7.58±6.14 (49.85±40.97 %)	7.81±3.01 (52.68±20.18%)	8.63±3.43 (57.53±22.86%)	8.29± 6.41 (54.76±43.16 %)	8.80±4.76 (58.35±31.73%)	p_{a-e} p=0.07 p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
	p=0.35	p=0.05	p =0.94	p=0.27	p=0.014	
Age ≤47 (RDA)	9.79± 5.89 (60.17±43.17 %)	10.02± 6.73 (66.76±44.88%)	8.80±3.78 (58.66±25.18%)	8.04± 5.58 (53.60±37.19 %)	10.17±4.64 (67.83±30.97%)	p_{a-e} = n.s. p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
Age >47 (RDA)	6.41± 3.22 (41.58±21.83 %)	8.29± 3.03 (56.18±20.26%)	7.85± 3.22 (52.30±21.44%)	9.76± 6.59 (64.41±44.63 %)	9.54±5.63 (63.15±37.73%)	p_{a-e} = n.s. p_{c-e} = n.s. p_{a-c} = n.s. p_{b-d} = n.s. p_{b-e} = n.s. p_{d-e} = n.s.
	p= 0.04	p=0.84	p= 0.73	p=0.46	p=0.18	

n.s.= non-significant.

Table 6. Vitamin K intake expressed in µg/day.

	a. IBD	b. Celiacs	c. Controls	
Total (RDA%)	88.04±75.62 (59.56±49.71%)	140.23±80.59 (98.78±56.86%)	197.33±131.52 (135±91.68%)	p_{a-b} <0.001 p_{a-c} <0.001 p_{b-c} <0.001
Males (RDA%)	73.83± 56.42 (50.10± 39.03%)	131.87±79.65 (90.58±54.32%)	187.86±111.56 (130.40±80.26%)	p_{a-b} <0.000001 p_{a-c} <0.000001 p_{b-c} = n.s.
Females (RDA%)	100.44± 87.71 (67.96±56.61%)	142.69±81.88 (101.19±58.16%)	204.82±145.84 (139.84±100.38%)	p_{a-b}<0.000001 p_{a-c} <0.000001 p_{b-c} = n.s.
	p=0.13	p=0.72	p =0.97	
Age ≤47 (RDA)	78.18±62.17 (55.83± 44.40%)	124.09± 78.64 (88.63± 56.17%)	188.40±128.57 (134.51±91.77%)	p_{a-b} =0.000001 p_{a-c} =0.000001 p_{b-c} =0.000001
Age >47 (RDA)	97.71±86.34 (63.28± 54.69%)	163.55± 79.74 (113.44±56.16%)	205.21±134.66 (136.64± 92.39%)	p_{a-b}=0.000001 p_{a-c} =0.000001 p_{b-c} = n.s.
	p=0.25	p=0.10	p=0.56	

n.s.= non-significant.

Table 7. Differences between Crohn's disease and ulcerative colitis.

	Crohn's disease	Ulcerative colitis	
Vitamin D levels*	15.42±7.20	18.63±6.92	p=0.08
Vitamin D levels‡	31.13±6.18	32.52±12.52	p= 0.67
Vitamin D intake* (RDA)	8.78±4.13 (58.20±27.96)	7.65±5.51 (46.08±38.83)	p=0.18
Vitamin D intake‡ (RDA)	9.87±3.74 (67.02±24.66)	7.87±6.66 (52.49±44.40)	p=0.005
Vitamin K intake (RDA)	80.75± 60.41 (55.14±40.64%)	95.77± 88.94 (64.15±57.72%)	p=0.65
Calcium intake (RDA)	1118.44±525.85 (103.70±48.08%)	1047.51±526.20 (96.43± 50.50%)	p=0.50

*: patients not supplementing vitamin D; ‡: patients supplementing vitamin D.

Table 8. Vitamin K and calcium intake in Crohn's disease according to disease localization.

Crohn's disease	Vitamin K Intake (RDA%)	p	Calcium Intake (RDA%)	p
L1	79.05± 67.66 (54.63± 45.37%)	p=0.67	1065.42± 518.24 (98.22± 46.04%)	p=0.54
L2	57.79± 40.59 (37.48± 23.38%)		1384.55± 734.55 (125.16± 62.91%)	
L3	85.40± 55.94 (58.28± 37.68%)		1117.01± 520.79 (105.54± 50.15%)	

Table 9. Vitamin K and calcium intake in ulcerative colitis according to disease localization.

UC	Vitamin K Intake (RDA%)	p	Calcium Intake (RDA%)	p
E1	97.47± 119.92 (69.62±85.65%)	p= 0.96	882.31 ± 235.84 (82.26 ± 32.02%)	p= 0.93
E2	100.96 ± 111.86 (64.92 ±68.08 %)		1027.28 ± 539.11 (92.83 ± 45.83%)	
E3	93.96 ± 73.23 (64.40 ±51.41%)		1071.33 ± 551.09 (99.42 ± 56.12%)	

UC= ulcerative colitis.

Table 10. Calcium intake expressed in µg/day.

	a. IBD	b. Celiacs	c. Controls	
Total (RDA%)	1084.01±524.64 (100.17±49.16%)	1127.29±513.16 (102.45±47.67%)	1142.42±645.94 (107.15±60.99%)	p_{a-b} <0.05 p_{a-c} <0.05 p_{b-c} <0.05
Males (RDA%)	1038.95±497.43 (99.36±47.81%)	1121.93±475.61 (108.13±50.17%)	1185.54±596.06 (114.39±59.88%)	p_{a-b} = n.s. p_{a-c} = n.s. p_{b-c} = n.s.
Females (RDA%)	1123.33±548.80 (100.88±50.74%)	1128.87±530.50 (100.72±47.55%)	1108.35±685.69 (101.44±61.73%)	p_{a-b}= n.s. p_{a-c} = n.s. p_{b-c} = n.s.
	p=0.39	p =0.98	p=0.17	
Age ≤47 (RDA)	1029.08±488.96 (101.62± 47.53%)	1071.76± 503.73 (104.85±51.31%)	1119.98±564.14 (111.99± 56.41%)	p_{a-b} = n.s. p_{a-c} = n.s. p_{b-c} = n.s.
Age >47 (RDA)	1137.89± 556.90 (98.75± 51.13%)	1207.49±530.48 (98.76±42.75%)	1162.20± 714.58 (102.89± 64.93%)	p_{a-b}= n.s. p_{a-c} = n.s. p_{b-c} = n.s.
	p=0.33	p=0.37	p=0.93	

n.s.= non-significant.

Table 11. Vitamin D levels in IBD expressed in ng/mL, according to disease activity.

	Clinical remission	Clinical activity	p		Endoscopic remission	Endoscopic activity	p
IBD (no supplements)	17.45±6.27	17.23±7.80	p=0.53		19.63±7.78	16.91± 6.96	p=0.42
IBD (supplements)	33.06±9.68	29.51±7.21	p=0.14		32.03±11.80	30.17± 7.19	p=0.88
	p<0.0001	p<0.0001			p<0.01	p<0.0001	

Table 12. Vitamin D intake in IBD expressed in µg/day, according to disease activity.

	Clinical remission	Clinical activity	p		Endoscopic remission	Endoscopic activity	p
IBD (no supplements)	8.44± 6.50 (53.15± 45.56%)	8.44± 6.50 (53.15± 45.56%)	p=0.94		9.47± 4.42 (53.60± 36.00%)	7.78± 5.12 (50.25± 35.36%)	p=0.19
IBD (supplements)	7.77± 4.32 (52.47± 29.24%)	10.96± 5.64 (73.03± 37.62%)	p=0.02		6.87± 2.87 (45.81± 19.16%)	10.12± 5.60 (68.62± 37.53%)	p=0.04
	p=0.95	p=0.65			p=0.19	p=0.70	

Table 13. Vitamin K and calcium intake in IBD expressed in µg/day, according to disease activity.

	Clinical remission	Clinical activity	p		Endoscopic remission	Endoscopic activity	p
Vitamin K	97.45±88.31 (66.57± 57.82%)	79.51± 61.52 (53.32± 40.70)	p=0.32		114.46±103.63 (75.56± 65.94%)	79.12± 61.82 (54.08± 41.94%)	p=0.12
Calcium	1000.76± 390.21 (92.26± 37.55%)	1159.55± 616.12 (107.35± 57.13%)	p=0.33		965.00± 445.69 (88.64± 43.53%)	1124.19± 545.52 (104.06± 50.59%)	p=0.14

Table 14. Seasonal changes in vitamin D levels (ng/mL) in patients not taking supplements.

	a. IBD	b. Celiac disease	c. Controls	
Winter	17.41±7.52	17.97 ±5.76	19.29±6.26	p_{a-b} = n.s. p_{a-c} = n.s. p_{b-c} <0.05
Summer	21.80±9.86	24.11±5.72	28.27±4.03	p_{a-b} = n.s. p_{a-c} <0.05 p_{b-c} <0.05
	p=0.59	p=0.07	p=0.001	

Table A. Bone remodeling biomarkers and vitamin K-dependent biomarkers in patients and controls.

	a. IBD	b. Celiac patients	c. Controls	
PINP ng/ml	9.16±8.84	90.92± 244.01	7.60± 8.84	P_{a-b}= 0.0005 P_{a-c}= 0.0005 P_{b-c}= 0.0005
BALP µg/l	12.02±5.42	14.01± 5.26	13.25± 5.74	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
TRAcP U/L	2.71±1.01	2.97± 1.37	2.92± 1.04	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
CTX ng/ml	0.43±0.27	0.40± 0.26	0.44± 0.30	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
ucOC ng/ml	0.70±0.88	0.64± 0.68	1.08± 1.44	P_{a-b}= 0.07 P_{a-c}= 0.07 P_{b-c}= 0.07

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table B. Bone remodeling biomarkers and vitamin K-dependent biomarkers in Crohn's disease -, ulcerative colitis patients, and controls.

	a. Crohn's disease	b. Ulcerative colitis	c. Controls	
PINP ng/ml	9.01±8.97	9.32± 8.79	7.60± 8.84	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
BALP µg/l	11.49±4.47	12.61± 6.30	13.25± 5.74	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
TRAcP U/L	2.60±1.12	2.83±0.87	2.92± 1.04	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
CTX ng/ml	0.41±0.30	0.46± 0.24	0.44± 0.30	P_{a-b} >0.05 P_{a-c} >0.05 P_{b-c} >0.05
ucOC ng/ml	0.75±0.76	0.63± 1.03	1.08± 1.44	P_{a-b}= 0.05 P_{a-c}= 0.05 P_{b-c}= 0.05

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table C. Bone remodeling biomarkers and vitamin K-dependent biomarkers in males vs. females.

	PINP ng/ml	BALP µg/l	TRAcP U/L	CTX ng/ml	ucOC ng/ml
IBD Males	4.42±3.78	12.18±5.60	2.69±0.96	0.46±0.22	0.73±0.72
IBD Females	13.70±9.90	11.87±5.29	2.73±1.07	0.40±0.31	0.65±1.06
	p= 0.0006	p= 0.97	p= 0.87	**p= 0.04**	**p= 0.02**
Celiac Males	147.06±316.57	13.95±5.81	3.27±1.66	0.49±0.29	0.70±0.56
Celiac Females	16.07±4.12	14.08±4.91	2.64±0.94	0.30±0.20	0.58±0.81
	p=0.48	p=0.84	p=0.32	**p=0.04**	p=0.29
Control Males	2.87±5.35	13.56±6.35	2.84±0.87	0.45±0.27	0.79±1.55
Control Females	11.94±9.22	12.97±5.17	2.99±1.17	0.43±0.32	1.33±1.30
	p=0.0002	p=0.96	p= 0.47	p= 0.20	**p= 0.02**

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table D. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to age and gender.

	Age	PINP ng/ml	BALP µg/l	TRAcP U/L	CTX ng/ml	ucOC ng/ml
IBD <47	**Males**	4.04±4.10	12.15±6.54	2.50±0.91	0.47±0.23	0.93±0.89
	Females	11.36± 10.97	10.54± 6.95	2.46± 0.88	0.40± 0.36	0.91±1.49
significance		p= 0.24	p= 0.41	p= 0.97	p= 0.11	p= 0.17
IBD ≥47	**Males**	4.93±3.35	12.23±4.26	2.94±0.98	0.45±0.22	0.50±0.33
	Females	15.10±9.09	12.72±3.81	2.91±1.15	0.40±0.27	0.53±0.78
significance		**p= 0.001**	p= 0.68	p= 0.91	p= 0.29	p= 0.24
Celiac <47	**Males**	13.77±9.25	15.27±6.13	3.20±1.65	0.47±0.16	0.68±0.68
	Females	15.06±3.96	14.47±8.57	3.07±1.69	0.30±0.26	0.39±0.37
significance		p= 0.51	p= 0.92	p= 1.0	p= 0.18	p= 0.64
Celiac ≥47	**Males**	333.67±448.17	12.10±5.39	3.35±1.86	0.52±0.44	0.73±0.39
	Females	16.57±4.46	13.88±2.50	2.48±0.58	0.30±0.17	0.68±0.96
significance		p= 0.92	p= 0.29	p= 0.43	p= 0.23	p= 0.29
Control <47	**Males**	1.48±3.87	12.90±5.76	2.75±0.82	0.54±0.36	0.57±0.33
	Females	10.21±8.59	11.08±3.91	2.49±0.83	0.42±0.35	1.57±1.37
significance		**p= 0.004**	p= 0.63	p= 0.59	**p= 0.05**	**p= 0.04**
Control ≥47	**Males**	3.88±6.10	14.05±6.82	2.90±0.91	0.39±0.20	0.90±1.90
	Females	13.47±9.65	14.64±5.62	3.43±1.27	0.44±0.28	1.16±1.24
significance		**p= 0.01**	p= 0.56	p= 0.13	p= 0.80	p= 0.27

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table E. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to gender and age.

	Age	PINP ng/ml	BALP µg/l	TRAcP U/L	CTX ng/ml	ucOC ng/ml
IBD Males	**<47**	4.04±4.10	12.15±6.54	2.50±0.91	0.47±0.23	0.93±0.89
	≥47	4.93±3.35	12.23±4.26	2.94±0.98	0.45±0.22	0.50±0.33
significance		p= 0.22	p= 0.22	p= 0.12	p= 0.70	p= 0.13
IBD Females	**<47**	11.36± 10.97	10.54± 6.95	2.46± 0.88	0.40± 0.36	0.91±1.49
	≥47	15.10±9.09	12.72±3.81	2.91±1.15	0.40±0.27	0.53±0.78
significance		p= 0.34	**p= 0.04**	**p= 0.05**	p= 0.50	p= 0.84
Celiac Males	**<47**	13.77±9.25	15.27±6.13	3.20±1.65	0.47±0.16	0.68±0.68
	≥47	333.67±448.17	12.10±5.39	3.35±1.86	0.52±0.44	0.73±0.39
significance		p= 0.22	p= 0.35	p= 1.0	p= 0.63	p= 0.63
Celiac Females	**<47**	15.06±3.96	14.47±8.57	3.07±1.69	0.30±0.26	0.39±0.37
	≥47	16.57±4.46	13.88±2.50	2.48±0.58	0.30±0.17	0.68±0.96
significance		p= 0.53	p= 0.37	p= 0.76	p= 0.93	p= 0.74
Control Males	**<47**	1.48±3.87	12.90±5.76	2.75±0.82	0.54±0.36	0.57±0.33
	≥47	3.88±6.10	14.05±6.82	2.90±0.91	0.39±0.20	0.90±1.90
significance		**p= 0.01**	p= 0.60	p= 0.60	p= 0.18	p= 0.49
Control Females	**<47**	10.21±8.59	11.08±3.91	2.49±0.83	0.42±0.35	1.57±1.37
	≥47	13.47±9.65	14.64±5.62	3.43±1.27	0.44±0.28	1.16±1.24
significance		p= 0.24	**p= 0.03**	**p= 0.01**	p= 0.11	p= 0.22

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table F. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to disease activity.

	Clinical remission	Clinical activity	p		Endoscopic remission	Endoscopic activity	p
PINP ng/ml	8.88± 9.23	9.43± 8.53	p=0.66		8.00±8.16	9.58±9.09	p=0.63
BALP µg/l	11.83± 4.55	12.21± 6.20	p=0.99		12.55±4.07	11.83±5.84	p=0.26
TRAcP U/L	2.59± 0.95	2.83±1.06	p=0.25		2.83±0.93	2.67±1.04	p=0.30
CTX ng/ml	0.36± 0.21	0.50±0.30	**p=0.02**		0.34±0.18	0.47±0.29	p=0.07
ucOC ng/ml	0.68± 0.89	0.71±0.89	p=0.42		0.75±1.02	0.67±0.83	p=0.97

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table G. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to clinical activity.

	Clinical remission	Mild clinical activity	Moderate clinical activity	Severe clinical activity	p
PINP ng/ml	8.88± 9.23	10.97±8.48	7.64± 8.30	n.a.	p=0.45
BALP µg/l	11.83± 4.55	12.59± 5.73	9.94± 6.73	n.a.	p=0.34
TRAcP U/L	2.59± 0.95	2.73± 1.02	2.72± 1.04	n.a.	p=0.77
CTX ng/ml	0.36± 0.21	0.50± 0.34	0.48± 0.23	n.a.	p=0.10
ucOC ng/ml	0.68± 0.89	0.53±0.41	1.12±1.56	n.a.	p=0.77

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; n.a.= no available data.

Table H. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to endoscopic activity.

	Clinical remission	Mild endoscopic activity	Moderate endoscopic activity	Severe endoscopic activity	p
PINP ng/ml	8.88± 9.23	11.20±9.29	8.16± 8.16	4.41±8.06	p=0.31
BALP µg/l	11.83± 4.55	11.21±3.99	11.51±8.16	11.86±6.46	p=0.48
TRAcP U/L	2.59± 0.95	2.52±1.11	2.53±0.844	2.85±0.98	p=0.43
CTX ng/ml	0.36± 0.21	0.37±0.24	0.54±0.35	0.54±0.23	**p=0.05**
ucOC ng/ml	0.68± 0.89	0.72±0.69	0.85±1.22	0.35±0.32	p=0.81

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table I. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to clinical activity and gender.

		PINP ng/ml	BALP µg/l	TRAcP U/L	CTX ng/ml	ucOC ng/ml
Clinical remission	**a. Males**	4.07± 2.88	12.11± 5.32	2.40± 0.74	0.40± 0.22	0.90± 0.86
	b. Females	12.92± 10.75	11.60± 3.90	2.74± 1.09	0.33± 0.20	0.47± 0.88
		p_{a-b}= 0.04	p_{a-b}= 0.90	p_{a-b}= 0.43	p_{a-b}= 0.20	**p_{a-b}= 0.001**
Clinical activity	**c. Males**	4.72± 4.43	12.24± 5.94	2.93± 1.06	0.52± 0.22	0.61± 0.59
	d. Females	14.54± 9.04	12.18± 6.60	2.73± 1.06	0.48± 0.38	0.87± 1.24
		p_{c-d}<0.0001	p_{c-d}= 0.88	p_{c-d}= 0.88	**p_{c-d}= 0.01**	p_{c-d}= 0.10
		p_{a-c}= 0.80 p_{b-d}= 0.74	p_{a-c}= 0.87 p_{b-d}= 0.93	p_{a-c}= 0.12 p_{b-d}= 0.83	p_{a-c}= 0.11 p_{b-d}= 0.15	p_{a-c}= 0.19 **p_{b-d}= 0.02**

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table L. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to clinical activity and gender.

		PINP ng/ml	BALP µg/l	TRAcP U/L	CTX ng/ml	ucOC ng/ml
Endoscopic remission	**a. Males**	4.42±2.55	11.62±4.56	2.77±0.88	0.37±0.20	0.83±0.98
	b. Females	11.87±10.33	13.55±3.38	2.90±1.02	0.29±0.16	0.66±1.12
		p_{a-b}=0.27	p_{a-b}=0.23	p_{a-b}=0.80	p_{a-b}=0.21	p_{a-b}=0.14
Endoscopic activity	**c. Males**	4.42±4.20	12.40±6.01	2.65±1.00	0.50±0.23	0.69±0.59
	d. Females	14.30±9.82	11.33±5.71	2.68±1.09	0.44±0.33	0.65±1.07
		p_{c-d}<0.0001	p_{c-d}=0.73	p_{c-d}=0.89	**p_{c-d}= 0.01**	p_{c-d}=0.76
		p_{a-c}= 0.77 p_{b-d}= 0.47	p_{a-c}= 0.94 p_{b-d}= 0.07	p_{a-c}= 0.53 p_{b-d}= 0.51	p_{a-c}= 0.17 p_{b-d}= 0.15	p_{a-c}= 1.0 p_{b-d}= 0.82

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table M. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to biomarkers of activity.

	CRP <0.5 ng/ml	CRP >0.5 ng/ml	p		FC <250 µg/g	FC >250 µg/g	p
PINP ng/ml	9.76± 9.06	8.04±8.44	p=0.38		9.51±8.94	6.98±8.16	p=0.15
BALP µg/l	12.58±5.46	10.98±5.27	p=0.09		12.16±5.29	11.15±6.35	p=0.63
TRAcP U/L	2.80±1.06	2.54±0.89	p=0.26		2.70±1.01	2.78±1.05	p=0.77
CTX ng/ml	0.47±0.30	0.37±0.19	p=0.13		0.40±0.26	0.64±0.25	**p=0.001**
ucOC ng/ml	0.67±0.94	0.75±0.76	p=0.20		0.61±0.73	1.26±1.46	p=0.06

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table N. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to therapy.

	a. anti-TNFα	b. UST	c. VDZ	d. Jak/AZA	e. No Therapy	
PINP ng/ml	8.64±8.78	4.91±7.29	11.92±9.05	8.19±7.36	8.17±9.02	p=0.23
BALP µg/l	12.63±5.26	11.42±3.47	11.18±6.19	9.84±4.77	12.53±4.23	p=0.49
TRAcP U/L	2.75±1.10	2.30±0.59	2.74±1.06	2.29±0.35	2.71±0.89	p=0.79
CTX ng/ml	0.43±0.26	0.39±0.20	0.46±0.33	0.47±0.19	0.38±0.23	p=0.90
ucOC ng/ml	0.88±1.07	0.72±0.30	0.60±0.95	0.57±0.60	0.60±0.48	p=0.57

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; TNF: tumor necrosis factor; UST: ustekinumab; VDZ: vedolizumab; Jak: Janus kinase inhibitors; AZA: azathioprine.

Table O. Correlation of bone remodeling biomarkers and vitamin K-dependent biomarkers with bone mineralometry.

	Femoral T-score	**Vertebral T-score**
PINP ng/ml (significance)	-0.050 (p=0.72)	-0.088 (p=0.53)
BALP µg/l (significance)	-0.325 **(p=0.01)**	-0.281 **(p=0.04)**
TRAcP U/L (significance)	-0.352 **(p=0.01)**	-0.350 **(p=0.01)**
CTX ng/ml (significance)	-0.184 (p=0.19)	-0.219 (p=0.11)
ucOC ng/ml (significance)	-0.078 (p=0.61)	-0.024 (p=0.87)

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin.

Table P. Bone remodeling biomarkers and vitamin K-dependent biomarkers according to vitamin D levels.

	Vitamin D levels				
	a. IBD patients		**b. Celiac patients**		
	No supplementation	**Supplementation**	**No supplementation**	**Supplementation**	
PINP ng/ml	8.10±8.10	12.14± 9.53	98.76±245.02	15.78±7.43	p_a=0.10 p_b=0.88
BALP µg/l	11.85±5.81	11.64±5.00	14.69±5.89	12.48±3.71	p_a=0.68 p_b=0.50
TRAcP U/L	2.59±0.90	2.69±1.06	2.67±0.70	2.72±1.39	p_a=0.69 p_b=0.49
CTX ng/ml	0.42±0.31	0.43±0.25	0.31±0.18	0.38±0.19	p_a=0.59 p_b=0.45
ucOC ng/ml	0.78±0.77	0.51±0.85	0.50±0.37	0.82±0.92	**p_a=0.03** p_b=0.50.

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table Q. Correlation of bone remodeling biomarkers and vitamin K-dependent biomarkers with vitamin D.

	Vitamin D levels	
	IBD patients	**Celiac patients**
PINP ng/ml (significance)	0.225 (p=0.04)	0.264 (p=0.30)
BALP µg/l (significance)	-0.021 (p=0.85)	-0.075 (p=0.75)
TRAcP U/L (significance)	0.172 (p=0.13)	0.012 (p=0.96)
CTX ng/ml (significance)	-0.087 (p=0.44)	0.313 (p=0.17)
ucOC ng/ml (significance)	-0.28612 (p=0.02)	0.157 (p=0.50)

PINP: procollagen type I N-terminal propeptide; BALP: bone-specific alkaline phosphatase; TRAcP: tartrate resistant acid phosphatase, CTX: type I collagen cross-linked C-telopeptide; ucOC: under-carboxylated osteocalcin; IBD: inflammatory bowel disease.

Table R. Correlation of vitamin K-dependent biomarkers with vitamin K RDA.

	Viatmin K RDA%		
	IBD patients	**Celiac patients**	**Controls**
ucOC ng/ml (significance)	-0.132 (p=0.29)	0.223 (p=0.40)	-0.123 (p=0.27)

ucOC: under-carboxylated osteocalcin; RDA: recommended daily allowance.

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