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Published in:
Pediatric Diabetes

DOI:
10.1111/pedi.12963

Publication date:
2020

Document version:
Accepted manuscript

Citation for polished version (APA):

Madsen, J. O. B., Herskin, C. W., Zerahn, B., Jensen, A. K., Jørgensen, N. R., Olsen, B. S., Svensson, J., Pociot, F., & Johannesen, J. (2020). Bone turnover markers during the remission phase in children and adolescents with type 1 diabetes. *Pediatric Diabetes*, 21(2), 366-376. <https://doi.org/10.1111/pedi.12963>

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Bone turnover markers during the remission phase in children and adolescents with type 1 diabetes

Running title:

Bone turnover during partial remission of T1D

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pedi.12963

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Abbreviations:

BMD: Bone mineral density

BMI: Body mass index

CSII: Continuous subcutaneous insulin infusion

CTX: C-terminal cross-linked telopeptide of type-1 collagen

DXA: Dual-energy X-ray absorptiometry

fBG: fasting blood glucose

IDAA1c: Insulin dose adjusted HbA1c

MDI: Multiple daily injections

MMTT: Mixed meal tolerance test

OCN: Osteocalcin

P1NP: Procollagen type-1 amino-terminal propeptide

SD: Standard deviation

T1D: Type 1 diabetes mellitus

T2D: Type 2 diabetes mellitus

TDD: Total daily insulin dose

Acknowledgements

The authors would like to thank Jette Høgsmose and Sussi Polmann for their comprehensive assistance with their expertise in blood sample acquisition and handling. Funding: The study has been financially supported by Aase og Ejnar Danielssens fond, Vissing Fonden, Dronning Louises Børnehospitals Fond, Poul og Erna Sehested Hansen Fonden and the research council at Herlev and Gentofte Hospital.

Author contributions

The study has been designed and modified by JOBM, JS and JJ. BZ and NRJ have contributed with assistance and expertise on bone examination and bone turnover and the interpretation of the results. BSO and FP have contributed with their important knowledge in both study development and study design. Finally, JS and CWH have collected the data together with JOBM. JOBM and JJ have been responsible for production of both first and final drafts of the paper. Each author have proof-read the paper and contributed with constructive criticism and all authors have approved the final manuscript.

Abstract

Background and aim: In rodents, Osteocalcin (OCN) stimulate insulin production and insulin sensitivity, both important factors during partial remission in humans with type 1 diabetes (T1D). However, decreased OCN has reported in both adult and pediatric T1D. This study aims at investigating bone turnover and partial remission in children and adolescents with recent onset T1D.

Subjects and methods: 99 individuals (33% girls) were recruited within 3 months of T1D onset and examined three times, 6 months apart. Outcome variables were bone formation markers OCN and procollagen type-1 amino-terminal propeptide (P1NP) and the bone resorption marker C-terminal cross-linked telopeptide of type-1 collagen (CTX). Dependent variables included IDAA1c (surrogate marker of partial remission), total-body bone mineral density (BMD) and stimulated C-peptide as representative of endogenous insulin production.

Results: OCN- and P1NP Z-scores were significantly decreased throughout the study, whereas CTX Z-scores were increased. None of the bone turnover markers changed significantly between visits. Total body BMD Z-score did not change during the study but was significantly higher than the reference population at visit 2 ($P=0.035$). There were no differences in the bone turnover markers for those in partial remission as defined by either C-peptide or IDAA1c at any visit. The individual change in CTX Z-score was negatively associated with the increase of IDAA1c ($P=0.030$) independent of C-peptide decline ($P=0.034$).

Conclusion: Bone turnover markers indicate increased bone resorption and decreased bone formation during the first year of T1D. The negative association between bone resorption and IDAA1c might represent compensatory mechanisms affecting insulin sensitivity.

Keywords: Diabetes Mellitus, Type 1; Osteocalcin; C-peptide; Bone remodeling; Bone density

INTRODUCTION:

The remission phase is a transient period of partial remission beginning shortly after initiation of insulin treatment in type 1 diabetes (T1D).¹ Partial remission is characterized by increased endogenous insulin production and improved insulin sensitivity, peaking in prevalence three months after diagnosis.^{1,2} Interestingly, animal studies have demonstrated a stimulating effect of the osteoblast secreted protein osteocalcin (OCN) on both insulin production and peripheral insulin sensitivity.^{3,4} A process regulated in part by insulin which has been shown to stimulate OCN release from osteoblasts controlled by different feedback system⁵

Several studies in adults with T1D have concluded that bone is negatively affected in T1D with impaired bone mineral density (BMD), increased fracture-risk and decreased bone turnover markers including decreased OCN.⁶⁻⁸ Alterations in the turnover of bone have been suggested as a cause of both impaired BMD and bone strength.⁹ Two recent studies concluded that BMD was not decreased in Danish children and adolescents with T1D.^{10,11} Despite having BMD comparable to healthy peers, markers of both bone formation and bone resorption were significantly decreased in children and adolescents with T1D for more than one year.¹² These findings supported the conclusions of a review and meta-analyses demonstrating that OCN was significantly decreased in children and adolescents with T1D¹³. Most of the studies included in the review consisted of cases with long-standing T1D.¹³

Evaluation of bone turnover markers in children and adolescents with recent onset T1D have been limited¹⁴⁻¹⁶. If OCN is already decreased during partial remission, the beneficial hormonal effects of OCN might be limited and stimulation of bone formation could be a potential intervention for bone-mediated prolongation of partial remission. A longer remission phase has in large studies been associated with lower HbA1c and fewer complications (hypoglycemic events, albuminuria and retinopathy).^{17,18} Besides prolonged insulin production capacity, improved insulin sensitivity might also positively influence the duration of partial remission due to improved glycemic control and decreased stress on the remaining beta-cells.¹⁹

The aim of this study was to describe the course of bone turnover during the first year after T1D diagnosis, using new national references for bone turnover markers. Furthermore, we aimed at

investigating the connection between bone turnover markers and endogenous insulin production (evaluated by stimulated C-peptide) and insulin dose-adjusted HbA1c (IDAA1c), a surrogate marker of partial remission also affected by insulin sensitivity.²⁰

SUBJECTS AND METHODS:

The primary cohort was recruited from the Department of Pediatrics and Adolescent Medicine at Herlev University Hospital between January 2015 and September 2017. A total of 203 children and adolescents were diagnosed with diabetes and 165 of these were eligible for the study (causes of exclusion: severe comorbidities (including psycho-social problems); non-T1D; follow-up elsewhere; below 3 years of age at T1D onset; participation in another projects; and language barriers (**Figure 1**). Participants were included within 3 months of T1D with the inclusion visit quickly hereafter. The following two visits were scheduled 6 months (visit 1) and 12 months (visit 2) after the baseline visit. In between research visits, participants followed their regular appointments in the outpatient clinic as needed.

The primary cohort was pooled with participants from a previous cohort, including 20 children and adolescents recruited from the same department between March 2012 and May 2014. This cohort was examined and tested in the same manner as the primary cohort, except for participants being put on a gluten free diet to test the implications of a gluten-free diet on the length of the remission phase. Before pooling, the previous cohort were compared with the new cohort and found compatible with no differences in the investigated parameters (see results).

Mixed meal tolerance tests

At each visit, a liquid mixed-meal tolerance test (MMTT) was performed in the morning following a minimum of 8 hours fast. Participants were instructed to withhold morning insulin (both bolus and basal insulin) if treated with multiple daily injections (MDI), or to withhold bolus insulin during the night and pause basal insulin infusion 1 hour before the test if treated with continuous subcutaneous insulin infusion (CSII). The insulin restrictions were based on guidelines from Immunology of Diabetes Society regarding MMTT, however, the one hour pause in basal insulin in CSII treated

individuals was included due to unknown influence of exogenous insulin on bone turnover markers.²¹ Prior to the MMTT participants had capillary measurements (Bayer Contour XT) of fasting blood glucose (fBG (mmol/mol)) and HbA1c (presented in both mmol/L and DCCT %) using a high-pressure liquid chromatographic method (Tosoh Bioscience, South San Francisco, CA, USA). The MMTT was only performed if fBG was below 12.0 mmol/mol. Otherwise the visit was rescheduled.

Fasting blood tests were drawn from the cubital vein before the MMTT. Stimulated C-peptide was measured 90 minutes after ingestion of the mixed meal to quantify endogenous insulin production in alignment with previous studies.^{22,23} Initially, participants were stimulated using Boost High Protein (6 mL/kg, max 360 mL, Nestlé Health Science, Bridgewater, NJ, USA; 100 mL 14.9 g carbohydrates, 6.3 g protein and 2.5 g fat, 101 kcal). Due to import regulations, the mixed meal had to be changed to Ensure Plus (6 mL/kg, max 360 mL, Abbott Nutrition, Maidenhead, Berks, United Kingdom; 100 mL contains 20.2 g carbohydrates, 6.25 g protein, and 4.92 g fat, 150 kcal). Since Ensure Plus contained more carbohydrates, the increases in blood sugar and stimulated C-peptide levels could be expected to be greater. However, as reported in the results section, the two types of mixed meal showed no differences in the outcome measurements.

Partial remission was defined by both insulin production (stimulated C-peptide >300 pmol/L) and IDAA1c. IDAA1c was calculated as HbA1c (in %) + 4 times the total daily insulin dose (TDD) in IE/kg/24 hours. A calculated IDAA1 < 9 is a validated surrogate marker of partial remission, however, IDAA1c reflects both insulin production and insulin sensitivity.^{20,24}

Biochemical evaluation

Due to postprandial suppression of especially bone resorption, fasting plasma was used for analysis of bone turnover markers.^{25,26} Aliquots were kept frozen at -80 degrees Celsius until analyzed and none had previously been thawed. C-terminal cross-linked telopeptide of type-1 collagen (CTX), a marker of osteoclast activity and bone resorption, was measured using the IDS-iSYS CTX (CrossLaps®) assay (Immunodiagnostic Systems, plc, Tyne and Wear, UK). OCN and procollagen

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type-1 amino-terminal propeptide (P1NP), both products of osteoblast activity, were analyzed as markers of bone formation. P1NP was measured using the IDS-iSYS intact P1NP assay (Immunodiagnostic Systems) and OCN was measured using the N-Mid Osteocalcin assay (Immunodiagnostic Systems). An automated analyzer, iSYS (Immunodiagnostic Systems) was used for all assays according to the manufacturer's instructions. All assays were chemiluminescence immunoassays and control specimens made available by the manufacturer verified the assays' performance. The intermediary precisions expressed as coefficients of variation for CTX were 5.3% (at CTX concentration 213 ng/L), 3.4% (869 ng/L), and 3.5% (2,113 ng/L) for iSYS. For P1NP the intermediary precisions were 5.4% (18.96 µg/L), 6.5% (48.48 µg/L), and 6.1% (122.10 µg/L) for iSYS. For osteocalcin the intermediary precisions were 3.0% (8.73 µg/L), 3.6% (27.6 µg/L), and 3.5% (68.7 µg/L). All results were converted into Z-scores based on a large, new Danish reference material covering ages 7.7 to 17.5 years.²⁷ The balance between formation and resorption was evaluated by OCN/CTX and P1NP/CTX ratios, also converted into Z-scores.

The remaining blood was shortly refrigerated at 4°C until analyzed by the hospital's clinical biochemical department. C-peptide was analyzed using Immulite® 2000, chemiluminescent immunometric assay. Results were reported in pmol/L with a reportable range of 33-6.620 pmol/L. Measurements <33 pmol/L were registered as being 0 pmol/L.

Puberty and Anthropometric measurements

At each visit a physician examined whether the participants had entered puberty by use of the Tanner criteria according to development of breasts for girls and genital development and testicular size for boys.^{28,29} Pubertal stages were then categorized as prepubertal (Tanner 1) or pubertal (Tanner 2-5). Height was measured by use of a stadiometer to the nearest mm. Using a digital scale, participants in light clothes and without shoes were weighed to the nearest 0.1 kg and body mass index (BMI) was calculated as weight divided by height squared (kg/m²).

Dual-energy X-ray absorptiometry scans

From April 2016 examination of BMD was included at inclusion and visit 2. Participants above the age of 5 years had whole body BMD evaluated by a Dual-energy X-ray absorptiometry (DXA) scanner (GE lunar iDXA, GE Healthcare Technologies, Madison, Wisconsin, US; software version 16). The DXA scans were performed with the participants in light clothes not containing metal, and radiation dose was limited to 3.0 μ Gy. Because of the subjects being children and adolescents in different maturation stages, BMD was evaluated with minimized influence of the head (total body less head), as recommended by the International society of Clinical Densitometry.³⁰ If metal objects, such as glucose monitors, could not be removed, the objects were point typed as artifacts, making sure they did not falsely influence the BMD. Following a standardized procedure, according to the instructions of the manufacturer, the DXA scanners were restarted and quality tested every morning without any significant shifts or jumps in the quality assessments.

Statistics

Height, weight and BMI are presented as Z-scores using a large Danish reference material.³¹

Similarly, BMD results from the DXA scan are presented as Z-scores using an unpublished pediatric reference material incorporated in the scanner software (GE Healthcare Technologies).

Due to non-normal distribution, C-peptide was logarithmically transformed and will be presented as log C-peptide. Descriptive statistics are presented as mean \pm standard deviation (SD) for continuous variables and frequencies and proportions for categorical variables.

At each visit, between-group comparisons (sex, treatment, diet) were done using t-tests. Deviation in Z-scores were used for comparison with the general population mean (Z-score = 0).

Data from multiple visits were modeled by linear mixed effects regression to account for the repeated measurements on the same individuals. Subject-specific slopes were estimated by prediction of the random effects and used in multiple linear regression in a two-step procedure.

Models with BMD Z-score as dependent variable, were adjusted for HbA1c, age, height- and weight Z-score as numerical variables, according to our previous findings¹⁰.

P-values < 0.05 were considered statistically significant.

Ethical approval and consent:

The study was approved by the local Ethics Committee (H-3-2014-052). Participants consented to participation and as all participants were younger than 18 years upon inclusion, the legal guardians (parents) gave their written consent. All could withdraw their consent at any point during the trial.

RESULTS:

Homogeneity of the cohort

Comparing the two pooled cohorts showed that at the inclusion visit, participants in the previous cohort were significantly younger (Age: 9.5 vs 11.4 years; $P=0.039$) and received significantly less insulin (TDD: 0.38 vs 0.55; $P=0.026$) than the primary cohort (**Table 1**). Regression analysis demonstrated that adjusted for the age difference, TDD was no longer significantly different between the two cohorts ($P=0.096$). After the 12 months (at visit 2) only the difference in age remained significantly different between the two cohorts ($P=0.007$). Participants completing intentional gluten-free diet had a significantly lower BMI Z-score at the inclusion visit ($P=0.045$). No differences were found in BMI z-score or any other variables (including bone turnover markers, stimulated C-peptide and IDAA1c) at visit 1 and visit 2 (data not shown).

Participant characteristics

A total of 99 patients were included in the cohort (33% girls). The average time from diagnosis until the inclusion visit was 2.4 ± 0.7 months. The average time between inclusion and visit 1 was 5.9 ± 0.9 months and 11.8 ± 0.8 months between inclusion and visit 2. At visit 2 the average T1D duration was 14.2 ± 1.1 months.

Not all participants completed the follow-up. Four participants (4%) dropped out before visit 1 and further, 11 patients (11%) dropped out before visit 2. Participants leaving the study after the inclusion visit had significantly higher HbA1c ($P=0.033$), higher TDD ($P=0.011$), resulting in higher IDAA1c ($P<0.001$) at the inclusion visit compared to those continuing in the study. Similarly, those who dropped out after visit 1 had significantly higher HbA1c ($P=0.004$) and IDAA1c ($P=0.002$) at visit 1 compared to those completing in the study.

Participant characteristics from the inclusion visit, are presented in **Table 1**. Participants had a mean age of 11.0 ± 3.6 years and a mean HbA1c of 54.1 ± 11.9 mmol/mol (DCCT: $7.1\% \pm 1.1$). No differences were seen between sexes. Four participants (4%) were treated with CSII at inclusion and the remaining 95 participants (96%) were treated with MDI. At visit 2, the number of CSII treated participants had increased to 30 (36%). There were no differences in log C-peptide, IDAA1c or any of the bone turnover markers between MDI- and CSII treated participants at visit 2.

Throughout the study, weight- and BMI Z-scores increased from inclusion to both visit 1 ($P=0.009$ and $P=0.016$, respectively) and from inclusion to visit 2 ($P<0.001$ for both). Height Z-score did not change during the study. At none of the three visits, height-, weight- and BMI Z-scores were significantly different between sexes or significantly different from healthy peers (data not shown).

The course of the remission phase

Partial remission, as defined by stimulated C-peptide above 300 pmol/L, was present in 80 (81%), 60 (63%) and 38 (45%) participants at inclusion, visit 1 and visit 2, respectively. Defined by an IDAA1c ($IDAA1c < 9$) partial remission was present in 53 (54%), 44 (46%) and 28 (33%) of participants at inclusion, visit 1 and visit 2, respectively.

Those in partial remission (defined by C-peptide) were significantly older at both visit 1 ($P<0.001$) and visit 2 ($P<0.001$) and had lower HbA1c ($P=0.041$ and $P<0.001$ at visit 1 and 2, respectively), compared with those not in partial remission. Defined by IDAA1c there were no difference in age between the two groups at either visit 1 or visit 2.

The decline in C-peptide production

Log C-peptide decreased during the remission phase and was significantly lower at visit 1 compared with the inclusion visit ($P<0.001$) and lower at visit 2 compared with visit 1 ($P<0.001$) (**Figure 2^a**). A linear estimation of the decline in log C-peptide had a mean slope of -0.058 (95% CI: $-0.065 - -0.050$) corresponding to a 6% monthly decrease in C-peptide. There were no differences in the average decline of log C-peptide between sexes whereas age was significantly associated to the slope of log C-peptide ($P<0.001$) indicating that older participants experienced a slower decline in endogenous insulin production.

Due to the different amounts of carbohydrates in the two types of mixed meals used in the study, we compared log C-peptide outcome between individuals receiving the different types. Log C-peptide stimulated with Ensure Plus had a tendency toward higher levels at each visit, however, the differences were not significant, nor were there any differences in log C-peptide between visits when the type of MMTT was included into the mixed models ($P=0.118$). Finally, no difference in bone turnover markers, IDAA1c or any other variables could be demonstrated between MMTT types at any visit (data not shown).

IDAA1c and HbA1c during the remission phase

The average IDAA1c did not change between inclusion and visit 1 ($P=0.884$), but IDAA1c at visit 2 was significantly higher than both at inclusion ($P=0.010$) and at visit 1 ($P=0.014$) (**Figure 2^b**). Again, no differences between sexes were observed ($P=0.319$). An approximated linear increase in IDAA1c (from inclusion to visit 2) had a monthly increase of 0.068 (95% CI: $0.049 - 0.088$). The individual slopes for log C-peptide and IDAA1c were not significantly associated ($P=0.579$). Age was not significantly associated to the slope of IDAA1c increase ($P=0.404$).

From inclusion and until visit 1, the average HbA1c decreased significantly ($P<0.001$). Although the change in HbA1c between visit 1 and visit 2 was not significant ($P=0.136$), the mean HbA1c at visit 2 was no longer significantly different from that at inclusion ($P=0.096$) (**Figure 2^c**). The mean TDD increased significantly between inclusion and visit 1 (0.10 IU/kg; $P<0.001$) and between visit 1 and visit 2 (0.06 IU/kg; $P=0.040$). TDD was not different between sexes ($P=0.841$).

Bone turnover markers

Measurements of bone turnover markers and BMD are presented in **Table 2**. The average OCN- and P1NP Z-scores were significantly decreased compared with the reference population in all three visits. Oppositely, the average CTX Z-score was significantly increased in all three visits.

CTX- and P1NP Z-scores increased significantly between inclusion and visit 1, as was seen with HbA1c (**Figure 2^{d-h}**). The changes in HbA1c during the first 6 months did not associate to the simultaneous changes in P1NP Z-score ($P=0.071$) or CTX Z-score ($P=0.222$). There were no independent influences of sex or pubertal status on the changes in bone turnover markers.

Bone turnover markers and stimulated C-peptide

There were no associations between the individual slopes of log C-peptide decline and the individual slopes of any of the bone turnover markers.

At the inclusion visit, multiple regression analyses showed that log C-peptide associated positively to OCN Z-score (0.16 (95% CI: 0.04 – 0.29); $P=0.013$) and the OCN/CTX Z-score (0.13 (95% CI: 0.00 – 0.27); $P=0.043$). As age was significantly associated with the log C-peptide decline, age was added to the regression analyses and log C-peptide no longer associated to OCN (0.11 (95% CI: -0.01 – 0.23); $P=0.080$) or OCN/CTX Z-score (0.08 (95% CI: -0.05 – 0.21); $P=0.242$), or any other variables at any visit.

No differences could be demonstrated for any bone turnover marker between those in partial remission defined no matter the definition used.

Bone turnover markers and IDAA1c

At the inclusion visit both bone formation markers associated positively to IDAA1c; OCN Z-score (0.49 (95% CI: 0.08-0.91); $P=0.021$) and P1NP Z-score (0.42 (95% CI: 0.06-0.77); $P=0.022$).

However, these associations were no longer significant after adjustment for age. No associations were found to CTX Z-score or the formation/resorption ratios at the individual visits.

The change in CTX Z-score (slope) associated negatively to the slope of IDAA1c (-0.496 (95% CI: -0.946 – -0.050); $P=0.030$) and remained significant after adjustment for age ($P=0.029$) and C-peptide decline ($P=0.034$). This finding indicated that larger increases in CTX Z-score during the 12 months associated to smaller increases in IDAA1c. None of the bone formation markers or the ratios associated to the slope of IDAA1c.

Bone turnover markers and bone mineral density

The average BMD Z-score did not change between inclusion and visit 2 ($P=0.649$) (**Figure 2i**).

However, at visit 2, the BMD Z-score was significantly increased compared with the reference population ($P=0.035$), which was not the case at inclusion. No associations were found between the individual changes in BMD Z-score and the individual changes in any of the bone turnover markers.

Multiple regression analyses were again performed for each visit, and after adjusting for HbA1c, age and height- and weight Z-score, the only bone turnover marker to associate with the BMD Z-score was P1NP/CTX Z-score at inclusion (-0.15 (95% CI: -0.29 – -0.00); $P=0.048$) and again at visit 2 (-0.20 (95% CI: -0.33 – -0.06); $P=0.005$).

DISCUSSION:

During the first 14 months after T1D onset markers of bone formation were decreased whereas the bone resorption marker was increased. Despite these indications of altered bone turnover, BMD was not decreased, nor did it change during the 12 months study. The bone turnover markers showed no associations to stimulated log C-peptide. However, there was a slower increase in IDAA1c with increasing bone resorption.

Most of the previous studies on bone turnover markers in T1D have been performed on patients with long lasting diabetes, and most often adults.^{8,13} This is, to our knowledge, the first investigation of bone turnover markers in relation to stimulated C-peptide and IDAA1c during the first year after T1D onset in children and adolescents.

Previously, OCN has been examined close to onset of T1D in just three smaller prospective studies including a maximum of 31 children and adolescents.¹⁴⁻¹⁶ In all three studies OCN was decreased within 11 days of onset and in all studies, OCN returned to values no different from the control groups. Two studies showed normalization of the OCN values within 15 days of insulin treatment, concluding that the decreases were due to insulinopenia and metabolic imbalance before initiation of insulin.^{15,16} The last study, following 17 individuals for 12 months, found that 3 and 12 months after onset of T1D, OCN was no different from the controls.¹⁴ In our study, OCN Z-scores remained decreased during the entire trial, however, bone turnover was not evaluated as close to T1D onset as in the other studies. No studies of children and adolescents with recent onset T1D have previously evaluated P1NP. The decreases found in OCN- and P1NP Z-scores were in alignment with previous findings in children and adolescents with long-standing T1D.^{12,13}

In the current study CTX Z-scores were increased through all three visits. This finding is supported by a previous cross-sectional study which demonstrated significantly increased CTX in 27 children and adolescents with T1D for less than one year, compared with both healthy controls and participants with T1D for more than one year.³² Other studies have found CTX to be decreased at onset of T1D, but equal to controls after 3 and 12 months whereas another study found CTX levels to be comparable to controls at onset of T1D, but decreased after 3, 6 and 12 months of T1D.^{14,33}

Importantly, the last study measured CTX-MMP, a specific subgroup of CTX, produced only from enzymatic activity of matrix metalloproteinase, making direct comparison difficult.³⁴

Interestingly, the CTX Z-score was found to be increased throughout the 12 months trial, and not decreased as demonstrated in long-standing T1D¹². The cause of this difference remains unknown.

However, an interesting theory has been proposed by Xuan et al. who found that in women with normal glucose tolerance, higher HbA1c associated to higher CTX levels.³⁵ Furthermore, the study demonstrated that CTX and OCN levels were higher in women with impaired glucose regulation compared with participants with normoglycemia or type 2 diabetes (T2D).³⁵ Xuan et al.

hypothesized, based on previously reported positive associations between CTX and insulin sensitivity, that the increase in CTX could be a compensatory mechanism to improve glucose homeostasis.^{35,36} The research group further hypothesized that exhaustion of these compensatory mechanisms could be an important cause of the increases in glucose levels leading to T2D.^{35,37}

This theory supports our findings of increased CTX Z-score during the remission phase of T1D.

Hypothetically, as CTX Z-score associated to IDAA1c decline and not C-peptide, the increased CTX Z-score may result in increased insulin sensitivity as a compensatory response to the declining endogenous insulin production. The hypothesis of a CTX mediated compensatory mechanism, was further supported by a 4-year follow-up showing that normo-glycemic individuals with the highest tertile of CTX at baseline had the highest prevalence of dysglycemia 4 years later (46.5%).³⁷

Finally, one study including adult men demonstrated significantly reduced odds of prevalent T2D for each standard deviation increase in CTX or undercarboxylated OCN.³⁸

The regulatory effects of osteoclast activity in the hormonal role of bone have been investigated in mice. One study examined both loss of function (osteoblast ablation) and gain of function (osteoprotegerin knock out) models of osteoclast activity.³⁹ In the mice with increased osteoclast activity higher levels of undercarboxylated OCN was observed, whereas the carboxylated form, secreted by osteoblasts, were not affected. The mice with increased osteoclast activity demonstrated higher glucose tolerance, higher insulin sensitivity but no changes in serum insulin or beta-cell mass.³⁹ The opposite, low glucose tolerance, low insulin sensitivity and low undercarboxylated OCN (without changes in beta-cells or osteoblast function), was seen in mice with diminished

osteoclast function.³⁹ Although the changes in these mouse models were induced, which potentially lead to other unreported side-effects, the models demonstrated an important role of bone resorption in regulation of glucose tolerance and insulin sensitivity, possibly through regulation of the degree of carboxylation of OCN.^{3,4,39} Some of the OCN deposited in the bone matrix during bone formation is released into the blood stream as undercarboxylated OCN during bone resorption.⁴⁰ The increased CTX Z-score (a marker of bone resorption) in our study, might be an indication of a shift towards a higher percentage of total OCN in the blood being undercarboxylated OCN. Unfortunately, we are not able to distinguish between the two forms of OCN in our results. Previous studies have demonstrated that individuals with HbA1c below 58 mmol/mol (DCCT: 7.5%) had significantly higher undercarboxylated OCN, but not carboxylated OCN, compared with those with higher HbA1c.⁴¹ Another study found undercarboxylated OCN to negatively associate to disease duration, which could support the theory of compensatory efforts burning out.⁴² The current study did not demonstrate any differences in bone turnover markers between those in and out of partial remission at any visit regardless of the definition of partial remission. Stimulated C-peptide as a definition of partial remission is solely a measurement of preserved endogenous insulin production, whereas IDAA1C accounts for the achieved metabolic control (HbA1c) in relation to the exogenous insulin administered (TDD). The IDAA1C outcome is not able to discriminate between the beneficial effects of endogenous insulin production and insulin sensitivity. The two measurements (C-peptide and IDAA1c) correlate well to one another as documented in several studies, however, there is a fraction of pubertal participants where the two measurements are not aligned.^{20,24} In teenagers there is a natural increase in insulin resistance, which can result in high IDAA1c (due to high insulin needs to reduce HbA1c) despite high levels of C-peptide.^{43,44} Interestingly, in our study, the slope of CTX Z-score associated inversely to the slope of IDAA1c after adjustment for age and C-peptide decline. These findings suggest that whereas higher CTX does not associate to prolonged C-peptide production, CTX associates to a slower IDAA1c increase, potentially by associations to improved insulin sensitivity, optimizing the effect of the total insulin (exogenous and endogenous) available.

Decreased markers of bone formation together with an increased marker of bone resorption implies a relative increase in bone resorption, supported by the significantly decreased OCN/CTX- and P1NP/CTX Z-scores. With relatively higher bone resorption than bone formation, a decline in BMD could be expected. In our study BMD did not change significantly during the trial, implying that BMD increased in a similar pace as in healthy children and adolescents. During the 12 months trial, the participants increased in both weight- and BMI Z-scores, indicating a higher weight gain than what could be explained by natural growth as seen in healthy peers. Higher weight (and BMI) are well known stimulators of BMD and could explain the BMD Z-score being higher than references only at visit 2.^{45,46}

The finding of a BMD Z-score no different from healthy peers at inclusion is supported by other studies demonstrating that in children and adolescents with recent onset T1D, BMD is not different from healthy peers.^{52,53}

Regression analysis demonstrated a negative association between P1NP/CTX Z-score and BMD Z-score at both inclusion and visit 2. The negative association implies that with relatively higher bone formation than bone resorption, BMD Z-score decrease. A likely explanation of this interesting finding has been presented in previous publications, demonstrating negative association between bone formation markers and BMD in healthy girls, attributed to changes in estradiol levels during puberty.⁴⁷⁻⁴⁹ Finally, P1NP and CTX are only markers and does not represent total bone formation and resorption, respectively. None of the other bone turnover markers associated to BMD Z-score as demonstrated previously in children and adolescents with T1D.^{50,51} Changes in bone turnover only very slowly affects BMD and longer follow-up studies are needed as meta-analysis have demonstrated that BMD is already decreased in children and adolescents younger than 20 years of age with T1D.⁷

Besides being larger than previous studies our study has several strengths. The most important strength is the unique use of Z-scores for bone turnover markers. The use of Z-scores allow us to separate changes due to T1D from changes naturally occurring in children and adolescents during one year of growth (maturation). However, the use of Z-scores also poses some limitations. The references used for bone turnover markers Z-scores in our study, only covered children and

adolescents aged 7.7-17.5 years of age, excluding some of the available bone turnover measurements. Similarly, the reference material for the DXA scans did not cover children younger than 5 years of age. A large national reference material covering both bone turnover markers and BMD would be a significant improvement of future studies.

Although the MMTT had to be changed during trial, differences in stimulated C-peptide or any other variable could not be demonstrated. We strongly believe that the exchange has not affected our results. The same is true for the pooling of the two cohorts. Since the two original cohorts were collected at the same out-patient clinic, in similar research setups, and none of the out-come variables differed between the two cohorts (or those following thru with the gluten-free diet), we are convinced that the validity of our results has only benefitted from the larger study group.

A final limitation to our study is the information lost to drop-out. Even though the numbers were small, the findings of lower stimulated C-peptide and higher HbA1c at the visit before drop-out demonstrate a selection towards more optimal treated participants with higher production of C-peptide. This selection could contribute to the higher percentages of participants in partial remission at visit 1 and visit 2 compared with previous reports.¹ However, in comprehensive observational studies drop-outs are expected, especially in studies including participants with recent life-changing diagnoses such as T1D.

In conclusion, by use of Z-scores in children and adolescents with T1D, we demonstrate that already within three months of T1D diagnosis, bone formation markers are decreased whereas the bone resorption marker is increased. These alterations continue beyond the first year of insulin treatment. Despite the findings of bone turnover markers favoring bone resorption, BMD Z-scores were not negatively affected. Although a direct association between OCN and the course of the remission phase could not be demonstrated, indications of a potential role of bone turnover in T1D remission remain interesting. Compensatory mechanisms might be present, as suggested by higher CTX Z-scores and the association between CTX Z-score and IDAA1c increase, however, further research is needed in the subject.

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Tables

Table 1: Participant characteristics

	All participants	Difference from healthy peers	Primary cohort	Previous cohort	Difference between cohorts
Number (%)	99 (100%)		79 (80%)	20 (20%)	
Age (years)	11.0 ±3.6		11.4 ±3.5	9.5 ±4.0	P=0.039
HbA1c (mmol/mol)	54.1 ±11.9		53.3 ±12.0	57.2 ±11.5	P=0.186
HbA1c (DCCT %)	7.1 ±1.1		7.0 ±1.1	7.4 ±1.1	P=0.186
Hba1c at T1D onset (mmol/mol)	101.5 ±23.3		103.1 ±23.2	95.7 ±23.4	P=0.211
Hba1c at T1D onset (%)	11.4 ±2.1		11.5 ±2.1	10.9 ±2.1	P=0.211
TDD (IE/kg/day)	0.5 ±0.3		0.6 ±0.3	0.4 ±0.4	P=0.026
Weight Z-score	-0.06 ±0.95	P=0.519	0.01 ±0.97	-0.35 ±0.85	P=0.136
Height Z-score	0.06 ±0.90	P=0.512	0.09 ±0.90	-0.06 ±0.90	P=0.516
BMI Z-score	-0.14 ±0.98	P=0.148	-0.06 ±1.00	-0.46 ±0.88	P=0.108

Table 1: Characteristics of participants at the inclusion visit and comparisons between the pooled cohorts. Results are presented as means with standard deviation (±SD) or number with percentage (%). TDD= Total daily insulin dose. HbA1c at T1D onset was measured upon hospitalization where insulin treatment was initiated. Z-scores are compared to the population mean to test for difference in the T1D population. P-values < 0.050 are considered significant.

Table 2: Bone turnover markers and bone mineral density divided by visit and compared with the reference population.

Variable	Inclusion			Visit 1			Visit 2		
	Mean	N (%)	P-value	Mean	N (%)	P-value	Mean	N (%)	P-value
BMD Z-score	0.18 ±0.79	40 (40%)	P=0.158				0.24 ±0.87	63 (75%)	P=0.035
OCN Z-score	-0.65 ±0.91	75 (76%)	P<0.001	-0.59 ±0.98	75 (79%)	P<0.001	-0.63 ±1.09	67 (80%)	P<0.001
CTX Z-score	-0.54 ±1.07	75 (76%)	P<0.001	-0.32 ±0.97	75 (79%)	P=0.005	-0.49 ±0.99	67 (80%)	P<0.001
CTX Z-score	0.37 ±0.95	75 (76%)	P<0.001	0.66 ±1.05	75 (79%)	P<0.001	0.41 ±1.08	67 (80%)	P=0.003
OCN/CTX Z-score	-1.12 ±0.91	75 (76%)	P<0.001	-1.28 ±0.96	75 (79%)	P<0.001	-1.13 ±0.97	67 (80%)	P<0.001
OCN/CTX Z-score	-1.04 ±1.23	75 (76%)	P<0.001	-1.04 ±1.15	75 (79%)	P<0.001	-1.08 ±1.38	67 (80%)	P<0.001

Table 2: characteristics of Z-score for bone turnover markers and bone mineral density (BMD) at the three study visits. Results are presented as means with standard deviation (±SD) and percentage of investigated participants (%). Z-scores are compared to the population mean to test for difference in the T1D population. P-values < 0.050 are considered significant.

Figure legends

Figure 1: Recruitment process for the study cohort

Figure 1: Flowchart of inclusion and merging with the previous cohort.

Figure 2: Changes in bone turnover markers during the 12 months study period

*Figure 2: Changes in mean values (with 95% CI) during the three visits; visit 0 (inclusion), visit 1 and visit 2. OCN = Osteocalcin, P1NP = Procollagen type-1 amino-terminal propeptide; CTX = C-terminal cross-linked telopeptide of type-1 collagen; BMD = bone mineral density * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$*



