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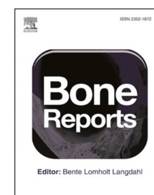

Bone marrow adiposity inversely correlates with bone turnover in pediatric renal osteodystrophy

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ABSTRACT

Bone marrow adiposity is associated with bone disease in the general population. Although chronic kidney disease (CKD) is associated with increased bone fragility, the correlation between marrow adiposity and bone health in CKD is unknown. We evaluated the relationship between bone marrow adipocytes and bone histomorphometry in 32 pediatric patients. We also evaluated the effects of growth hormone and calcitriol (1,25 $(OH)_2D_3$)—two therapies commonly prescribed for pediatric bone disease—on marrow adiposity and bone histomorphometry. Finally, the adipogenic potential of primary human osteoblasts from CKD patients was assessed *in vitro*, both alone and in the presence of 1,25$(OH)_2D_3$. In cross-sectional analysis, marrow adipocyte number per tissue area (Adi.N/T.Ar) correlated with bone formation rate/bone surface (BFR/BS) in patients with high bone turnover ($r = -0.55$, $p = 0.01$) but not in those with low/normal bone turnover. Changes in bone formation rate correlated with changes Adi.N/T.Ar on repeat bone biopsy($r = -0.48$, $p = 0.02$). *In vitro*, CKD and control osteoblasts had a similar propensity to transition into an adipocyte-like phenotype; 1,25$(OH)_2D_3$ had very little effect on this propensity. In conclusion, marrow adiposity correlates inversely with bone turnover in pediatric patients with high turnover renal osteodystrophy. The range of adiposity observed in pediatric patients with low/normal bone turnover is not explained by intrinsic changes to precursor cells or by therapies but may reflect the effects of circulating factors on bone cell health in this population.

1. Introduction

Bone disease is a universal complication of end-stage kidney disease that is associated with increased fracture rates and boney deformities (Denburg et al., 2016). Increased bone turnover, driven largely by alterations in serum parathyroid hormone (PTH) levels, is a significant component of CKD-mediated bone disease (renal osteodystrophy) (Bakkaloglu et al., 2010). This particular aspect of renal osteodystrophy is controlled by the administration of activated vitamin D analogues, of which, calcitriol (1,25$(OH)_2D_3$) is most commonly used in pediatric CKD. However, despite normalization of bone turnover, skeletal morbidity, including growth failure, fractures and deformities, persist. Chronic exposure to the uremic milieu itself appears to cause cellular changes that affect bone health and epigenetic modifications have been identified in osteoblast precursors from CKD patients that interfere with their maturation and that associate with persistent bone disease in this population (Pereira et al., 2018).

Bone marrow adiposity has been associated with clinical bone disease in patients without kidney disease (Cohen et al., 2012; Justesen et al., 2001; Fazeli et al., 2012; Syed et al., 2008); human studies have shown that an increase in marrow adiposity is inversely associated with bone formation in senile osteoporosis, in growth hormone deficient men, and in obese women (Bredella et al., 2014; Verma et al., 2002; Bravenboer et al., 2005). Inverse correlations have also been identified between marrow adiposity and cortical bone mass in imaging studies (Wren et al., 2011). Adipocytes are derived from the same mesenchymal precursor as osteoblasts and the cellular changes that affect osteoblast precursors may also affect marrow adipocytes in CKD. Although the association between skeletal disease and marrow adiposity has not been evaluated in the CKD population, it is interesting to note that

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insensitivity to insulin—at least in peripheral fat stores—is a feature of CKD (Spoto et al., 2016). Such an insensitivity, if also present in marrow cells, might impair adipogenesis and contribute to bone disease in CKD patients.

Growth failure—due largely to growth plate resistance to the actions of growth hormone and IGF1—is a hallmark of CKD in children. The administration of supra-physiologic doses of recombinant human growth hormone overcomes some of this growth plate resistance and is the current standard of care for pediatric CKD patients with short stature (Haffner et al., 2000; Vimalachandra et al., 2006; Hokken-Koelega et al., 1991). In addition to its effects on the growth plate, growth hormone and the IGF1 axis have direct effects on mineralized bone, muscle mass, and energy utilization (Ivanovski et al., 2000). We have shown that recombinant growth hormone increases bone turnover in children with end-stage kidney disease (Bacchetta et al., 2013). While growth hormone response involves insulin signaling through IGF1, how (and if) growth hormone therapy affects marrow adiposity in CKD is unknown.

In order to evaluate the relationship between marrow adiposity, bone histology, and renal osteodystrophy therapies in the CKD population, we examined the relationship between numbers of marrow adipocytes and bone histomorphometric parameters of turnover and mineralization in pediatric dialysis patients *in vivo*. We also assessed the effects of $1,25(OH)_2D_3$ and growth hormone—two widely used therapies for pediatric renal osteodystrophy which affect bone cell biology and protein/cal utilization (Sinha et al., 2013; Ho et al., 1996)—on marrow adipocyte number and bone formation rate *in vivo* in these same patients. We also used an *in vitro* model of primary CKD osteoblasts to evaluate whether intrinsic maturation defects, embued by the uremic milieu, alter the adipogenic potential of primary osteoblasts alone or in the presence of $1,25(OH)_2D_3$.

2. Materials and methods

2.1. In vivo analysis of bone histology and marrow adipocyte numbers in pediatric patients with advanced CKD

2.1.1. Patients and bone biopsies

The bone biopsy material used in this analysis was obtained from a previous clinical trial which assessed the effects of recombinant human growth hormone on bone histology in pediatric dialysis (*i.e.* end-stage kidney disease) patients (Bacchetta et al., 2013). This previously performed clinical trial was conducted at the David Geffen School of Medicine at the University of California, Los Angeles between July 1994 and May 1999. As described, all pediatric patients treated with maintenance continuous cycling peritoneal dialysis were eligible for that trial. Exclusion criteria included non-adherence; parathyroidectomy within the preceding 12 months; a closed epiphyseal growth plate; and ongoing or recent immunosuppressant therapy. After a 4-week withdrawal period from vitamin D therapy, enrolled patients were admitted to the UCLA General Clinical Research Center and a baseline bone biopsy was obtained from the anterior iliac crest after double tetracycline labeling. Patients were then stratified according to bone formation rate as measured on bone histomorphometry (high turnover *versus* normal/low bone turnover) and randomized in a 1:1 allocation ratio to receive recombinant human growth hormone (0.05 μg/kg/day) plus standard of care *versus* standard of care alone. All patients were dialyzed with a 2.5 mEq/L calcium dialysate and received calcium-based binders for phosphate control. As per standard of care, all patients with high bone formation rates received $1,25(OH)_2D_3$ doses adjusted to maintain normal serum calcium and phosphorus concentrations (Bacchetta et al., 2013). A bone biopsy was repeated after 8 months of treatment. Informed consent was obtained from all individual participants in the study.

The current retrospective analysis used all bone biopsy specimens from this previous clinical trial which contained an intact marrow (cancellous) compartment and two cortices (Bacchetta et al., 2013).

2.1.2. Biochemical and bone histomorphometric measurements

Biochemical determinations of serum calcium, phosphorus, alkaline phosphatase, IGF1 and PTH were obtained at the time of bone biopsy. Serum calcium, phosphorus, alkaline phosphatase, IGF1 and parathyroid hormone (PTH) levels were measured by ELISA (IGF1: Diagnostic Systems Laboratories, Webster, TX; first-generation PTH Nichols assay: Nichols Institute Diagnostics, San Juan Capistrano, CA). Serum calcium, phosphorus, and alkaline phosphatase levels were measured using standard laboratory methods.

Bone quantitative histomorphometry was performed as previously described. Baseline and final bone histomorphometric variables for the trial have been previously reported (Bacchetta et al., 2013). In this manuscript, we report the changes in bone histomorphometric variables only in the samples used specifically for the current analyses. The terminology established by the Nomenclature Committee of the American Society for Bone and Mineral Research was used to report all histomorphometric variables (Parfitt et al., 1987).

2.1.3. Bone marrow adipocyte measurements

Adipocyte numbers were assessed in all samples by two trained observers (OS and RCP) who were blinded as to patient demographics and treatment. Five micron bone sections were prepared with Goldner stain and marrow adipocyte number (Ad.N) was counted under 100× magnification using the Osteomeasure^R analysis system (Osteometrics Inc., Decatur, IL). The entire trabecular area (6–8 fields) of each biopsy was measured; cortical areas were excluded. Fields with artifact were also excluded. All adipocytes greater than two millimeters in diameter were included in the analysis. Numbers of adipocytes (Ad.N) in each biopsy sample were normalized by total tissue area (T.Ar).

2.2. Characterization of the direct, independent, effects of CKD, $1,25(OH)_2D_3$, and IGF1 axis activation on marrow adipogenesis in vitro

2.2.1. Primary osteoblasts: an in vitro model of CKD-mediated human osteoblast dysfunction

Primary osteoblasts and adipocytes are members of the mesenchymal cell lineage and primary osteoblasts can be induced to differentiate into adipocytes upon activation of the insulin/IGF1 axis (James, 2013)—the same axis that is activated by recombinant human growth hormone treatment (Tonshoff et al., 1990). We thus used these primary cells to evaluate whether intrinsic defects which affect osteoblast maturation also contribute to altered marrow adipogenesis or to altered growth hormone response in CKD. We then treated these cells with varying concentrations of $1,25(OH)_2D_3$ to evaluate the effect of renal osteodystrophy therapy on osteoblast-adipocyte plasticity.

The primary human osteoblasts used in this study were obtained from trabecular bone of five pediatric patients with end-stage kidney disease who were treated with chronic cycling peritoneal dialysis, as previously described (Pereira et al., 2015). Separate isolates were obtained from 3 patients with low bone turnover, defined by a bone formation rate/bone surface below the normal range (<8 um^3/um^2/year) and decreased osteoblast numbers on bone histomorphometry and from two patients with high bone turnover, defined as a bone formation rate/bone surface greater than the normal range (>73.4 um^3/um^2/year). Isolates from healthy controls were obtained from trabecular bone of three individuals between the ages of 14 and 16 years whose characteristics have also been previously described (Pereira et al., 2015). The acquisition of bone cells and their subsequent use in *in vitro* studies were approved by the UCLA Institutional Review Board and consent/assent was obtained from all patients and their parents.

2.2.2. Transition of primary cells to an adipocyte-like phenotype

Primary osteoblasts were transitioned to an adipocyte-like phenotype in the presence of insulin (1 μM), dexamethasone (10^{-6} M), and isobutylmethylxanthine (0.5 mM) (standard adipogenic conditions). The effect of $1,25(OH)_2D_3$ in response to pro-adipogenic stimuli was

assessed in osteoblasts cultured under pro-adipogenic conditions with and without the addition of $1,25(OH)_2D_3$ (0, 1, 10 and 100 nM). After 2 weeks under pro-adipogenic conditions, cultures were evaluated for fat accumulation using Oil red O staining. Images of each plate were obtained and oil accumulation quantified first by Image J analysis of red staining. Each isolate was evaluated in quadruplicate; the coefficient of variation in values of Oil Red O staining between replicates by Image J analysis was between 5 and 18%. Next, Oil Red O dye was extracted from each isolate in 100% isopropanol and then quantified by absorbance at 525 nm. The coefficient of variation between technical replicates by extracted dye absorbance was 7–10%. The averages of the independent isolates and the standard errors of these values are reported.

2.2.3. RNA analysis

Total RNA was extracted from confluent osteoblasts using RNeasy (Qiagen) according to the manufacturer's instructions. Measurement of RNA yield was performed using a NanoDrop 1000A Spectrophotometer (Thermo Fisher Scientific). A reverse transcription reaction was performed for cDNA synthesis using High Capacity cDNA RT kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) amplification was performed using QuantiTect ® Probe PCR kit (Qiagen) to assess adipsin/complement factor D (*CFD*) (Hs00157263_m1), CCAAT/enhancer-binding protein alpha (*CEBPA*) (Hs00269972_S1), lipoprotein lipase (*LPL*) (Hs00173425_m1), runt-related transcription factor 1 (*RUNX*) (Hs00231692_m1), vitamin D receptor (*VDR*) (Hs01045840_m1), 25-hydroxyvitamin D3 1-alpha-hydroxylase (*CYP27B1*) (Hs00168017_m1), cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) (Hs00167999_m1) expression. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* was used as an endogenous control for data normalization. Samples were assayed in triplicate for these analyses and the coefficient of variation between technical replicates was less than or equal to 2%. Relative quantification studies of threshold cycle (Ct) were performed with Sequence Detector software (Applied Biosystems).

2.3. Statistical analysis

For both parts of the study, normally distributed data are presented as mean ± standard error (SE) and non-normally distributed values as the median (interquartile range, IQR). Between group differences were assessed using the independent sample *T*-Test or Mann-Whitney *U* test. Differences between baseline and final bone measures are reported as absolute values and as percent changes from baseline and statistically significant differences were identified using the paired *T*-Test or Mann-Whitney *U* Test. Spearman correlation coefficients were used to evaluate the association between marrow adipocytes and bone histomorphometry. Multivariable linear regression was performed to assess the relationship between changes in bone formation rate and changes in adipocytes over time, independent of treatment group. All statistical analyses were performed using SPSS version 23.0 (IBM Corp) and a two-sided alpha cut-off of 0.05 was used to determine statistical significance for all tests.

3. Results

3.1. In vivo effects of CKD, growth hormone, and $1,25(OH)_2D_3$ on bone histomorphometry and adipocyte numbers

3.1.1. Patients and bone biopsy characteristics

Of the 36 dialysis patients who underwent baseline bone biopsy analysis prior to entry into the trial (Bravenboer et al., 2005), bone turnover was high in 20 and normal/low in 16. Two baseline samples, both from patients with normal/low bone turnover, had marrows that were inadequate for adipocyte measurement. Thus, baseline adipocyte numbers were assessed 20 samples with high bone turnover and in 14 samples with normal/low bone turnover (Table 1). Three of these patients were lost to follow up during the study period, six terminated early due to kidney transplantation, and one post-treatment sample was inadequate for adipocyte measurement. Thus, samples from 24 patients (n = 14 with high baseline bone turnover and n = 10 with normal/low baseline turnover) were evaluated in paired, before-and-after treatment, analysis (Table 2). Of these 24 individuals, 12 had received treatment with recombinant human growth hormone (high baseline bone turnover: n = 6; normal/low baseline bone turnover: n = 6). The remainder (n = 12) had received standard of care, which, in the case of patients with high bone turnover, included $1,25(OH)_2D_3$, the dosing of which was previously described (Bravenboer et al., 2005).

3.1.2. Circulating concentrations of mineral ions and bone regulatory hormones

In the cross-sectional analysis of 34 baseline bone biopsies, median alkaline phosphatase and PTH levels were higher in patients with high bone turnover than in those with low/normal bone turnover (Table 1). A similar finding was observed in baseline biochemical of the 24 patients included in the pre-/post- treatment analysis (Table 2). As previously reported (Bravenboer et al., 2005), serum calcium and phosphorus values did not change over the course of treatment in patients receiving growth hormone or in patients receiving standard of care alone. Serum alkaline phosphatase and PTH levels increased in patients with underlying normal/low bone turnover who received recombinant human growth hormone.

Table 1
Patient characteristics and biochemical values: cross-section of patients with high and normal/low bone turnover.

	Baseline high bone turnover (N = 20)	Baseline normal/ low bone turnover (N = 14)	Normal range
Demographic data			
Age (yr) (median (IQ range)	12.5 (8.5, 14.8)	7.9 (4.9, 11.6)	NA
Gender			NA
-Female (N, %)	9, 45	8, 57	
Ethnicity (N, %)			NA
-White	4, 20.0	4, 28.6	
-Hispanic	13, 65.0	9, 64.3	
-Black	2, 10.0	-	
-Asian	1, 5.0	1, 7.1	
Duration of dialysis (yr) (median (IQ range)	0.4 (0.3, 0.7)	0.5 (0.3, 0.9)	NA
Z-score height (median (IQ range)	−1.9 (−2.9, −0.9)	−2.2 (−2.7, −1.2)	NA
Z-score weight (median (IQ range)	−1.7 (−2.2, −0.7)	−1.6 (−2.3, −0.6)	NA
Biochemical values			
Calcium (mg/dl) (mean ± SE)	9.3 ± 0.9	9.8 ± 0.5	8.6–10.4
Phosphorus (mg/dl) (mean ± SE)	5.8 ± 5.1	6.2 ± 1.8	3.6–5.8 (6 to 12 years) 2.3–4.5 (13–20 years)
Alkaline phosphatase (IU/L)[a] (median (IQ range)	374 (262, 628)	234 (175, 292)	60–450 (6–12 years) 40–180 (13–20 years)
PTH (pg/ml)[a] (median (IQ range))	722 (433, 945)	110 (93, 150)	10–65

PTH: parathyroid hormone; SE: standard error; N: number; yr: year; IQ: interquartile.

[a] Significant difference between patients with high turnover and normal/low turnover at baseline ($p < 0.05$).

Table 2
Patient characteristics and baseline biochemical values in patients included in the pre/post treatment assessment.

	High bone turnover (N = 14)	Normal/low bone turnover (N = 10)	Normal range
Demographic data			
Age (yr) (median (IQ range)	12.5 (10.7, 14.7)	8.8 (5.8, 11.3)	NA
Gender			NA
-Female (N, %)	5, 36	3, 30	
Ethnicity (N, %)			NA
-White	3, 21.4	3, 30	
-Hispanic	9, 64.3	6, 60	
-Black	2, 14.3	–	
-Asian	–	1, 10	
Duration of dialysis (yr) (median (IQ range)	0.4 (0.3, 0.7)	0.5 (0.3, 0.8)	NA
Z-score height (median (IQ range))	−1.8 (−2.9, −0.9)	−2.2 (−2.7, −1.2)	NA
Z-score weight (median (IQ range))	−1.7 (−2.2, −0.6)	−1.6 (−2.2, −0.6)	NA
Biochemical values			
Calcium (mg/dl) (mean ± SE)	9.2 ± 1.0	9.9 ± 0.5	8.6–10.4
Phosphorus (mg/dl) (mean ± SE)	5.5 ± 0.9	5.6 ± 1.7	3.6–5.8 (6 to 12 years) 2.3–4.5 (13–20 years)
Alkaline phosphatase (IU/L)[a] (median (IQ range))	372 (265, 702)	211 (132, 316)	60–450 (6–12 years) 40–180 (13–20 years)
PTH (pg/ml)[a] (median (IQ range)	626 (422, 935)	107 (88, 133)	10–65

PTH: parathyroid hormone; SE: standard error; N: number; yr: year; IQ: interquartile.
[a] Significant difference between patients with high turnover and normal/low turnover at baseline ($p < 0.05$).

3.1.3. *Bone histomorphometry and adipocyte number*

By definition, bone formation rate per bone surface (BFR/BS) was higher in patients with high bone turnover than in patients with normal/low bone turnover ($p \leq 0.001$). The median number of adipocytes per tissue area (Adi.N/T.Ar) did not differ between patients with high *versus* those with normal/low bone turnover at baseline. A correlation between Adi.N/T.Ar and BFR/BS was observed in patients with high bone turnover ($r = -0.55$, $p = 0.01$) although not in those with low/normal turnover at baseline (Fig. 1a). Because such a wide variation in adiposity was observed in the adynamic/normal bone turnover population at baseline, we examined the underlying biochemical and bone histomorphometric characteristics of patients with low/normal turnover with high (>200/T.Ar) numbers of adipocytes *versus* low (<200/T.r) adipocyte numbers. Neither PTH levels nor bone formation rate differed between these groups; however, alkaline phosphatase levels tended to be lower ($p = 0.144$) while osteoid maturation times were higher ($p = 0.034$) in patients with low turnover who had fewer marrow adipocytes (Supplementary Table).

Changes in bone histomorphometric parameters are shown in Table 3. As previously reported, bone formation increased from baseline in patients with normal/low bone turnover who received growth hormone (Bacchetta et al., 2013). A similar increase in bone formation was not observed in patients with high bone turnover who received concomitant treatment with both growth hormone and $1,25(OH)_2D_3$. In all patients included in the pre/post treatment adipocyte analysis, changes in BFR/BS tended to correlate with changes Adi.N/T.Ar ($r = -0.42$, $p = 0.052$) (Fig. 1b). In multivariable analysis, the change in BFR/BS predicted changes in Adi.N/T.Ar independent of whether

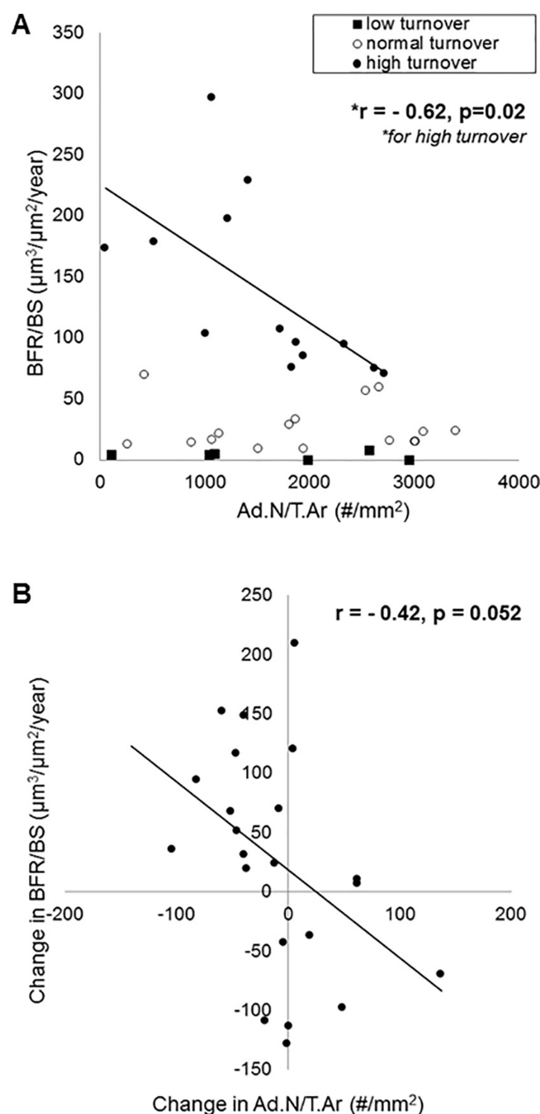


Fig. 1. Correlation between marrow adipocyte and bone formation (A) Ad.N/T.Ar and BFR/BS in patients at baseline. Patients with low bone turnover are indicated by the closed squares. Patients with normal bone turnover are indicated by the open circles. Patients with high bone turnover are indicated by the close circles. (B) Correlation between change in BFR/BS and change in marrow adipocytes (Ad.N/T.Ar).

patients were treated with $1,25(OH)_2D_3$ (Table 4a) and also independent of whether they received treatment with growth hormone (Table 4b). Despite the observed associations with bone formation rate, Adi.N/T.Ar did not correlate with osteoid accumulation (osteoid volume, thickness, or surface).

3.1.4. *Effects of CKD and $1,25(OH)_2$vitamin D_3 on primary osteoblast sensitivity to insulin-induced adipogenesis*

The primary cells used in this experiment were passaged cells that can be induced to differentiate into osteoblasts by culture in vitamin C and β glycerol-phosphate. When treated with a combination of insulin, dexamethasone, and isobutylmethylxanthine (IBMX), which are standard conditions for inducing the differentiation of marrow precursors into an adipocyte-like phenotype, fat accumulation (as defined by oil red O staining) could be detected in all isolates (Fig. 2). Phenotypic variation was observed between individual cell isolates cultured under these conditions, but no consistent difference was observed in oil red O staining between CKD and controls or between adipocyte-like cells from

Table 3
Changes in bone histomorphometry and marrow adiposity with rhGH and calcitriol treatment. Values are expressed as medians (IQ range).

Changes	High bone turnover		p-value between groups	Normal/low bone turnover		p-value between groups
	Calcitriol (N = 8)	Calcitriol + growth hormone (N = 6)		No growth hormone (N = 4)	Growth hormone (N = 6)	
Turnover						
Bone formation rate/bone surface ($\mu m^3/\mu m^2$/year)	−43.0 (−98.1, 67.8)*	10.6 (−118.7, 43.6)	0.81	31.7 (24.1, 69.9)	134.5 (16.1, 166.8)*	0.44
Mineralization						
Osteoid surface/bone surface (%)	4.9 (−13.3, 8.9)	−2.4 (−18.7, 6.4)	0.44	30.6 (3.7, 41.9)	19.7 (13.6, 28.2)	0.29
Osteoid volume /bone volume (%)	0.5 (−7.9, 3.9)	−0.4 (−5.5, 2.9)	0.69	9.1 (2.3, 13.9)	6.9 (3.0, 8.5)	0.39
Osteoid thickness (μm)	0.5 (−4.5, 5.2)	2.1 (−0.1, 5.7)	0.37	3.5 (−0.0, 8.0)	5.0 (1.5, 7.9)	0.83
Osteoid maturation time (d)	2.9 (−3.3, 8.6)	4.5 (−0.2, 8.2)	0.61	0.2 (−3741.9, 3.5)	2.5 (−4.2, 5.7)	0.39
Mineralization lag time (d)	31.5 (7.0, 85.9)*	−2.8 (−49.9, 10.8)	0.03	−142.6 (−3795.5, −16.1)	−53.7 (−100.3, −35.4)*	0.67
Volume						
Bone volume /tissue volume (%)	6.3 (−6.2, 8.6)	8.6 (2.6, 14.2)	0.30	1.1 (−6.6, 3.4)	0.5 (−1.8, 7.2)	0.83
Trabecular thickness (μm)	10.4 (−17.1, 34.1)	36.3 (−1.9, 64.5)	0.38	−1.1 (−27.5, 21.0)	20.8 (−18.5, 50.9)	0.39
Trabecular separation (μm)	−26.1 (−199.7, 78.5)	−98.7 (−321.4, −43.5)	0.11	−0.1 (162.0, 2.6)	36.9 (−113.4, 97.1)	1.00
Trabecular number (per mm^2)	−0.0 (−0.4, 0.5)	0.3 (−0.1, 0.7)	0.56	−0.0 (−0.3, 0.1)	−0.1 (−0.3, 0.3)	0.83
Adipocyte number/tissue area (Ad.N/T.Ar)						
Absolute change (#/mm^2)	10.2 (−39.5, 43.7)	−33.1 (−113.2, 15.3)	0.19	−25.5 (−44.4, −9.1)	−16.2 (−44.0, 19.9)	0.39
Percent change (%)	112.5	−25.9		−12.9	−14.1	

Values are expressed as median (IQ range). The p values represent the differences between GH treated and GH not-treated patients within each group (high *versus* normal-low turnover). The asterisk indicates a significant ($p < 0.05$) change from baseline.

Table 4
Multivariable prediction of delta Adi.N/T.Ar: bone biopsy analysis. Given the limited sample size (n = 24), two separate analyses were performed to avoid over-fitting. A) Relationship between change in BFR/BS and change in Adi.N/T.Ar controlling for the presence/absence of calcitriol therapy. B) Relationship between change in BFR/BS and change in Adi.N/T.Ar controlling for the presence/absence of growth hormone therapy.

A

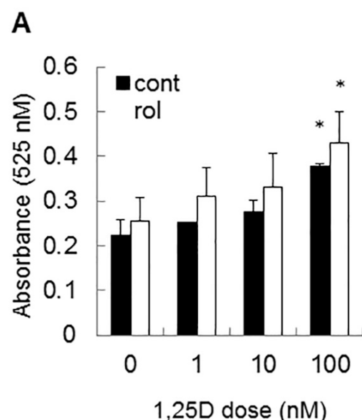
	Parameter estimate	Standard error	p-value
Change in BFR/BS	- 0.33	0.15	0.04
Calcitriol therapy (yes v. no)	25.23	27.86	0.38

B

	Parameter estimate	Standard error	p-value
Change in BFR/BS	- 0.24	0.12	0.06
Growth hormone therapy (yes v. no)	1.97	22.4	0.93

patients with high *versus* low bone turnover. To evaluate the effect of renal osteodystrophy therapy on osteoblast/adipocyte plasticity, the active vitamin D sterol 1,25(OH)$_2$vitamin D$_3$ (1,25(OH)$_2$D$_3$) was added to confluent cultures for two weeks. At high doses (100 nM), 1,25 (OH)$_2$D$_3$ increased lipid deposits by 49.9 ± 7.7% ($p < 0.01$ overall); a significant effect was not observed with lower concentrations, however. Consistent with the fat accumulation phenotype, expression of adipogenic markers could be detected in all cells. Importantly, expression of the early osteoblast marker *RUNX2* was undetectable in cells while *CEBPA*, a key regulator of adipogenesis, was present in both control and CKD adipocyte-like cells. Expression of *CEBPA* tended to increase with the highest dose (100 nM) of 1,25(OH)$_2$D$_3$ (Fig. 3a), as did expression of the adipocyte markers *LPL* (Fig. 3b), and *CFD* (Fig. 3c). Interestingly, *LPL* and *CFD* expression were higher in healthy control cells than in CKD cells at some doses of 1,25(OH)$_2$D$_3$ but these differences in gene expression did not associate with any phenotypic differences between control and CKD osteoblast isolates.

No differences in *VDR*, *CYP27B1, or CYP24A1* expression were observed between CKD and control osteoblasts cultured in the absence



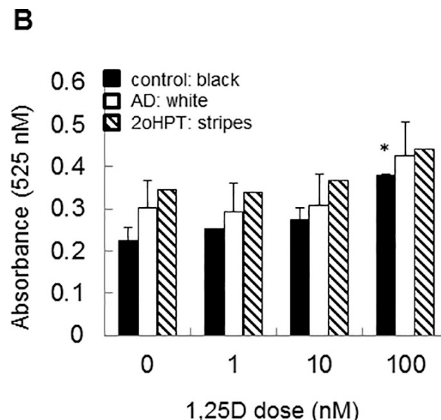


Fig. 2. 1,25(OH)$_2$vitamin D$_3$ increases fat accumulation in primary osteoblasts. (A) After 2 weeks under pro-adipogenic conditions, fat accumulation, as assessed by absorbance of extracted oil red O dye at 525 nm, was similar in healthy control cells (black bars) and in CKD cells (open bars). At high concentrations (100 nM), 1,25(OH)$_2$D$_3$ increased fat accumulation in all cells. (B) No differences were observed in fat accumulation or response to 1,25 (OH)$_2$D$_3$ in osteoblasts from dialysis patients with high turnover (white bars) *versus* low turnover (striped bars) cultured under pro-adipogenic conditions. The asterisk (*) indicates a difference ($p < 0.05$) from no-vitamin D treatment.

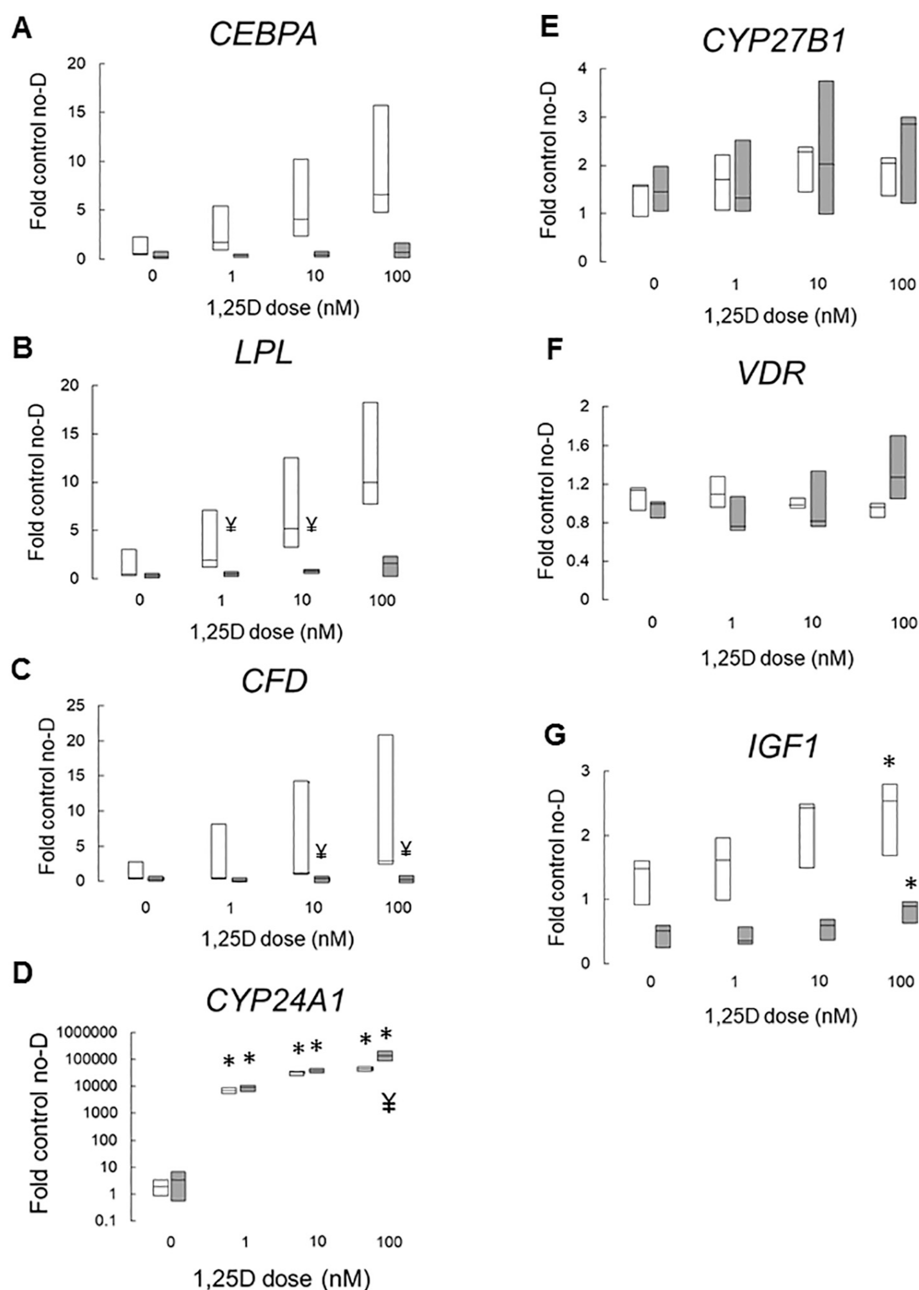


Fig. 3. Adipocyte markers (A) *CEBPA*; (B) *LPL*; and (C) *CFD* are expressed in primary osteoblasts transitioned to an adipocyte phenotype. D) *Cyp24A1* expression; (E) *Cyp27B1* expression; and (F) *VDR* expression are expressed in both CKD and control osteoblasts which have been transitioned to adipocytes. (G) *IGF1* expression increases in response to $1,25(OH)_2D_3$. Boxplots depict median and interquartile ranges. White boxes depict healthy controls; grey depict CKD patients. Gene expression is relative to un-treated normal controls. The asterisk (*) indicates a difference ($p < 0.05$) from no-vitamin D treatment. The double dagger (¥) indicates a difference between control and CKD osteoblasts at individual vitamin D doses.

of $1,25(OH)_2D_3$. Consistent with known actions of $1,25(OH)_2D_3$, *CYP24A1* expression increased even with low doses of $1,25(OH)_2D_3$ while *CYP27B1* and *VDR* expression remained unchanged (Fig. 3d, e, and f). *IGF1* expression was also similar between CKD and control cells. *IGF1* expression increased with the highest dose (100 nM) of $1,25(OH)_2D_3$ in control cells (Fig. 3g).

4. Discussion

In the current study, we found that bone formation rate, a measure of osteoblast activity, correlated inversely with adipocyte numbers in the bone marrow of pediatric patients with high turnover renal osteodystrophy. This inverse correlation affirms the reciprocal association between osteoblasts and adipocytes which has been previously reported in both animals and humans with normal kidney function (Gevers et al., 2002; Appiagyei-Dankah et al., 2003; Menagh et al., 2010). An increase in adipocyte parameters is inversely associated with bone formation in senile osteoporosis, in growth hormone deficient men, and in obese women (Bredella et al., 2014; Verma et al., 2002; Bravenboer et al., 2005). Moreover, inverse correlations have been identified between marrow adipocyte and cortical bone mass in imaging studies (Wren et al., 2011). Despite the observed associations between adipocyte number and bone formation rates in children with high turnover renal osteodystrophy, adipocyte numbers failed to correlate with osteoid accumulation in this same population. The reason behind this apparent paradox remains unclear, particularly as osteoblast surface might be expected to represent osteoblast number and function as well as bone formation rate. However, it is notable that a high prevalence of mineralization defects has been identified in pediatric patients with CKD, occurring in patients with low, normal, and high bone turnover alike (Bakkaloglu et al., 2010). It thus appears that matrix mineralization is to a large degree uncoupled from matrix deposition in the context of CKD (Bakkaloglu et al., 2010), and that osteoid accumulation may be a poorer marker of osteoblast number and activity in this population than

in individuals with normal kidney function. In addition, our previous reports have demonstrated that primary osteoblasts from CKD patients have delayed maturation along an osteoblast lineage (Pereira et al., 2018), suggesting that the presence of CKD induces intrinsic changes in bone cell maturation that impair osteogenesis. In the current study, we noted similarities in the ability of control and CKD osteoblast cursors to transition to an adipocyte-like phenotype, despite differences in adipocyte marker expression (particularly of *LPL*, but also in *CEBPA* and *CFD)*. Thus, the epigenetic changes that prevent effective osteogenesis appear to not as significantly affect adipogenesis in CKD bone.

Marrow adiposity was not consistently increased in pediatric patients with low turnover osteodystrophy. Many factors may contribute to low bone turnover in CKD patients and, indeed, low bone turnover is considered the underlying histology in animals and patients with CKD who do not have secondary hyperparathyroidism (Lund et al., 2004; Wesseling-Perry et al., 2012; Graciolli et al., 2017). In adults, diabetes mellitus contributes to much of the prevalence of low turnover renal osteodystrophy. Diabetes is much rarer in children and was not present in any of the patients in this study; however, other uremic toxins that "poison" bone cell function may contribute to adynamic bone disease in this population, as may high doses of calcium and vitamin D supplements (Kuizon et al., 1998). The similar adipogenic potential of osteoblasts from patients with high and low bone turnover osteodystrophy *in vitro* suggests that differences in adipocyte numbers *in vivo* are more related to factors in the circulating milieu than to any intrinsic changes induced by CKD. Although numbers became very small on sub-group analysis, the wide range of marrow adipocyte numbers in patients with low/normal bone turnover was associated with some serological and bone histomorphometric differences; those with lower adipocyte numbers tended to have higher alkaline phosphatase levels and faster mineralization times, suggesting more effective osteoblast function in this subgroup. Thus, although likely not a viable marker for prediction of underlying bone turnover by itself, assessment of marrow adiposity, in conjunction with assessment of bone turnover, may be an important indicator of underlying bone cell health. Whether the subgroup of patients with lower marrow adipocyte numbers might be less prone to issues associated with bone fragility is unknown but warrants further investigation.

While changes in bone formation rate associated with changes in marrow adipocyte number over time, treatment doses of $1,25(OH)_2D_3$ and recombinant growth hormone themselves appeared to have little effect on marrow adipocyte numbers *in vivo*, independent of their effects on bone formation. IGF1 is downstream of growth hormone, mediating its pro-growth effects *in vivo*, including stimulation of osteoblast proliferation and differentiation (Zhang et al., 2012). In the current study, osteoblast precursors exposed to insulin expressed similar amounts of *IGF1* and high doses of $1,25(OH)_2D_3$ (100 nM) increased *IGF1* expression to a similar degree in all cells. This is consistent with data that demonstrate that various forms of vitamin D interact with the IGF1 axis to promote bone formation. Indeed, cholecalciferol therapy increases circulating IGF1 in vitamin D-deficient children (Bereket et al., 2010; Soliman et al., 2008) while $Vdr^{-/-}$ mice consistently exhibit reduced IGF1 blood levels (Song et al., 2003). In our study, the increase in pro-osteogenic IGF1 with $1,25(OH)_2D_3$ might have counteracted the pro-adipogenic effects of this hormone *in vitro*, explaining why no phenotypic differences were seen in response to $1,25(OH)_2D_3$ despite increasing expression of adipocyte markers at high doses. Alternatively, the increase in *CYP24A1* expression, particularly in CKD cells, may have enhanced degradation of $1,25(OH)_2D_3$, preventing the full effect of $1,25(OH)_2D_3$ on adipogenesis.

We acknowledge that this study has certain limitations, predominantly concerning the small number of bone biopsy samples available for analysis and the lack of healthy controls for marrow adipocyte number assessment. It is also important to note that further studies will be needed to confirm this relationship, particularly in the adult population, since no patients in the current study had diabetes mellitus, a condition which contributes to a large proportion of the kidney disease observed in the adult population, which affects bone marrow adiposity, and which is associated with low-turnover osteodystrophy. Secondly, we counted adipocyte numbers but did not, due to technical issues, record the diameter of each adipocyte cell. Thus, we were not able to assess the effect of recombinant human growth hormone or calcitriol on adipocyte size or on the expansion of total fat volume. In addition, we exclusively analyzed trabecular bone from the iliac crest; whether similar changes can be identified in the long bones or spine is unknown but is of clinical importance, given the high rate of fractures in this population. We also acknowledge certain limitations intrinsic to the *in vitro* study of primary osteoblasts. Although striking differences in osteoblast maturation and mineralization have previously been observed between healthy control osteoblasts and primary osteoblasts from CKD patients, no differences in adipogenic potential were observed between CKD and control cells. Whether a larger sample size could reveal subtle differences not detected in the current study is unknown and warrants further investigation. However, considering the rarity of these samples and the lack of knowledge in this specific subject we believe that our unique data offers valuable insights into the effects of an important therapy for childhood renal osteodystrophy on bone biology.

5. Conclusion

Marrow adiposity correlates inversely with bone turnover in pediatric patients with high turnover renal osteodystrophy. The range of adiposity observed in pediatric patients with low/normal bone turnover is not explained by intrinsic changes to precursor cells or by therapies but may reflect the effects of circulating factors on bone cell health in this population.

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CRediT authorship contribution statement

OS, RCP, IBS, and KWP participated in the conception and design of the study. OCP, RCP, JB, and KWP analyzed and interpreted the data. OS and KWP drafted the article; all authors revised it. All authors provided intellectual content of critical importance to the work and provided approval for publishing the final version.

Transparency document

The Transparency document associated with this article can be found in online version.

Declaration of competing interest

None.

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