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Daily oligofructose-enriched inulin intake impacts bone turnover markers but not the cytokine profile in pediatric patients with celiac disease on a gluten-free diet: Results of a randomised, placebo-controlled pilot study

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ABSTRACT

Background: Bone metabolism disturbances are commonly observed in patients with newly diagnosed celiac disease (CD). The only available treatment for CD–the intake of a gluten-free diet (GFD)–has been found to be insufficient in effectively improving bone health in some patients. Therefore, there is an urgent need to modify the GFD so as to allow for the provision of all the necessary nutrients and improved absorption. Prebiotics intake reportedly improves the absorption of bone-related vitamin D and calcium as well as bone metabolism. The effect of prebiotic intake on bone health in CD patients has not been studied yet. This study aimed to evaluate the effect of oligofructose-enriched inulin intake on bone metabolism and immune response in children with CD on a GFD.

Methods: A total of 34 children with CD were randomised into two groups receiving 10 g of oligofructose-enriched inulin (Synergy 1) or a placebo (maltodextrin) for three months, together with a strict GFD. The children's bone metabolism marker levels and cytokine profiles were analysed before and after the intervention.

Results: After supplementation, the concentration of osteocalcin increased significantly in children receiving Synergy 1, while the concentration of bone alkaline phosphatase increased in both groups, independent of supplementation. After the intervention, the level of pyridinoline increased significantly in the placebo group, resulting in a concentration that was two times higher than that in the Synergy 1 group, in which it remained stable. Moreover, the plasma concentrations of N-terminal telopeptides of type I collagen decreased in both the groups, whereas the tartrate-resistant acid phosphatase 5b level increased particularly in the Synergy 1 group. The intervention did not lead to immunological response changes.

Conclusions: The proposed supplementation beneficially altered bone metabolism, through increased bone formation rates and decreased bone resorption process rates. Supplementation of GFD with prebiotic oligofructose-enriched inulin may be a promising auxiliary therapy for bone metabolism improvements in children with CD.

1. Introduction

Celiac disease (CD) in a gluten-related immune-mediated disease of the small intestine observed in genetically predisposed individuals [1]. It leads to intestinal villi atrophy and, subsequently, to reductions in the intestinal absorption surface, resulting in the malabsorption of macro- and micronutrients.

Pathological bone alterations, including reduced bone mineral content and bone mineral density (BMD), are frequently reported in patients newly diagnosed with CD [2–4]. While the pathogenesis of bone metabolism deterioration in CD patients is not fully understood, it is most likely the result of chronic intestine inflammation, villus

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atrophy and malnutrition as well as deficiencies in the levels of bone-related vitamins (vitamin D) and mineral compounds (calcium, phosphate, magnesium). Calcium deficiency may occur additionally due to the elimination of dairy products from the diet owing to secondary lactose intolerance, which is highly prevalent in CD [5]. The imbalance of osteoclastogenesis-regulating parameters may also be involved in bone metabolism dysregulation in CD [6]. Pro-inflammatory cytokines, such as interleukin 1 (IL-1), IL-6 and tumour necrosis factor (TNF) were found to stimulate bone resorption, mainly via the stimulation of osteoclast formation. IL-10 inhibits the formation of osteoblasts and consequently suppresses bone mineralization, whereas IL-12 indirectly suppresses the development of osteoclasts [7,8].

Currently, a strict adherence to a gluten-free diet (GFD) is the only available treatment for CD. The complete elimination of gluten from the daily diet reverses CD symptoms, restores intestinal mucosa and normalizes nutrient absorption in CD patients [9]. Besides, the intake of such a diet improves the degree of bone mineralization in a majority of CD patients [10–12]. However, some trials revealed a lower BMD in CD patients following the intake of a GFD, compared to the healthy population [11,13,14]. This may be a consequence of poor GFD compliance and/or imbalanced diet. GFD intake by itself may result in further deficiencies, including those of calcium phosphate and vitamin D [15,16], negatively impacting bone health.

Maintenance of an appropriate BMD is particularly important in young children, as bone health disturbances can lead to osteoporosis and increased fragility fracture rates. The best method for the assessment of bone metabolism rate is histomorphometry of the iliac crest; however, as it is an invasive procedure, it is not routinely used [12]. Non-invasive dual-energy X-ray absorptiometry requires a relatively long time for the identification of pathological alterations in bone metabolism. On the contrary, bone turnover markers are able to mirror the ongoing bone metabolism in shorter spans and at a molecular level. OC and BALP are the most commonly used markers of bone formation, while DPD, CTx and NTx are markers of bone resorption [17]. Previous studies that analysed the level of bone turnover biomarkers confirmed a progressive GFD intake-related improvement in bone metabolism in CD [12,14]. In particular, in pediatric patients with CD, in whom the level of bone formation markers is usually decreased and that of resorption markers is elevated at diagnosis [17], a strict adherence to a GFD resulted in the stimulation of bone formation, although the level of bone resorption markers did not normalize even after long-term GFD therapy [10,18]. These observations suggest that in some CD patients a GFD alone may be insufficient or too slow for effective bone health improvements during intensive growth and puberty. Therefore, there is an urgent need to modify the GFD so as to allow for the provision of all the necessary nutrients, minerals and vitamins, or compounds that improve their absorption.

Prebiotics, through their positive effect on intestinal microbiota characteristics [19], reportedly improve the absorption rates of bone-related nutrients, in particular those of vitamin D [20] and calcium [21,22]. Numerous studies have shown the positive effect of the intake of prebiotics, especially inulin-type fructans, on bone mineralization and bone metabolism [22–25]. Moreover, prebiotic intake was shown to reduce serum pro-inflammatory cytokine levels [26–29]. The mechanism of action of prebiotics is mainly indirect and related to the stimulation of the metabolic activity of gut microbiota; this consequently has a beneficial effect on immune response and cytokine production [27,30]. To investigate the impact of prebiotic intake on bone metabolism and cytokine levels in CD, this pilot, randomised, placebo-controlled nutritional intervention was designed to allow for the evaluation of the effect of oligofructose-enriched inulin on bone turnover markers and immune response in children with CD on a GFD. We hypothesized that daily intake of oligofructose-enriched inulin, that has the reported beneficial impact on bone-related nutrients absorption, will bring health benefits to children suffering from CD on GFD, in particular, it will affect favourably bone metabolism without any side effects. To the best of our knowledge, the effect of prebiotic intake on bone metabolism in CD patients has not been studied till date.

2. Materials and methods

2.1. Study protocol

A pilot clinical trial with nutritional intervention was conducted with the participation of pediatric CD patients ($N = 34$) from the Department of Pediatrics, Gastroenterology and Nutrition of the Regional Specialized Children's Hospital in Olsztyn (Poland), who were following a strict GFD for at least 6 months. Participants were randomly assigned to the Synergy 1 ($N = 18$; 11 girls; average age 10 years (range: 5–17 years)) or placebo group ($N = 16$; 10 girls; average age 10 years (range: 4–16 years)). Stratified randomisation based on sex and age was conducted to create comparable groups. At the baseline, the experimental groups were statistically similar in term of age, body mass and height [20,31]. Height expressed as a standard deviation score (SDS-height) was calculated according to OLAF calculator [32]. The durations of GFD adherence were similar, at 3.00 ± 2.17 and 2.63 ± 2.03 years in the Synergy 1 and placebo groups, respectively. The proportions of participants in the pubertal stage did not differ (50% of children with Tanner stages I and II in both groups). Participants did not have any bone fracture incidents. Growth velocity was 5.13 ± 3.36 and 7.10 ± 4.05 cm/year in Synergy 1 and placebo group, respectively. All participants met the inclusion criteria: CD confirmed by serological marker presence and duodenal biopsy assessment. The exclusion criteria included: use of antibiotics in the month preceding the study, use of drugs that can affect bone metabolism (glucocorticosteroids, antiepileptic drugs, thyroid hormone, anticoagulants, bisphosphonates), use of probiotics, prebiotics or fibre supplements, poor or average overall health, as determined by a gastroenterologist, participation in other trials, and having undergone surgery recently. The nutritional intervention lasted three months. During this period, patients in the Synergy 1 group ingested 10 g of Synergy 1 (Orafti® Synergy 1, Beneo, Tienen, Belgium) daily, while those in the placebo group ingested maltodextrin. Children in both groups continued their adherence to a GFD, which was controlled by the measurement of the levels of anti-tissue transglutaminase antibodies (tTG) on each clinical visit, as reported in our previous study [33]. Study product, Synergy 1 or placebo, was provided to participants during the first visit in powder form, in single-use, pre-weighted packets. Participants were asked to record their daily intake of the provided study product and any adverse events occurring during the trial on the observation chart provided to each participant. At the baseline and after the intervention, anthropometric measurements (body weight and height) were performed and plasma and urine samples were collected, which were then aliquoted and stored at −80 °C till analysis. Study visits were coordinated with regular visits (every three months) to the gastroenterology clinic to avoid multiple blood sampling. Parents or caregivers of the study participants were aware of the potential benefits and risks of the nutritional intervention and signed an informed consent form. The study was approved by the Bioethics Committee of the Faculty of Medical Sciences of the University of Warmia and Mazury in Olsztyn, Poland (agreement No: 23/2015). This study was a part of a larger study that has been registered in the US National Library of Medicine under the number NCT03064997 [34]. Details on the study protocol, patient characteristic and sampling procedures have been described in our previous study [31].

2.2. Measurement of bone metabolism markers

Bone turnover markers were assessed with enzyme-linked immunosorbent assay (ELISA) using commercial kits. The plasma levels of osteocalcin (OC) and bone alkaline phosphatase (BALP) were analysed using: Human OC/BGP (Osteocalcin) Kit (Elabscience, Bethesda, USA)

with a detection range of 1.25–80 ng/mL, sensitivity of 0.75 ng/mL and coefficient of variation < 10% and Human BALP (Bone Alkaline Phosphatase) ELISA Kit (Elabscience, Bethesda, USA) with a detection range of 78.13–5000 pg/mL, sensitivity of 46.88 pg/mL and coefficient of variation < 10%, respectively. The evaluation of bone resorption markers included plasma concentrations of tartrate-resistant acid phosphatase 5b (TRAP 5b) and the N-terminal telopeptides of type I collagen (NTx), as well as the urinary excretion of the C-terminal telopeptides of type I collagen (CTx) and cross-links of mature type I collagen in bone, including pyridynoline (PYR) and deoxypyridinoline (DPD). TRAP 5b analysis was conducted using MicroVue™ TRAP5b EIA Kit (Quidel, San Diego, USA) with a detection range of 1.3–16.0 U/L, sensitivity of 0.2 U/L and a coefficient of variation < 3%. NTx levels were determined using OSTOMARK NTx Serum (EMERGO EUROPE, The Hague, The Netherlands) and expressed in nanomoles bone collagen equivalents (nM BCE). The detection range and coefficient of variations were 5–40 nM BCE and < 7%, respectively. CTx levels were measured using Urine CrossLaps (CTXI) EIA (Immunodiagnostics Systems Limited, Boldon, UK) with a detection range of 87–7858 μg/L, sensitivity of 50 μg/L and a coefficient of variation < 10%. PYR levels were analysed using MicroVue™ PYD EIA (Pyridinium cross-links; PYD + DPD) EIA CE IVD (Quidel, San Diego, USA), with a detection range of 15–750 nmol/L, sensitivity of 7.5 nmol/L and a coefficient of variation < 12%. These levels were was calculated by the extraction of a DPD value obtained using a corresponding test, MicroVue™ DPD EIA (Deoxypyridinoline cross-links) urine CE IVD (Quidel, San Diego, USA) with a linear range of 1–100 nmol/L, sensitivity of 0.5 nmol/L and a coefficient of variation < 16%. All the urinary markers were normalised for urine dilution by the creatinine content. Urinary creatinine levels were analysed according to standard clinical methods by the Diagnostic Laboratory of Regional Specialized Children's Hospital in Olsztyn.

In the analyses of the levels of all the bone metabolism markers, Biochrom® Asys UVM 340 Microplate Reader (Biochrom Ltd, Cambridge, UK) was used. All ELISA kits were used according to the protocols described by the manufacturers.

2.3. Measurement of insulin-like growth factor 1 (IGF-1)

Serum IGF-1 levels were measured using the sandwich ELISA method with a commercial kit (IGF-1 ELISA, Mediagnost®, Reutlingen, Germany), in accordance with the manufacturer's protocol. The inter-assay and intra-assay coefficients of variation for serum IGF-1 were 6.79% and 6.65%, respectively and the detection range was 1–50 ng/mL.

2.4. Measurement of plasma cytokine levels

A BD Cytometric Bead Array Human Inflammatory Cytokines kit (BD Biosciences, San Jose, USA) was used for the simultaneous measurement of the levels of cytokines: IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α in the plasma samples according to the manufacturer's instructions. Briefly, 50 μL of capture bead suspension, 50 μL of the test sample or standard, and 50 μL of detection reagent were mixed in a separate tube and incubated at room temperature for 1.5 h. Samples were then washed with 1 mL of wash buffer and centrifuged at 400 × *g* for 5 min. The supernatant was discarded, and 50 μL of the Human Inflammatory Cytokine PE Detection Reagent was added to each tube. Samples were incubated at room temperature for 1.5 h, protected from light. After another washing step, the supernatant was aspirated and discarded, and the pellet was suspended in 300 μL of wash buffer. Samples were read on a flow cytometer BD LSR-Fortessa Cell Analyzer (Erembodegem, Belgium) using the Diva Version 6.2 software (BD Bioscience, USA). The cytokine concentrations in each sample were calculated using the FCAP Array™ software (BD Bioscience, USA). The standard curve range was from 20 to 2500 pg/mL for each cytokine. The manufacturer declared an intra-assay precision value between 2 and 10% for each cytokine, measured for three concentrations–80, 625 and 2500 pg/mL. BD Cytometric Bead Array Human IFN-γ Flex Set was used for the analysis of interferon γ (IFN-γ). IFN-γ was excluded from the statistical analyses because the values obtained were under the detection limit of the assay.

The plasma level of IL-1ra was evaluated using the Quantikine® ELISA Human IL-1ra Immunoassay, according to the manufacturer's instructions (DRA00B, R&D System Inc., Minneapolis, USA). This assay uses quantitative sandwich ELISA with two very specific monoclonal antibodies against human IL-1ra. The detection range is 40–640 pg/mL, and the intra-assay precision value, as declared by the manufacturer, ranges from 4.1 to 5.7%.

2.5. Statistical analysis

Descriptive statistics were calculated for the anthropometric indices and data are expressed as means ± standard deviation. All analytical measurements were performed in duplicates. The normal distribution of the data was evaluated using a Shapiro-Wilk *W* test. Quantitative variables with a normal distribution are expressed as mean ± standard deviation, while quantitative variables that showed a non-normal distribution are expressed as median (P25-P75). The comparison between the experimental groups was performed using *t*-Student's tests or Mann-Whitney *U* tests, as appropriate. Comparisons within the groups, between the baseline and after the intervention, were performed using *t*-Student's test for dependent variables or Wilcoxon signed-rank tests, as appropriate. Only the CTx, PYD and DPD values were normally distributed, so they were evaluated with parametric tests; the other parameters were compared using non-parametric tests. Correlations between the bone metabolism markers, cytokines and anthropometric features were analysed using a Pearson correlation coefficient test. In correlation analysis, also an axial z-score from past densitometry was included. All analyses were performed using Statistica software (v. 12, StatSoft, Tulsa, OK, USA) or XLSTAT statistical software for Microsoft Office, as appropriate. Statistical significance thresholds were set at *p*-value < 0.05 (*) and $p < 0.01$ (**).

3. Results

Of the 34 CD patients enrolled in this study, 30 were included in the final analysis. Two patients were excluded owing to antibiotic use during the intervention and two due to non-compliance (compliance rate lower than 80%) with the recommended daily study product consumption level.

3.1. Anthropometric indices

As expected, three months after the nutritional intervention, the body mass and height of all the CD patients increased (Fig. 1), but there were no statistical differences between the Synergy 1 and placebo groups. In addition, in both groups, the anthropometric parameter values analysed after the intervention did not differ significantly compared to those at the baseline (T0).

3.2. Bone metabolism

The concentrations of BALP, OC, CTx, DPD, PYD, TRAP 5b and NTx before and after the intervention in the placebo and Synergy 1 groups are presented in Table 1.

Analyses of the bone formation markers indicated that the three-month supplementation with prebiotics in the Synergy 1 group resulted in a significant ($p < 0.05$) increase in the plasma OC concentration. Additionally, the level of BALP increased after the intervention in both the experimental groups (Table 1).

The dietary intervention did not affect the concentration of urinary

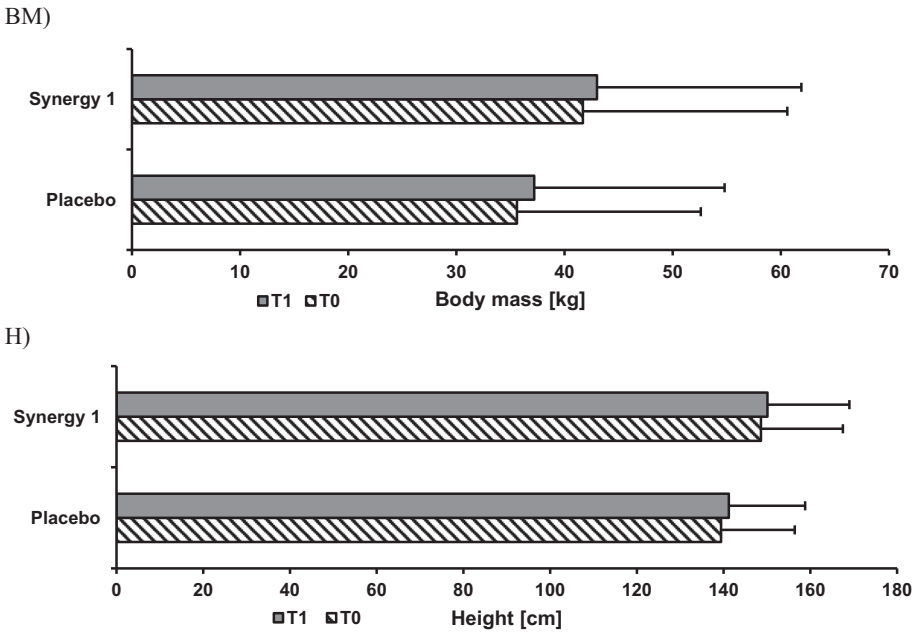


Fig. 1. Anthropometric indices before (T0) and after the intervention (T1). (BM) – body mass; (H) – height.

bone resorption markers, except for PYD (Table 1). The level of PYD increased significantly ($p < 0.01$) in the placebo group, making its concentration after the intervention two times higher than that in the Synergy 1 group, in which it remained stable. In the Synergy 1 group, a decreasing tendency was observed for the levels of all the urinary bone resorption markers. After the intervention, the plasma concentration of NTx decreased in both the experimental groups, whereas the TRAP 5b level increased, particularly in the Synergy 1 group (Table 1).

The concentration of IGF-1 did not change in either group after the intervention; however, an increasing tendency was observed in the Synergy 1 group (Fig. 2).

3.3. Cytokine level

The concentrations of IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12p70 and TNF-α before and after the intervention are presented in Table 2. No significant differences in the plasma cytokine level were observed between the placebo and Synergy 1 groups before and after the intervention.

3.4. Association between parameters

The correlations between the markers of bone metabolism and cytokines, and anthropometric indices, as well as the GFD intake duration, were evaluated and presented in Fig. 3. Not surprisingly, strong positive

Table 1
Bone metabolism parameters in patients with celiac disease at the baseline (T0) and after 3-months supplementation (T1) expressed as a mean ± SD or median (P25-P75), depending on normal distribution.

	Body fluid	Placebo group ($N = 15$)		Synergy 1 group ($N = 16$)		Placebo: T0 vs.T1	Synergy 1: T0 vs. T1	T1: Placebo vs Synergy 1
		T0	T1	T0	T1			
BALP[a] [ng/mL]	P[b]	0.48 (0.44–0.61)	1.00 (0.57–1.37)	0.49 (0.24–0.57)	0.88 (0.78–0.98)	**0,008****	**0,005****	0,597
OC[c] [ng/mL]	P	4.77 (3.38–6.42)	6.35 (4.33–9.61)	2.73 (1.63–5.95)	9.86 (9.37–14.75)	0,241	**0,018***	0,105
CTx[d] [μg/mmol creatinine]	U	993.52 ± 113.20	1149.92 ± 134.39	971.02 ± 109.11	906.48 ± 146.14	0,105	0,179	0,243
DPD[e] [nmol/mmol creatinine]	U	27.32 ± 2.49	25.57 ± 3.79	22.11 ± 2.72	17.43 ± 2.52	0,479	0,076	0,110
PYD[f] [nmol/mmol creatinine]	U	77.23 ± 8.84	113.93 ± 14.21	75.98 ± 9.24	61.80 ± 10.13	**0,009****	0,150	**0,014***
NTx[g] [nM BCE][h]	P	74.19 (62.54–87.40)	57.16 (45.97–60.50)	63.54 (56.54–76.88)	54.71 (37.92–62.61)	**0,028***	**0,038***	0,829
TRAP 5b[i] [U/L]	P	14.91 (14.36–15.92)	16.74 (13.88–19.30)	12.59 (12.08–16.51)	16.14 (12.16–18.90)	0,070	**0,047***	0,576

[a] Bone alkaline phosphatase.
[b] Measured in plasma (P) or urine (U).
[c] Osteocalcin.
[d] C-terminal telopeptides of type I collagen.
[e] Deoxypyridinoline.
[f] Pyridinoline.
[g] N-terminal telopeptides of type I collagen.
[h] Nanomoles bone collagen equivalents per liter.
[i] Tartrate-resistant acid phosphatase 5b.
* $p < 0.05$.
** $p < 0.01$.

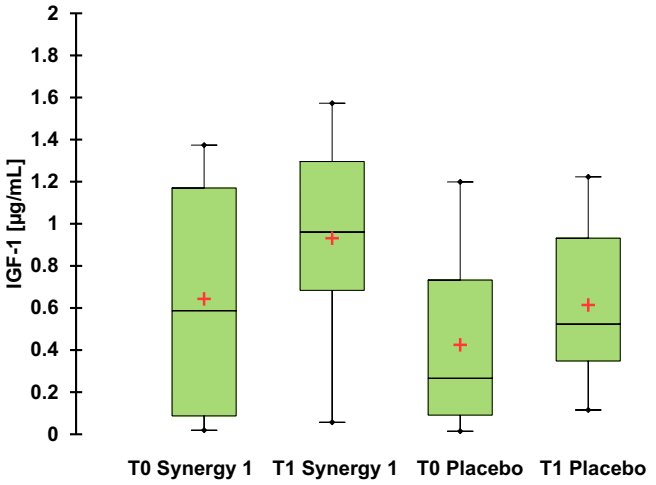


Fig. 2. Plasma level of insulin-like growth factor 1 (IGF-1) before (T0) and after the intervention (T1). Cross – mean, horizontal line – median, box – values between P25-P75, whiskers – minimum/maximum values.

correlations ($r > 0.7$) were observed between anthropometric indices. Moderate negative correlations ($-0.7 < r < -0.5$) were observed between age and CTx, DPD, PYD and TRAP 5b, between height and DPD and PYD, between Tanner stage and CTx and PYD, as well as growth velocity and DPD. The strongest correlation was determined between growth velocity and TRAP 5b. Additionally, strong negative correlations ($r < -0.7$) were observed between weight and CTx and PYD, between height and CTx as well as between growth velocity and NTx. From bone formation markers, the only correlation was observed between BALP and the GFD intake duration. The presented results show that neither sex, axial z-score nor the SDS height correlated with the bone metabolism parameters. The bone resorption markers correlated positively with each other, with the strongest correlation observed between CTx and PYD.

Cytokine levels were not so dependent on the analysed parameters. Moderate positive ($0.5 > r > 0.7$) correlations were observed between gender and TNF-α, between the GFD intake duration and IL-10 as well as between axial z-score and IL-12p70. Moreover, a moderate negative correlation was determined between BALP and IL-10.

The concentrations of OC, IL-1β, IL-1ra, IL-6, IL-8 and IGF-1 not correlated with the bone metabolism markers, anthropometric indices, or cytokines.

4. Discussion

In this randomised, single-centre, pilot study, the effect of a three-month dietary intervention with oligofructose-enriched inulin in addition to a GFD intake on bone formation, resorption markers, and cytokine profiles in pediatric patients with CD was examined, and the results were compared to those of individuals receiving a placebo.

In the present study, we evaluated participants' complete bone metabolism analysing the concentrations of the spectrum of markers involved in the opposite bone turnover processes: BALP and OC, as bone formation markers, and DPD, PYD, NTx, CTx, as bone resorption markers. In addition, TRAP 5b – the enzyme produced by osteoclasts – was analysed.

We found that a three-month intake of Synergy 1 together with a strict GFD resulted in a significant increase in OC concentrations, suggesting that the process of bone formation could be stimulated by dietary supplementation with prebiotics in CD patients. However, the increased level of plasma OC observed in the Synergy 1 group alone may be a result of the prebiotic-stimulated increase in 25-hydroxyvitamin D concentrations, as reported in our previous study [20], as vitamin D is involved in OC synthesis [35]. The level of BALP was increased in both the experimental groups, suggesting a continuous beneficial effect of GFD compliance on bone formation, as also observed by Mora et al. [12]. Barera et al. [10] concluded that a six-month treatment with GFD intake was sufficient to balance the BALP concentrations in children with CD to the levels determined in the healthy controls.

Interestingly, in the Synergy 1 group, the concentrations of CTx and PYD, bone resorption markers, tended to decrease, contrary to the results determined in the placebo group. This change may suggest the prebiotic-induced inhibition of bone resorption processes. We also found that the concentration of NTx decreased after the three-month intervention independent of supplementation. The decreasing NTx levels determined in both the experimental groups may suggest a decrease in the bone resorption rates, as high levels of NTx are correlated with a low BMD [6]. Similar to NTx, the level of DPD decreased in both the groups. This trend may also indicate an association between good GFD compliance and lower bone loss rates.

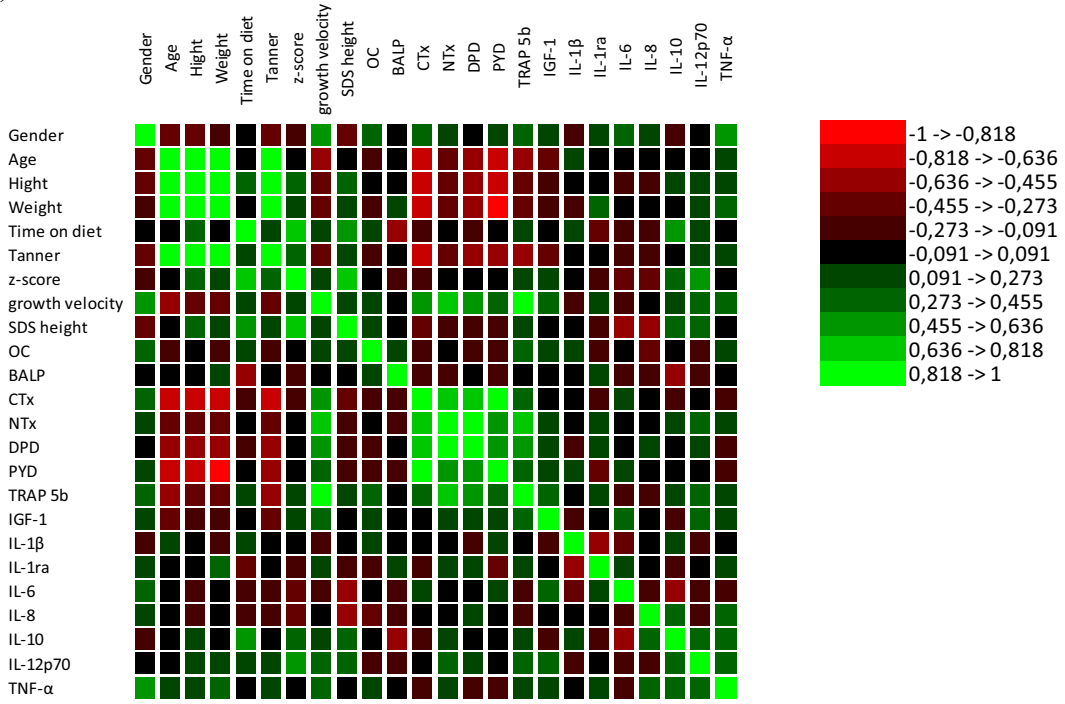
Previously, TRAP 5b was suggested to be a marker of bone resorption; however, recently, it was demonstrated that it reflects the osteoclast number, rather than bone resorption [36]. TRAP is important for bone formation too [37]. In the present study, the level of TRAP 5b increased in all the patients. The high levels of TRAP 5b can be explained by the active growth observed in childhood and adolescents, especially if adherence to the GFD is maintained. During childhood, bone metabolism processes are focused mainly on bone formation, related to an increase in size, while in adults, bone metabolism is required for the maintenance of bone strength, through bone remodelling processes [10]. A lack of TRAP 5b resulted in the impairment of long-bone development in young mice, mainly owing to the effect on the development of the growth plate and metaphysis [37]. Shidara et al. [38] reported a significant correlation of TRAP 5b with BALP and OC, and the strongest correlation ($r = 0.821$) with NTx. In this study too, we found a significantly strong correlation between TRAP 5b and NTx; however, we did not observe a correlation between TRAP 5b and bone

Table 2
Plasma profile of pro- and anti-inflammatory cytokines in celiac disease children from placebo and Synergy 1 group before (T0) and after the intervention (T1). Values are expressed in pg/mL and presented as median (P25-P75). No statistical difference was observed between cytokine levels.

	Placebo group ($N = 15$)		Synergy 1 group ($N = 16$)	
	T0	T1	T0	T1
IL-1β[a]	1.74 (1.59–1.88)	1.63 (1.44–1.75)	1.74 (1.63–1.82)	1.69 (1.55–1.75)
IL-1ra	247.3 (139.5–690.9)	196.6 (129.7–492.7)	156.3 (136.4–187.5)	131.7 (116.4–187.7)
IL-6	0.50 (0.41–1.11)	1.06 (0.70–1.63)	0.89 (0.73–1.13)	1.01 (0.49–1.42)
IL-8	4.22 (3.57–4.79)	3.65 (3.57–4.41)	3.74 (3.34–4.12)	3.82 (3.39–4.71)
IL 10	3.07 (2.95–3.26)	3.02 (2.81–3.10)	3.10 (2.71–3.30)	3.12 (2.85–3.44)
IL-12p70	1.79 (1.64–2.00)	1.45 (1.19–2.04)	1.55 (1.32–1.86)	1.69 (1.33–1.75)
TNF-α[b]	0.91 (0.56–1.17)	0.59 (0.48–0.89)	0.95 (0.53–1.24)	0.83 (0.52–1.15)

[a] IL – interleukin.
[b] Tumour necrosis factor α.

a)



b)

Variables	Gender	Age	Hight	Weight	Time on diet	Tanner stage	Z-score	growth velocity	SDS height	BALP	CTx	NTx	DPD	PYD	TRAP 5b	IL-10	IL-12p70
Age	-0.31																
Hight	-0.32	**0.88***															
Weight	-0.19	**0.88***	**0.87***														
Time on diet	-0.04	-0.02	0.28	-0.01													
Tanner stage	-0.42	**0.93***	**0.94***	**0.86***	0.11												
Z-score	-0.23	0.02	0.40	0.15	**0.75***	0.28											
growth velocity	0.47	**-0.58***	-0.34	-0.38	0.19	-0.43	0.17										
SDS height	-0.35	-0.02	0.41	0.15	**0.61***	0.27	**0.79***	0.16									
BALP	-0.06	-0.01	0.04	0.13	**-0.57***	0.06	-0.13	-0.04	0.08								
CTx	0.33	**-0.65***	**-0.73***	**-0.81***	-0.14	**-0.64***	-0.24	0.50*	-0.43	-0.17							
NTx	0.22	-0.42	-0.37	-0.37	-0.08	-0.37	-0.03	**0.70***	-0.27	-0.14	**0.68***						
DPD	0.03	**-0.55***	**-0.57***	-0.46	-0.27	-0.47	-0.09	**0.56***	-0.26	0.07	**0.68***	**0.83***					
PYD	0.14	**-0.66***	**-0.67***	**-0.85***	0.02	**-0.60***	-0.04	0.45	-0.23	-0.18	**0.90***	**0.56***	**0.56***				
TRAP 5b	0.37	**-0.57***	-0.31	-0.39	0.20	-0.46	0.14	**0.90***	0.14	-0.04	0.45	**0.76***	**0.57***	0.43			
IL-10	-0.17	-0.01	0.14	0.04	**0.53***	0.02	0.36	0.15	0.37	**-0.51***	-0.22	0.10	-0.07	-0.07	0.18		
IL-12p70	0.02	0.02	0.26	0.15	0.23	0.13	**0.52***	0.31	0.36	-0.20	-0.08	0.31	0.10	0.04	0.36	0.30	
TNF-α	**0.57**	0.19	0.25	0.32	-0.01	0.13	0.03	0.28	-0.04	0.05	-0.19	0.12	-0.17	-0.23	0.20	0.35	0.35

(*) - significant correlations ($p < 0.05$).

Fig. 3. Correlations matrix between all anthropometric indices, bone metabolism markers and cytokine levels. (a) Heat map of all the correlations. (b) Pearson correlation (r) values of the parameters for which at least one *r*-value was > 0.5 (bold).

formation markers. Several studies have suggested the association between bone turnover markers and anthropometric indices [39–41]. In our study, we also observed these correlations, however only in the markers of resorption. Even though we determined positive correlation between anthropometric indices and bone resorption markers, further dividing of groups to interpret the data in terms of height, weight and growth velocity would be impossible because of low number of participants. Besides that, our main goal was to evaluate the effect of GFD supplementation with prebiotics on bone metabolism, irrespectively on the initial body characteristic.

Prebiotic supplementation can have a beneficial effect on bone health, as recently summarised by Whisner and Castillo [42]. The results obtained in our study, regarding the improvements in the bone turnover markers after prebiotic intake, are in agreement with those of previous studies [22,23,25]. A study on post-menopausal Chinese women showed that dietary supplementation with fructooligosaccharide-fortified milk for 12 weeks significantly reduced the bone resorption rates and improved the vitamin D status [23]. Holloway

et al. [22] evaluated the effect of oligofructose-enriched inulin intake on bone metabolism in a postmenopausal woman and found a progressive increase in the OC level, while the bone resorption marker did not demonstrate consistency. The authors showed that the level of DPD decreased after three weeks of supplementation but increased after six weeks, exceeding the values from the baseline. Similarly, other authors reported a decrease in both the formation and resorption markers but concluded that supplementation with calcium and fructooligosaccharides reduced the rate of total-body and spinal bone loss in postmenopausal women with osteopenia [25]. Practically, studies on bone metabolism are easier to conduct in growing children, as the remodelling period is much shorter than that in adults [43]. A study on healthy girls (age 9–12 years) showed that supplementation with oligofructose-enriched inulin for 11 weeks improved the BALP and serum calcium status and decreased the parathormone levels [44]. Contradictory results were found in a study conducted on 28 adolescent girls, in which a 12-week intervention with a progressing dose of soluble corn fibre (0, 10 and finally 20 g) did not show any effect on the bone turnover markers (BALP, OC, NTx and parathormone) [45]. The authors observed a weak positive correlation between the levels of BALP and calcium, concluding that the consumption of soluble corn fibre may increase bone density. Although data concerning the effect of prebiotics on bone metabolism in children are limited, especially in CD, the previous findings on a postmenopausal woman and our results suggest the need for continued research for the identification of an effective dose to obtain maximal bone mass acquisition.

Of the various regulatory molecules that govern skeletal growth, IGF-1 is a critically important factor in the regulation of bone growth and bone formation [46]. Several studies conducted on transgenic mice indicated that IGF-1 stimulates the activity of osteoblasts and increases the rate of mineralization [47,48]. In children with CD, at diagnosis, the levels of IGF-1 were observed to be much lower than those in healthy children and increased after the introduction of a GFD [49]. In a study on adult CD patients, a positive correlation between IGF-1 levels and BMD was observed [50]. In our study, the level of IGF-1 did not change significantly after the three-month supplementation period. However, an increasing tendency was observed in the Synergy 1 group. The lack of statistical significance can be related to the very wide range of results. The sample size of the present study, although comparable with that of other nutritional intervention studies involving prebiotics, is fairly small; the inclusion of a larger sample size may have allowed for the observation of a significant effect of prebiotic intake on IGF-1. The effect of prebiotic intake on IGF-1 is not well-established in the literature. The only animal study on the topic showed that the addition of inulin to the diet increased the expression levels of IGF-1 in broiler chicken, resulting in better growth [51].

Increased bone resorption as a result of cytokine imbalance has been reported previously [6]. In our study, Synergy 1 supplementation did not affect plasma cytokine concentrations. It is possible that the immune systems of the children were already functioning well, as the GFD had been followed for at least six months, and based on the decreasing tTG trend [33], the adherence to the diet was good. The existing literature does not show consistency in the results on the effect of prebiotics on cytokine levels. A 12-week intervention with a synbiotic comprising fructooligosaccharides in combination with multispecies probiotic strains resulted in a significant decrease in the production of proinflammatory cytokines IL-6 and IL-1β, when the peripheral blood mononuclear cells were incubated with lipopolysaccharide [52]. In in vitro studies using Caco-2 cells, treatment with oligosaccharides decreased the secretion of IL-12 and gene expression of IL-12p35, IL-8, TNF-α and NF-κB, and the anti-inflammatory effect was explained by the stimulation of the nuclear receptor PPARγ [26]. In contrast, supplementation with Synergy 1 for four weeks in healthy humans did not show any effect on cytokine levels; however, a bifidogenic effect was observed [53].

The present study has certain limitations. First, the sample size of the experimental group was relatively small. There was a lack of statistical power and sample size calculation for this trial; however, both limitations are related to the pilot design of the study. Moreover, to avoid misinterpretation, we decided to analyse a relatively wide range of bone metabolism parameters. The lack of dietary evaluation is another limitation. However, dietary control through the use of validated food frequency questionnaires [54,55] was performed, even though details on the same have not been presented in this manuscript. Additionally, the non-inclusion of a healthy control group is another limitation. We decided against including such a group as the main goal of this study was to evaluate the effect of the dietary modification in a certain group of patients. Finally, the study does not contain the bone age. This limitation is related to the limited resources, not allowing for performing this analysis for all children. Despite these limitations, our study is the first to attempt to evaluate the effect of oligofructose-enriched inulin intake on bone metabolism in children with CD following a GFD. As this is a pilot and exploratory study, the results presented may provide information on sample size calculation for future studies.

5. Conclusions

To the best of our knowledge, this study is the first to recognize the effect of oligofructose-enriched inulin (Synergy 1) intake on the bone remodelling markers and cytokine levels in children with CD who were on a GFD. The present study indicated that the supplementation of GFD with oligofructose-enriched inulin was well-tolerated, did not slow down the development of the children and did not cause changes in their immunological response. The main finding of this study is that the proposed GFD supplementation beneficially altered bone metabolism, increasing the rates of bone formation, and decreasing those of the bone resorption process. Taking into account the results obtained, supplementation of a GFD with prebiotic oligofructose-enriched inulin may be a promising auxiliary therapy for the improvement of bone metabolism in children with CD. However, further prospective multicentre research studies with a larger number of participants are required to confirm the obtained results.

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Author contributions

UK-K conceived and supervised the study; UK-K, ND, EJ-C were involved in designing the study, analysing results, interpreting the data; ND and EJ-C collected samples; ND performed the main part of experiments, performed the statistical analysis, wrote the manuscript draft; DZ participated in the flow cytometry analysis; EJ-C, DZ, PA and UK-K provided feedback for the further writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

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