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# **Inhibition of osteoclast differentiation by 1.25-D and the calcimimetic KP2326 reveals 1.25-D resistance in advanced CKD**

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**Running title:** vitamin D, calcimimetics and human osteoclasts in CKD

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## **Abstract**

Active vitamin D analogs and calcimimetics are the main therapies used for treating secondary hyperparathyroidism (SHPT) in patients with chronic kidney disease (CKD). Peripheral blood mononuclear cells (PBMCs) of 19 pediatric patients with CKD1-5D and 6 healthy donors (HD) were differentiated into mature osteoclasts with RANK-L and M-CSF. The effects of single or combined treatment with active vitamin D (1.25-D) and/or calcimimetic KP2326 were evaluated on osteoclastic differentiation and osteoclastic-mediated bone resorption. Although 1.25-D inhibited osteoclastic differentiation, a significant resistance to 1.25-D was observed when glomerular filtration rate (GFR) decreased. A significant albeit less important inhibitory effect of KP2326 on osteoclastic differentiation was also found both in cells derived from HD and CKD patients, through a putative activation of the Erk pathway. This inhibitory effect was not modified by CKD stage. Combinatorial treatment with 1.25-D and KP2326 did not result in synergistic effects. Last, KP2326 significantly inhibited osteoclast-mediated bone resorption.

Both 1.25-D and KP2326 inhibit osteoclastic differentiation, however to a different extent. There is a progressive resistance to 1.25-D in advanced CKD that is not found with KP2326. KP2326 also inhibits bone resorption. Given that 1.25-D has no effect on osteoclastic resorption activity and that calcimimetics have direct anabolic effects on osteoblasts, there is an experimental rationale that could favor the use of decreased doses of 1.25-D with low-doses of calcimimetics in SHPT in dialysis to decrease the risk worsen of osteodystrophy. However, this last point deserves confirmatory clinical studies.

### **Key-words**

Chronic kidney disease

Secondary hyperparathyroidism

Osteoclast

Etelcalcetide

Vitamin D

## Introduction

Changes in mineral metabolism during chronic kidney disease (CKD) induce secondary hyperparathyroidism (SHPT), characterized by parathyroid gland hyperplasia and subsequent high serum parathyroid hormone (PTH) levels. SHPT has also been associated with impaired longitudinal growth, fractures, anemia, left ventricular hypertrophy, and increased mortality [1]. The primary bone lesion in pediatric CKD, at least in pediatric patients reaching end-stage renal disease (ESRD) without any previous management, is the high-turnover osteodystrophy (*osteitis fibrosa*), because of SHPT with low 1-25 OH<sub>2</sub> vitamin D (1.25-D) levels [2]. SHPT results from global disturbances in mineral metabolism, and notably hypocalcemia, hyperphosphatemia and decreased 1.25-D levels, contributing to the complex CKD associated-mineral and bone disorders (CKD-MBD), including ectopic calcifications and renal osteodystrophy [2].

The optimal management of pediatric CKD-MBD is a daily challenge, consisting mainly of correcting native vitamin D deficiency, keeping nutritional calcium within the age-recommended daily intake, decreasing phosphate levels (using nutritional management and phosphate binders), and keeping PTH levels within the target range [3]. Active vitamin D analogs and calcimimetics (such as cinacalcet or etelcalcetide) are cornerstones of SHPT management, and their use has been recently delineated in the European pediatric guidelines [4][5]. Conversely, low turnover osteodystrophy (*adynamic bone*) can be observed in children undergoing maintenance dialysis who receive too much calcium and/or vitamin D analogs [6], therefore requiring a close follow-up with regular therapeutic adjustments [7].

The direct effects on bone of SHPT remain nevertheless to be determined, especially the effects of the two main therapies used in daily practice, namely vitamin D analogs and calcimimetics. We have already reported the effects of 1.25-D on osteoclastic differentiation and bone resorption in healthy volunteers, demonstrating a significant and major inhibitory

effect on differentiation with no effect on resorption activity [8]. In addition, a progressive resistance to 1.25-D in osteoblasts derived from patients with CKD has been reported when CKD worsens: in this context, 1.25-D decreases proliferation but increases mineralization [9]. The Calcium-Sensing Receptor (CaSR) plays a crucial role in calcium homeostasis; it is mainly expressed in parathyroid and tubular renal cells, but also in bone cells such as chondrocytes, osteoblasts and osteoclasts [10]. In bone, the CaSR regulates growth plate formation and bone remodelling [11]. In animal models, mature osteoclasts undergo apoptosis in the presence of high levels of calcium [12]; a dominant-negative CaSR construct abrogates this effect [13]. In young animal models, the activation of CaSR in osteoclasts inhibits bone resorption, thus increasing bone anabolism [11]. In contrast, in older animal models, the activation of Ca-SR activates osteoblasts and bone formation, but also RANK-L expression and therefore bone resorption by osteoclasts [11].

In a murine model of SHPT due to 1-alpha hydroxylase deficiency, the calcimimetic cinacalcet corrects PTH levels, normalizes osteoblastic activity and increases osteoclastic number and activity [14]. It was shown recently that cinacalcet has no effect on osteoclastic differentiation; however, it inhibits resorption activity in osteoclasts derived from healthy human peripheral blood mononuclear cells (PBMCs) [15].

We recently developed and validated a non-invasive technique to differentiate PBMCs into mature and functional osteoclasts from blood samples using only 15mL of total blood allowing us to design patients-derived experimental studies [16]. The objectives of this study were therefore to evaluate the direct effect *in vitro* of single or combined treatment with 1.25-D and etelcalcetide (and more precisely its peptide analog KP2326 [17]) on human osteoclasts derived from PBMCs in both healthy controls and pediatric patients with CKD. We hypothesized that when glomerular filtration rate (GFR) decreases, bone cells acquire a

defective response or resistance to with 1.25-D and etelcalcetide treatments that could explain the adynamic bone observed in some patients.

## **Patients and methods**

**Patients:** the inclusion criteria were the following: pediatric patients between 2 and 18 years of age followed in our tertiary center for CKD stage 1-5D, with a body weight above 10 kg. The exclusion criteria were the following: renal disease known to induce a specific bone impairment (namely primary hyperoxaluria, cystinosis, Pierson syndrome and paracellin mutations), and ongoing therapy with growth hormone, bisphosphonates or teriparatide. The following clinical data were recorded: age, height, body weight, therapies (and notably active vitamin D analogs or cinacalcet). The following biological data were recorded: creatinine allowing the calculation of the estimated glomerular filtration rate (eGFR) using the bedside 2009 Schwartz formula, calcium, phosphate (expressed as absolute value but also SDS for age), alkaline phosphatase (ALP), PTH (electrochemiluminescence immunoassay, Roche, local reference values 15-65 ng/L) and 25OH vitamin D (chemiluminescence immunoassay, IDS-Isys-Multi-Discipline Automated System, 75-120nmol/L). The RENOCLAST study (ID-RCB 2017-A03241-52) was approved by the IRB (*Comité de Protection des Personnes Nord-Ouest II*, session December 14th, 2017, reference 2017/84). All parents and patients were informed and oral consent was obtained. Total blood from healthy controls was also obtained, with no record of clinical and biological parameters.

**Primary osteoclast culture:** Monocytes were purified from 15-20 ml of peripheral blood. Osteoclasts were obtained as previously reported [16]. Osteoclasts were obtained by incubating monocytes with 20 ng/mL M-CSF (Monocyte Colony-stimulating Factor, PeproTech, Rocky Hill, NJ) and 35 ng/mL of RANKL (Receptor Activator of Nuclear factor Kappa-B Ligand, PeproTech) from day 1 to the end of differentiation. All PBMCs were

treated with a combination of RANK-L and M-CSF in order to differentiate them into functioning osteoclasts. From the first day on, osteoclastic precursors were treated with 1.25-D (Enzo life sciences®) (0.1 nM; 0.5 nM; 1nM; 2.5 nM; 5nM) and/or KP2326 (Amgen®) ( $10^{-9}$ M;  $10^{-8}$ M;  $10^{-7}$ M;  $10^{-6}$ M;  $10^{-5}$ M;  $10^{-4}$ M) during the differentiation process. KP-2326 (Ac-c(C)rrrar-NH<sub>2</sub>) is an active metabolite of the calcimimetic etelcalcetide (Ac-c(C)arrrar-NH<sub>2</sub>), which are related but clearly distinct branched peptides (C,Cystein; A,Alanin; R,Arginin)[17][18]. In fact, both of them are pro-drugs in the sense that the disulfide between D-Cys and L-Cys at the branch-point must be cleaved before the main chain D-Cys residue (c) can bind to the CaSR VFT residue Cys-482 [19].

***Osteoclast functional assays:*** at the end of the osteoclastic differentiation protocol, the cells were harvested for real-time PCR analysis or were fixed with 4% of PFA and stained with leukocyte acid phosphatase (TRAP) staining kit according to the manufacturer's instructions (Sigma-Aldrich). Stained positive cells (>3 nuclei) were then counted. Osteoclast activity was assessed as previously described [8]. Increasing of KP2326 ( $10^{-9}$ M;  $10^{-6}$ M) were added at day 2 and maintained until the end. At day 7, to measure the surface of the resorbed matrix, OCs were washed off with distilled water, and then the matrix was stained with a 5% (wt/vol) silver nitrate solution; the pits produced by osteoclasts appeared as white spot under a light microscope: their surfaces were quantified by ImageJ software.

***Reverse Transcription and Real-time quantitative PCR (qRT-PCR):*** RNA isolation and purification were obtained as previously described [16]. Systematic controls were generated without iScript and amplified; qPCR was carried out in triplicate for each data point. Primers for the amplification used in human osteoclasts were *hCaSR* (Calcium Sensing Receptor), *hVDR* (Vitamin D Receptor). Expression of these genes was quantified and normalized to corresponding RNA L32 values and expressed as relative expression using the 2<sup>(-Delta Ct)</sup> method.

**Western blot analyses:** After osteoclast differentiation, pre-osteoclastic cells were serum starved in fetal bovine serum 0.1% during two hours at 37°C. Then, they treated with KP2326 [10<sup>-6</sup>M] at different times T0 (control), T10 and T30. Cells were lysed and different steps used of western blot as previously reported [8]. Membranes were incubated respectively with ERK antibody (ref 4695, 1/1000, Cell Signaling Technology®) and P-ERK antibody (1/1000, ref 4695, 1/1000, Cell Signaling Technology®) overnight at 4°C. The membrane was then washed 3 times with TBS-T and incubated with anti-rabbit Cayman (1/20000, Sigma®) secondary antibody for 1 hour at room temperature. After washing the membrane 3 times with TBST-5% milk, then images revealing by radiography with the chemiluminescence revealing kit (ECL Amersham and ECL Prime Western Blotting detection reagent Ge Healthcare). For each Western blot, the loading was controlled using a monoclonal anti-βtubuline antibody (clone TUB 2.1, Sigma®). Western Blot analyses were quantified using ImageJ software (NIH, Bethesda MD, USA).

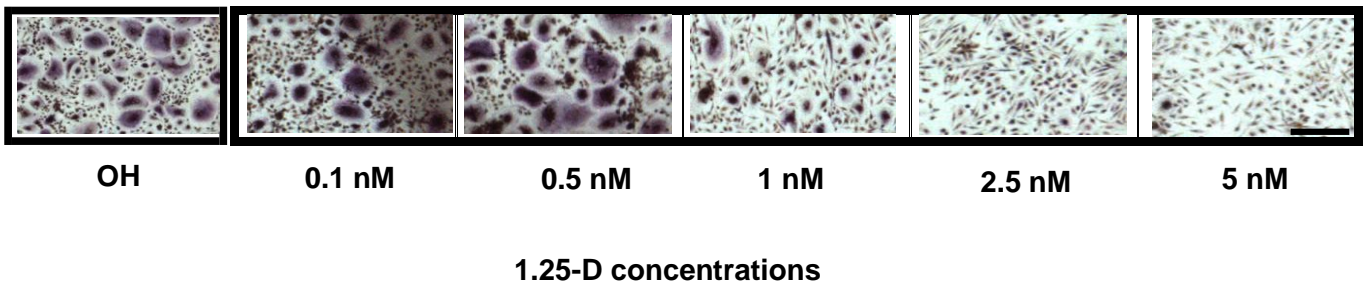
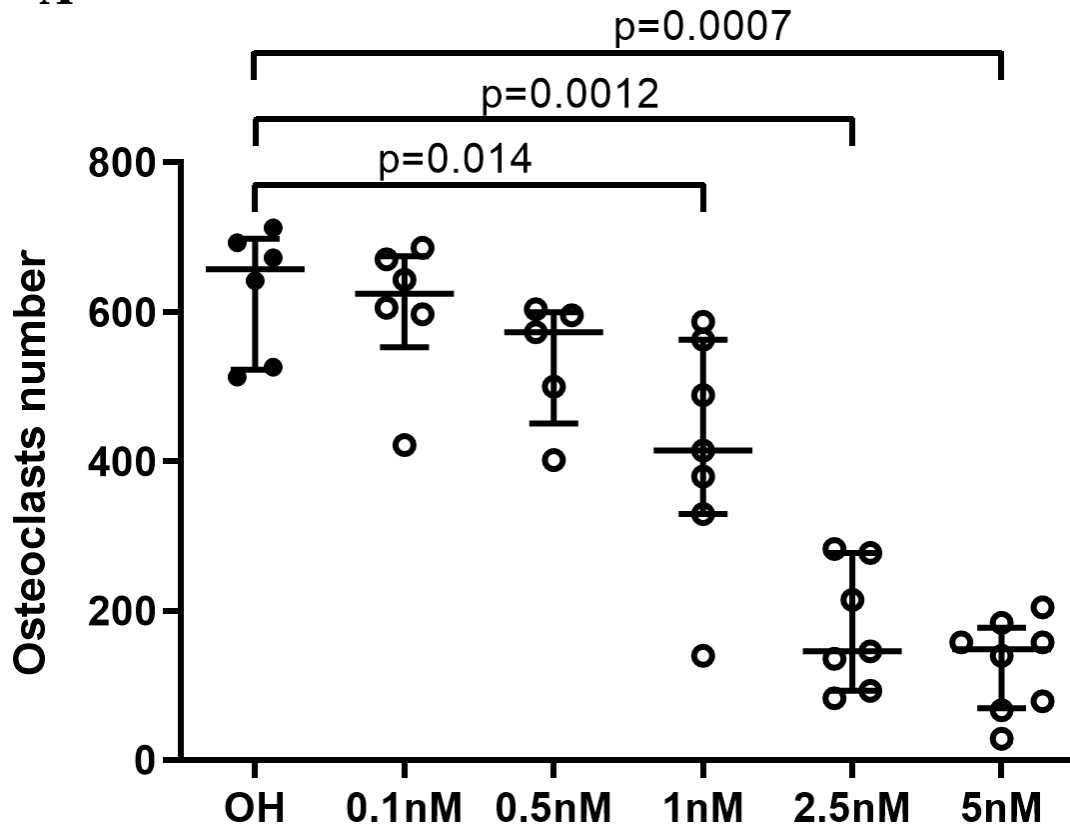
**Statistical analyses:** statistical analyses for experimental data were performed using Prism 5.03 (GraphPad, La Jolla, CA, USA). Mann-Whitney tests were used to analyze data from experiments that comprised two groups. Kruskal-Wallis tests with Dunn's test for multiple comparisons were used to analyze data from experiments which contained more than two groups. Bivariate analyses were performed using Spearman correlations. In all cases, p<0.05 was considered significant. Values were expressed as median with interquartile, or median (min-max) for biochemical routine parameters.

## **Results**

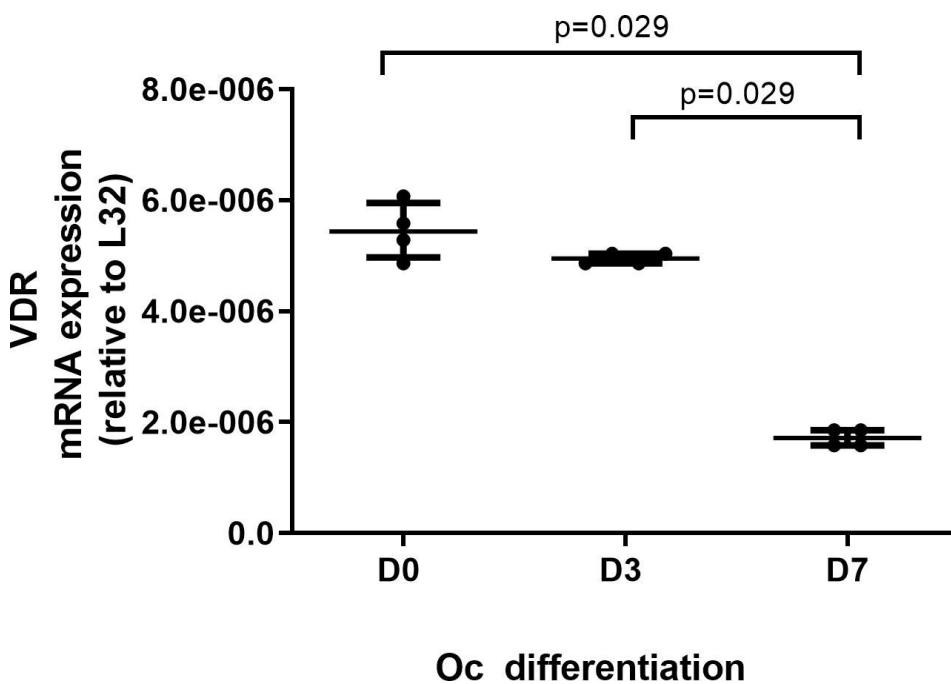
Nineteen pediatric patients with CKD1-5D and six healthy donors (HD) were included in this study: five patients were CKD I-II, four CKD III, five CKD IV-V and five were undergoing maintenance dialysis. There were no differences for age amongst the different sub-groups.



1 A



B



Among CKD IV-V and 5D patients, no patients received calcimimetics, but five and three patients received active vitamin D analogs, respectively. **Table 1** summarizes the main clinical and biological data obtained in patients that are relevant for the analysis of the *in vitro* studies presented below.

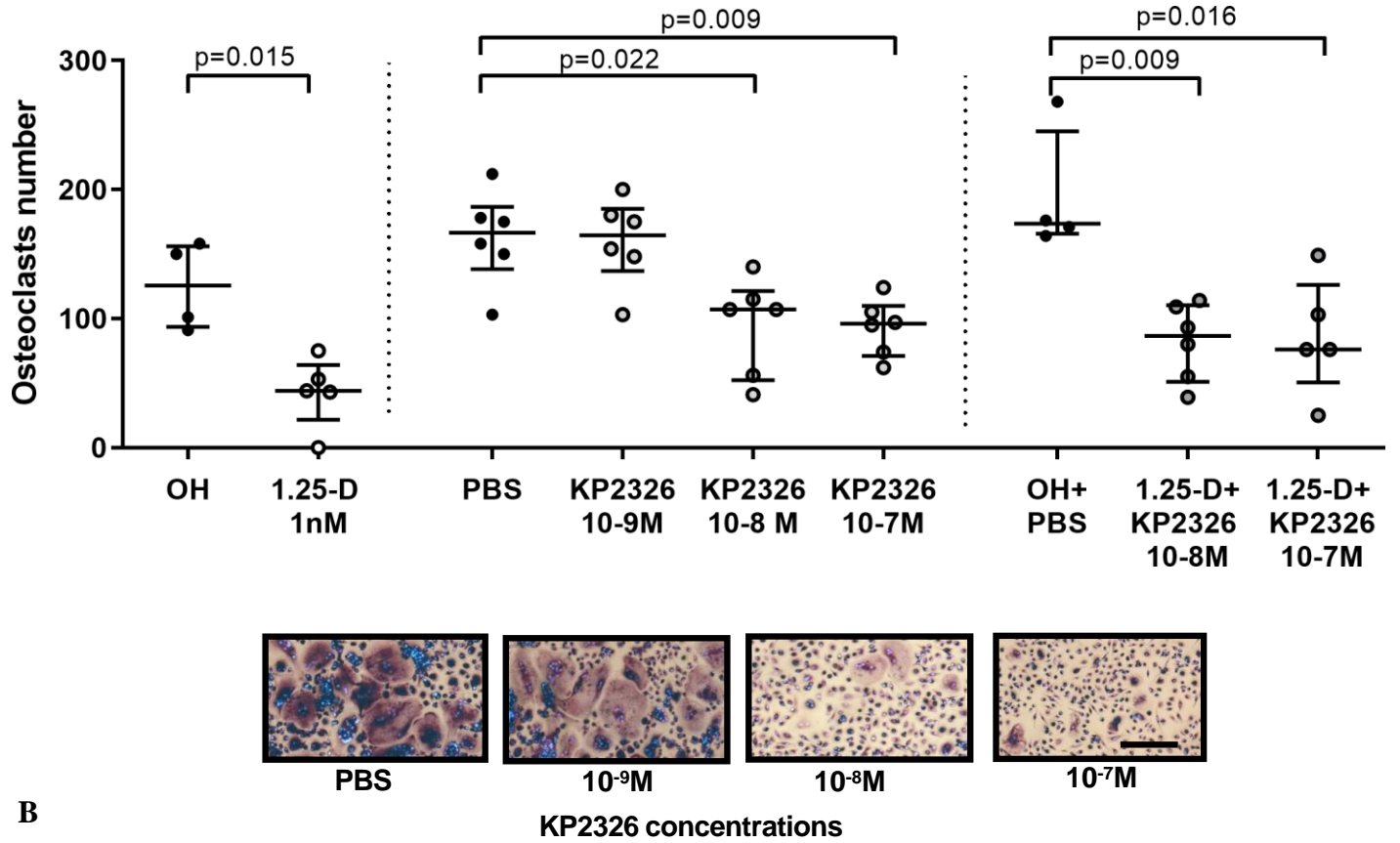
### **Optimal 1.25-D concentration to combine with calcimimetics for dual treatment of bone cells**

The dual effect of 1.25-D and calcimimetics has not been tested directly on blood-derived monocytes. We have previously shown that 1.25-D had an inhibitory effect on osteoclast differentiation. Our aim here was to find an optimal 1.25-D concentration generating a significant inhibition of differentiation, without suppressing it completely: a 1.25-D dose response on osteoclastogenesis was performed, as illustrated in **Figure 1A**. Treatment at 1nM of 1.25-D led to an average 50%-decrease of osteoclastogenesis; consequently, this moderate concentration was chosen for further co treatments. **Figure 1B** illustrates the progressive decrease in VDR expression with osteoclastic differentiation, indicating that osteoclast progenitors are potentially more responsive than mature osteoclasts to 1.25-D treatment.

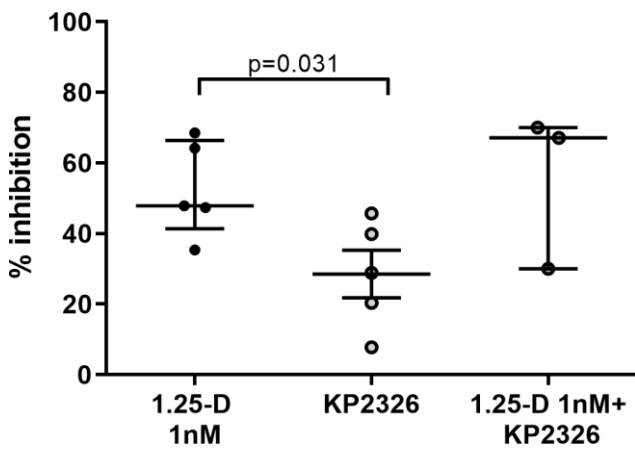
### **KP2326 inhibits osteoclastic differentiation and bone resorption through the Erk pathway**

To evaluate to which extent the KP2326 calcimimetic directly impacts osteoclastogenesis, a dose response experiment has been conducted. Blood mononuclear cells from healthy donors were differentiated in presence of M-CSF and RANK-L with increasing concentration of KP2326 (**Figure 2A**). KP2326 significantly inhibited osteoclastic differentiation; however, the concentration responsible for the maximal inhibition differed greatly between five controls, from  $10^{-9}$ M to  $10^{-4}$ M, with a maximal inhibition of osteoclastogenesis varying from 8% with KP2326  $10^{-5}$ M to 46% with KP2326  $10^{-4}$ M. Globally, 1-25D was more effective to inhibit osteoclastic differentiation than KP2326 (**Figure 2B**). Western-blot analyses of the

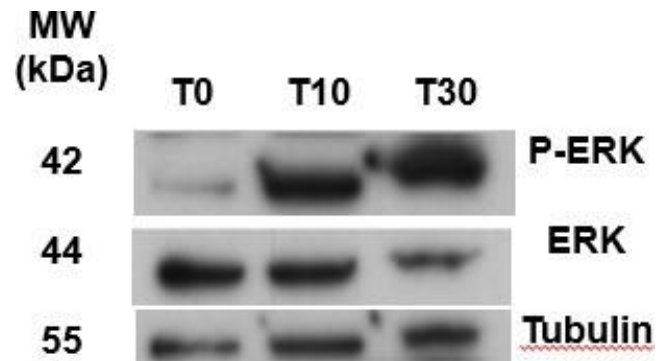
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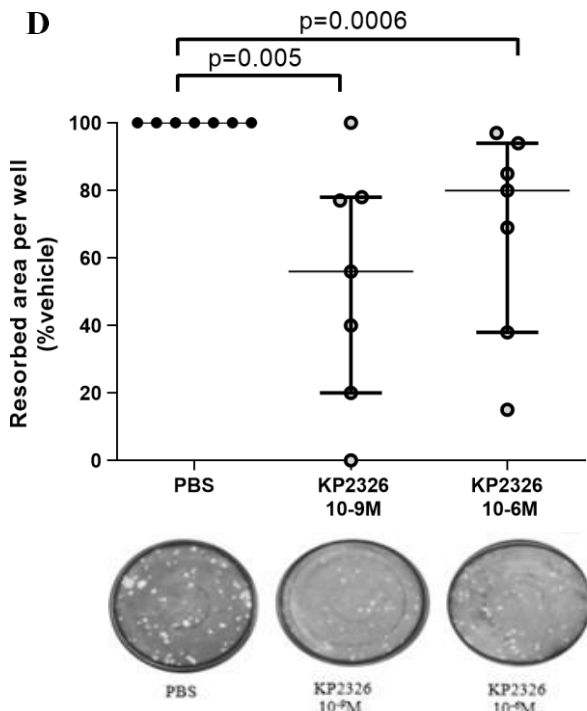
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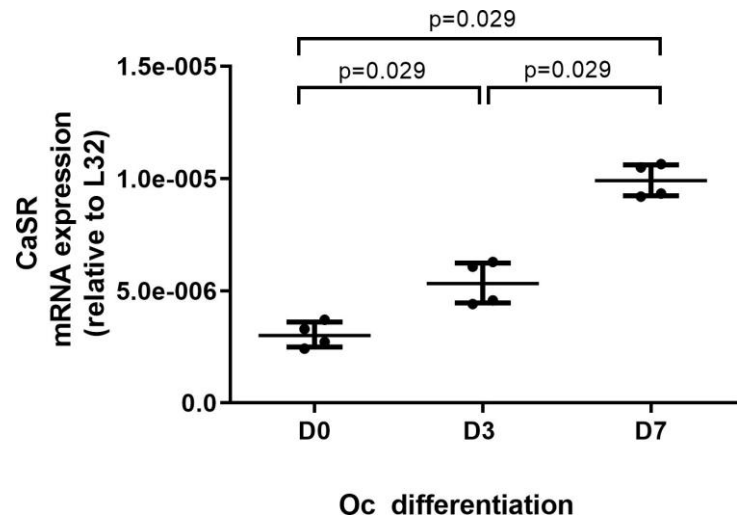
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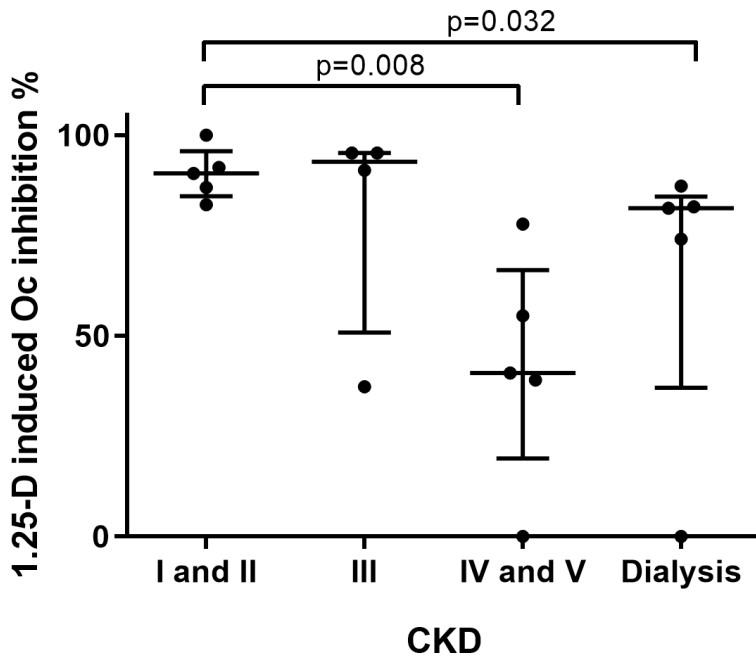
phosphorylation pathways at early time points after treatment demonstrated an activation of the Erk pathway at 10 and 30 minutes with KP2326 (**Figure 2C**). Combinatorial treatment with 1.25-D and KP2326 did not result in synergistic effects on osteoclastic differentiation (**Figure 2B**). Last, to explore the effect of KP2326 on osteoclastic activity, a resorption assay was performed. Mature osteoclasts generated under standard conditions were lifted and the same number of osteoclasts was seeded on mineralized plates with or without KP2326. Both low concentration ( $10^{-9}$ M) and high concentration ( $10^{-6}$ M) of KP2326 impaired resorption. Thus, unlike 1.25-D, KP2326 impaired both osteoclastic differentiation and resorption (**Figure 2D**). As illustrated in **Figure 2E**, CaSR is regulated throughout Oc differentiation reaching a significant peak of expression at the latter stage in healthy controls.

### **There is a progressive resistance to 1.25-D on osteoclastic differentiation when GFR decreases**

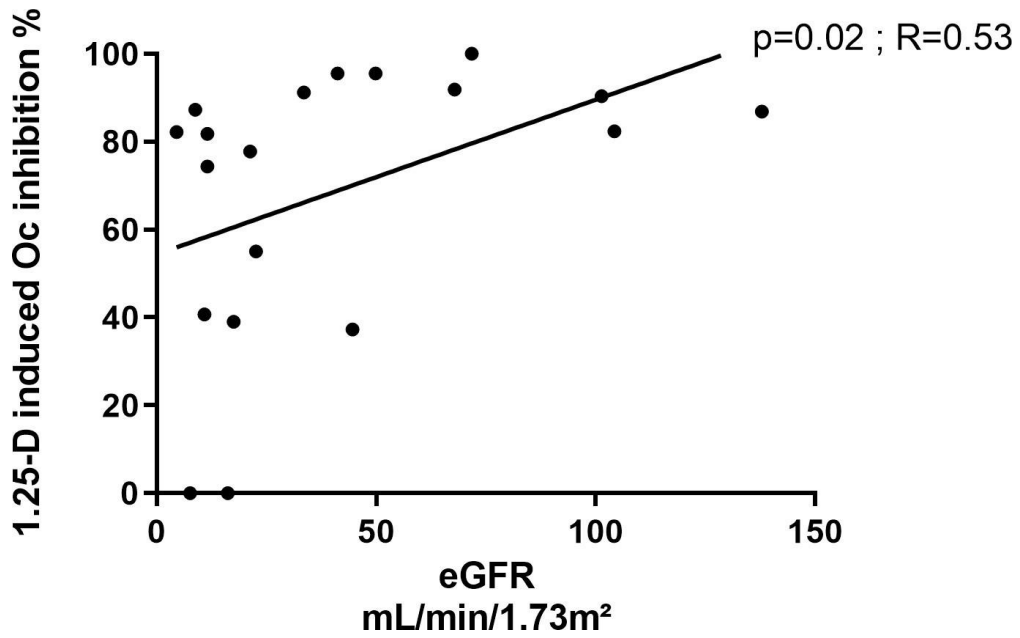
Osteoclasts derived from PBMCs of CKD children were differentiated in the presence of 1.25-D. The percentage of osteoclastogenesis inhibition obtained for each CKD stage was calculated using the control OH treatment (i.e., 100%). As illustrated in **Figure 3A**, a significant decreased inhibition in CKD stage IV-V children as compared to CKD stage I-II was observed ( $p=0.008$ ). For dialysis patients, there was no difference in comparison to CKD IV-V. This resistance to 1.25-D is supported by Spearman bivariate analyses, showing a significant positive association between GFR and the percent of inhibition of differentiation induced by 1 nM of 1.25-D ( $R=0.53$ ,  $p=0.02$ ) (**Figure 3B**). To further investigate the 1.25-D resistance, the expression of 1.25-D receptor (VDR) in osteoclasts was quantified when there was enough material (**Figure 3C**): VDR expression significantly decreased when GFR decreased, as a possible explanation for 1.25-D resistance in osteoclasts.

3

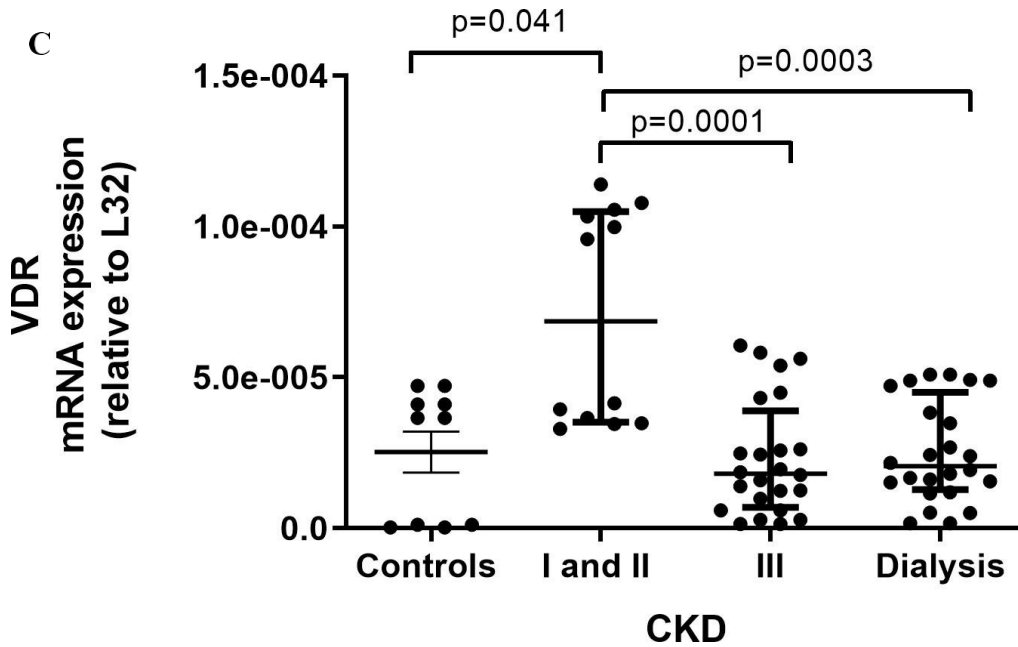
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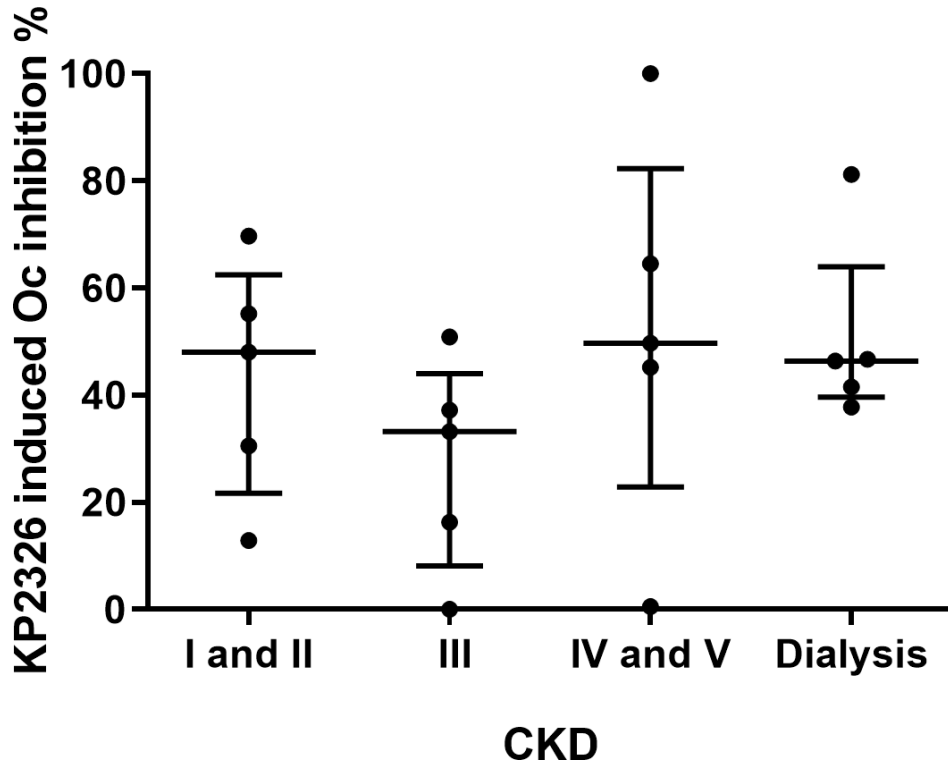
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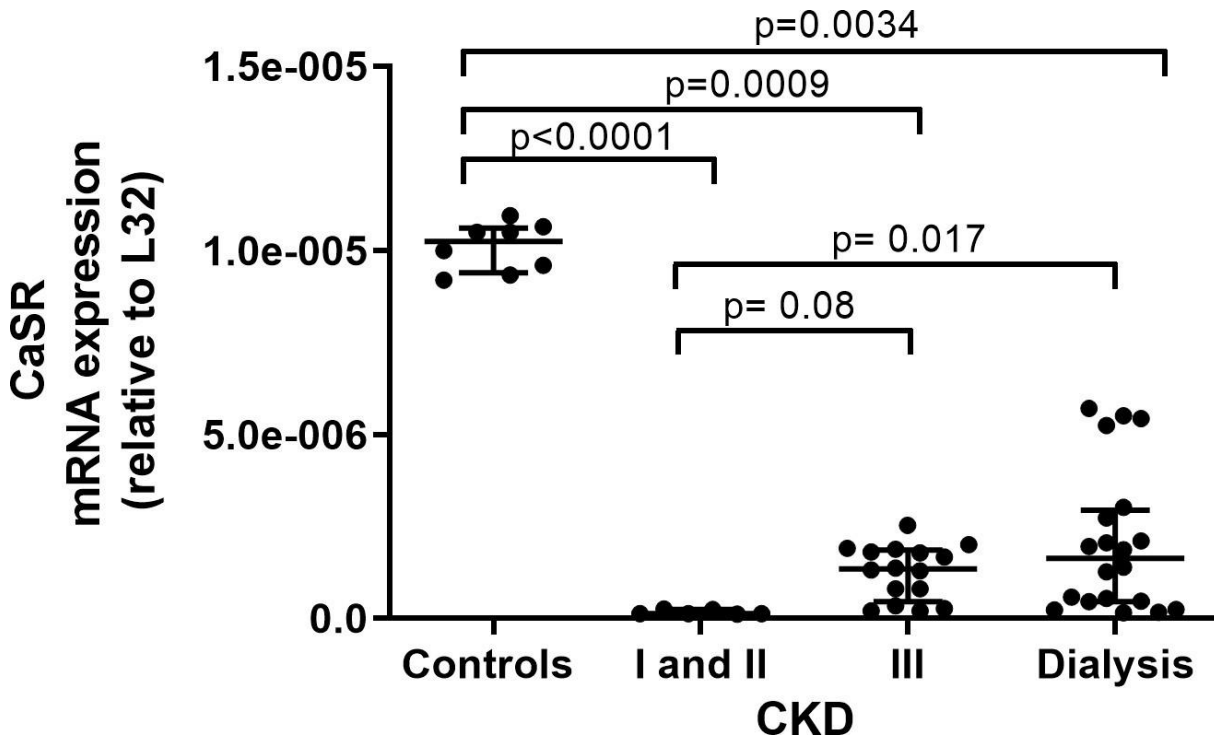
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4 A



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### **KP2326 inhibits osteoclastic differentiation to a lesser extent than 1.25-D and independently of the degree of renal impairment**

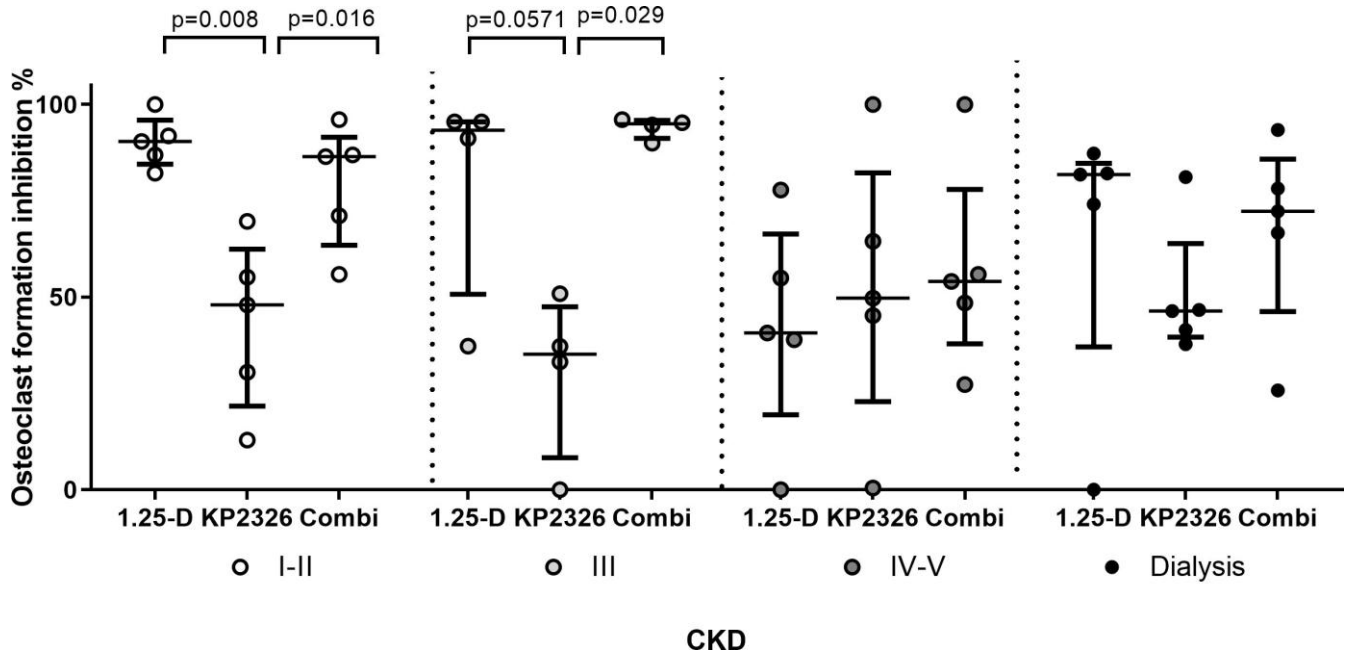
As shown in **Figure 4A**, there was a significant inhibition of osteoclastic differentiation in CKD children, independently of the degree of renal impairment. The expression of CaSR in osteoclasts was quantified when there was enough material. As illustrated in **Figure 4B**, the expression of CaSR was strikingly dropped off in CKD osteoclasts compared to controls even though it is significantly increased as renal function worsened. There was also a trend towards a positive association between PTH and CaSR expression ( $R=0.41$ ,  $p=0.43$ ), however on a small number of subjects ( $N=6$ ) (Data not shown).

### **In CKD children, the combination of 1.25-D and KP2326 inhibits osteoclastic differentiation, without synergistic effect**

As shown in **Figure 5**, the adjunction of KP2326 to 1.25-D in culture did not significantly modify the response in terms of osteoclastic differentiation. Combinatorial treatment had the same effect than 1.25-D in CKD I-II and CKD III, however with a significant difference with KP2326 alone. In CKD IV-V and in dialysis, the combinatorial treatment was not different from 1.25-D or KP2326 alone.

## **Discussion**

This is the first *in vitro* study evaluating the direct impact on human PBMCs differentiated into functioning osteoclasts of the two most frequently used treatments in SHPT, namely 1.25-D and/or calcimimetics, both in healthy controls and pediatric patients at different CKD stages. The main results are the following: there is a resistance to 1.25-D when GFR decreases, with a significant decreased inhibitory effect of 1.25-D on osteoclastic differentiation in advanced CKD. There is a significant albeit less important inhibitory effect





of KP2326 on osteoclastic differentiation through an activation of the Erk pathway, observed for both healthy controls and patients, which is not modified by CKD stage. There is no synergistic effect when co-treating cells with both 1.25-D and KP2326. Last, KP2326 also inhibits human osteoclast-mediated bone resorption unlike 1.25D.

Here, as previously described [8], we confirm that 1.25-D is a powerful inhibitor of osteoclastogenesis on a dose dependent manner in healthy controls. There is a progressive resistance to 1.25-D that appears when GFR decreases. Despite optimal therapy using vitamin D analogs, mineralization and bone defects persisted in a cohort of 60 pediatric patients undergoing maintenance dialysis despite a suppressive effect on PTH levels, thus questioning the overall positive effects of active vitamin D analogs on bone [6]. The resistance to 1.25-D in osteoclasts observed here with physiological doses of active vitamin D is in line with what has been described by Pereira *et al.* in osteoblasts [9], and is logically associated with a decreased expression of VDR in osteoclasts when GFR decreases, even though we have to acknowledge that only qRT-PCR was used as a quantitative technique. Indeed, we obtained rare samples from pediatric patients with CKD, without the confounding factors that would be associated in adults with CKD. However, the limited amount of material at our disposal, did not allow protein quantification of VDR.

This ‘imprinting’ theory is nevertheless not fully explained to date, and it is surprising that there are no significant differences between CKD IV-V and dialysis patients in terms of inhibition of osteoclastic differentiation. We could even discuss whether cells derived from patients undergoing chronic dialysis improve their response to 1.25-D, but this is not statistically significant. We first hypothesized that this observation could be explained by the proportion of patients receiving alfacalcidol but patients in dialysis as well as those in stage IV-V received this drug. However, it is noteworthy that our patients undergoing chronic hemodialysis are well controlled, with PTH levels at the lower limit of the “recommended”

target range, depending on which guidelines are taken into account [20]. This good control of SHPT in dialysis may have blunted the results, and it would be interesting to evaluate the responses in cells from dialysis patients with uncontrolled SHPT. However, the effects on the skeleton may differ between the different compounds used as vitamin D analogs. Indeed, it has been shown that doxercalciferol led to a greater improvement of eroded surface when compared to calcitriol in pediatric dialysis patients with skeletal lesions of SHPT [6], and the authors hypothesized a direct effect of doxercalciferol on osteoclastogenesis. It would be interesting to assess such compounds with the current *in vitro* system. In contrast, our CKD stage IV-V are less well controlled, if we consider that at this CKD stage PTH levels should be at the upper limit of the local normal range (15-65ng/L), and this latter fact may explain the observed resistance to 1.25-D observed in pre-dialysis. Last, we also observed a progressive decrease of VDR expression during osteoclastic differentiation in healthy controls; this point confirms that at the early stages of differentiation, cells may be very responsive to 1.25-D whilst in mature osteoclasts, 1.25-D does not affect bone resorption [8].

The second cornerstone of SHPT management in dialysis is the use of calcimimetics. Etelcalcetide is a CaSR positive allosteric peptide agonist, binding to an extracellular domain and acting through a different mechanism as compared to the first-generation calcimimetic cinacalcet. Indeed, it does not require the binding of calcium on CaSR to be active and to inhibit PTH synthesis. Thus, it activates the CaSR independently of extracellular calcium but its activity is, however, less important when extracellular calcium is low or absent [19]. A pharmacokinetic and pharmacodynamic study confirmed that KP2326, the peptide analog of etelcalcetide for experimental studies, was an allosteric activator of CaSR [21]. Based on the different distribution volumes of etelcalcetide (blood 50L, superficial tissue 120L, deep tissues 625L), the expected  $C_0$  concentrations in each compartment after an intravenous infusion of 5 mg of etelcalcetide (i.e., the dose commonly prescribed in clinical practice in

adults undergoing hemodialysis) would rise to  $9.5 \times 10^{-8} \text{M}$ ;  $4 \times 10^{-8} \text{M}$  and  $7.7 \times 10^{-9} \text{M}$ , respectively [21, 22]. These pharmacological estimations led us to test different concentrations of KP2326 *in vitro*, namely in a range of  $10^{-9} \text{M}$  and  $10^{-5} \text{M}$ , with heterogeneous results not in terms of biological effect but in terms of the inter-individual concentration required to observe such biological effects. Calcimimetics binding to CaSR leads to structural rearrangements. It was also described a biased agonism, defined as the ability of the ligand to stabilize receptor conformation subsets, coupled to specific signaling pathway [23]. This phenomenon could explain inter-individual variability in our experiments.

We demonstrate here an inhibitory effect of KP2326 on osteoclastic differentiation that is significant both in healthy controls and in CKD patients, without any resistance to KP2326 in advanced CKD: as a likely compensatory mechanism, the expression of CaSR significantly increases only in advanced CKD and in dialysis. However, the mechanisms behind this compensation remain unclear. Several signaling pathways are involved during the activation of CaSR in different cells. In osteoblasts, extracellular calcium activates both the phospholipase C [24], the Akt [25], and the ERK1/2 [26] pathways, this later stimulating the proliferation of osteoprogenitor cells and preventing apoptosis. Recently, the direct inhibitory effect of prostaglandin E2 (PGE2) on the differentiation of osteoclastic precursors has been shown, albeit with no effect on mature osteoclasts [27]. After activation of CaSR, the ERK/pERK pathway also activates phospholipase A2, thus stimulating the formation of arachidonic acid and PGE2 [28]. Therefore, one may hypothesize that the decrease in osteoclasts number is related to increased PGE2 levels via the ERK-pERK pathways secondary to the activation of CaSR by KP2326, but this remains to be investigated.

We also demonstrate an inhibiting effect of KP2326 on bone resorption. Thus, *in vitro*, KP2326 inhibits both osteoclastic differentiation and resorption activity; this provides a strong rationale for a global positive effect of KP2326 on bone remodeling. Even though we

provide some mechanistic insights to explain the direct effects of KP2326 on osteoclasts, notably by demonstrating an activation of the Erk pathway with KP2326, we still need to delineate why the concentrations required to obtain the maximal effect vary so widely between individuals, as illustrated in **Table 1**. Several explanations can be hypothesized to explain this modulation of CaSR at different concentrations. First, calcium concentrations can modify receptor cooperativity within the bone microenvironment and balance bone remodelling [29], but culture conditions were standard and similar for all experiments. Second, CaSR is also able to respond to other extracellular stimuli, such as inorganic cations (strontium, aluminium), polyamines, antibiotics (aminoglycosides) and also magnesium [28], but, again, culture conditions were standard and similar for all experiments. Third, an inter-individual variability for CaSR genotypes and expression may exist. Fourth, the expression of CaSR vary at the beginning and during the differentiation process, with a progressive increased expression of CaSR along differentiation. Last, in healthy controls, we did not have biological data such as calcium and 25OH vitamin D levels, that may influence CaSR expression.

We here present data on the effects of KP2326 on osteoclasts, but bone remodeling corresponds to an exquisite balance between bone formation and bone resorption, also known as the “osteoblast/osteoclast” crosstalk. In this study, we present only osteoclastic data, and it was ethically difficult to propose a bone biopsy or an adipose tissue sample (so as to trans-differentiate adipocytes into osteoblasts) since the RENOCLAST study was totally non-invasive. A recent paper evaluated the effect of AMG-641, after CASR activation, on osteoblasts in a very elegant study in normal and uremic rats undergoing a PTH clamp [30]. Indeed, Diaz-Tocados et al. showed that this calcimimetic, analog of cinacalcet which bind in the transmembrane spanning domains of CaSR, has a direct anabolic effect on bone, through the promotion of an osteogenic differentiation and mineralization in human mesenchymal

stem cells in vitro. Moreover, they also found that the administration of AMG641 increased osteoblast number and osteoid number in normal rats under a PTH clamp; additionally, in uremic rats exposed to PTH at 6-fold the usual replacement dose, the administration of the calcimimetic increased osteoblast number, osteoid surface and bone formation [30]. Another recent paper also showed that etelcalcetide reduced bone remodelling and mineralization defects by improving cortical bone structure in an animal model of renal osteodystrophy, namely the 5/6<sup>th</sup> nephrectomized rat with SHPT [31]. After 6 weeks, this treatment significantly decreased bone turnover, attenuated cortical bone deterioration, mineralization defect and marrow fibrosis and preserved bone strength. Taken together, these results on osteoblasts and osteoclasts show a beneficial effect of calcimimetics on bone, clearly favoring bone formation. These collective results can be of utmost importance in clinical practice, especially in dialysis adults who are also prone to osteoporosis [32].

It is also very interesting to note that there is no synergy when co-treating cells with 1.25-D and KP2326. The 2017 KDIGO guidelines recommend to use either vitamin D analogs or calcimimetics or both in case of SHPT in dialysis, without any additional details [7]. In the recent paper from Li *et al.* in which the beneficial effect of etelcalcetide on cortical bone structure was described, they showed that etelcalcetide significantly decreased not only PTH level but also FGF23 (Fibroblast Growth Factor-23) levels [31]. In the setting of global systematic deleterious effects of high FGF23 levels in CKD patients on one hand [33], and keeping in mind that active vitamin D analogs stimulate FGF 23 production on the other hand [33], all these results could therefore favor the use of lower doses of 1.25-D with low-doses of calcimimetics in dialysis in order to control SHPT without inducing a sharp inhibition of osteoclastic differentiation, and therefore decreasing the risk of adynamic osteodystrophy and further vascular calcifications. The clinical application of these experimental data obviously remains to be evaluated, but this strategy could also decrease (or at least stabilize) FGF23

levels, and therefore be less deleterious on the long term. This point can be of utmost importance, especially since KP2326 significantly inhibits bone resorption at all CKD stages whilst 1.25-D does not have any effect on bone resorption [8]. These hypotheses obviously need confirmatory clinical trials with more mechanical experiments to explore osteoclastic regulation and osteoblast/osteoclast crosstalk.

In conclusion, 1.25-vitamin D and KP2326 inhibit osteoclastic differentiation in healthy controls and in CKD patients, however to a different extent. There is a progressive resistance to 1.25-D when CKD worsens that is not found with KP2326.

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### **Disclosures of interest**

JB has been investigator for the Amgen-sponsored trials on the use of cinacalcet and etelcalcetide in pediatric dialysis. JB is the coordinator of the Amgen-sponsored international registry on the use of cinacalcet in pediatric dialysis.

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## Legends of figures

### Figure 1: 1.25-D inhibits osteoclast differentiation in healthy controls

A. The monocytes derived from adult healthy controls were cultured in  $\alpha$ -MEM medium and 10% FSB with M-CSF (20ng/mL) and RANKL (35ng/mL). Medium and cytokines were changed every 72 hours. Ethanol (OH) represents control vehicle of 1.25-D. Cells were treated with OH or 1.25-D (0.1 nM, 0.5 nM, 1 nM, 2.5 nM, 5nM) throughout osteoclastogenesis. Osteoclast differentiation was stopped at day 7. 1.25-D significantly inhibits osteoclast differentiation in a dose dependent manner. Box plots represent median with interquartile range of total TRAP positive cells relative to OH control. For each condition, 5 to 8 wells were analyzed (Kruskal-Wallis with Dunn's test). The results are presented for one healthy control (osteoclastic inhibition with 1 nM: 38.3% as compared to vehicle); similar results were obtained with 4 other healthy controls. Representative TRAP images at the end of the differentiation process are presented for the different conditions below the graph. The black line scale corresponds to 500  $\mu$ m.

B. Human osteoclasts were obtained after 7 days of differentiation under standard conditions with M-CSF (20ng/mL) and RANKL (35ng/mL) from two healthy controls. Systematic controls were generated without iScript and amplified; qPCR was carried out in duplicate for each data point. VDR expression was quantified and normalized to corresponding RNA L32 values and expressed as a relative expression using the  $2^{-\Delta\Delta Ct}$  method. VDR expression was evaluated along the differentiation process at Day 0, Day 3 and Day 7, showing a significant decreased expression in healthy donors at Day 7. Statistical analyses were performed with non-parametric tests (Kruskal-Wallis with Dunn's test).

**Figure 2: In healthy controls, the calcimimetic KP2326 inhibits osteoclastic differentiation through an activation of the Erk pathway; it also inhibits bone resorption**

**A.** The monocytes derived from a healthy control were cultured in  $\alpha$ -MEM medium and 10% FBS with M-CSF (20ng/mL) and RANKL (35ng/mL). Medium and cytokines were changed every 72 hours. Ethanol (OH), PBS and OH+PBS represent respective control vehicles of 1.25-D, KP2326 and 1.25-D+KP2326. Cells were treated with vehicle, 1.25-D (1nM), KP2326 ( $10^{-9}$ M,  $10^{-7}$ M,  $10^{-6}$ M) or combinatorial treatment of 1.25-D and  $10^{-8}$ / $10^{-7}$ M of KP2326 throughout osteoclastogenesis. Osteoclast differentiation was stopped at day 7. There is no synergistic effect when co-treating cells with both 1.25-D and KP2326. Box plots represent median with interquartile range of TRAP-positive cells. For each condition, 5 to 8 wells were analyzed (Mann-Whitney Kruskal-Wallis with Dunn's tests). The results are presented for one healthy control: the percentage of osteoclastic inhibition with KP2326  $10^{-8}$ M and  $10^{-7}$ M was 31 and 40% respectively). A representative photo merge of DAPI and TRAP images at the end of the differentiation process is represented for the different conditions below the graph. The black line scale stands for 500  $\mu$ m.

**B.** Each dot on the graph represents the individual result for each condition expressed as the percent of inhibition of osteoclastic differentiation relative to vehicle treatment that was considered arbitrarily to be at 0%). Ethanol (OH), PBS and OH+PBS represent respective control vehicles of 1.25-D (1nM), KP2326 and 1.25-D (1nM) KP2326. 1.25-D is significantly more effective than KP2326 to inhibit osteoclastogenesis ( $p=0.031$  between 1.25-D and KP2326, Mann-Whitney test). Combinatorial treatment has the same effect than 1.25-D ( $p=0.8$ ).

**C.** After 5 days of differentiation, osteoclasts were treated with KP2326  $10^{-6}$ M at 0, 10 and 30 minutes. KP2326 at a concentration of  $10^{-6}$ M activates the ERK/p-ERK pathway at 10 and 30 minutes. T0: 0 minute; T10: 10 minutes; T30: 30 minutes; MW: molecular weight in kDa

**D.** Human osteoclasts obtained after 6 days of differentiation under standard conditions with M-CSF (20ng/mL) and RANKL (35ng/mL) were re-plated on mineralized plates and treated with PBS (vehicle) and KP2326 ( $10^{-9}$ M and  $10^{-6}$ M) for 3 days. After osmotic lysis, slices were stained in silver nitrate to quantify the total resorbed areas. KP2326 significantly inhibits bone resorption. Relative results are expressed as the percentage of the resorbed area per well as compared to the control PBS (resorbed area per well with KP2326  $10^{-6}$ M: 57.8%  $p=0.0098$ ; osteoclast inhibition with KP2326  $10^{-9}$ M: 60.4%  $p=0.0024$ ). Micrographs of silver nitrate matrix staining are representative for the three conditions. Statistical analyses were performed with non-parametric paired tests (Kruskal-Wallis with Dunn's tests). Experiments were performed in batches from three different donors; each condition for each donor was done in quadruplicates in culture. We present here the representative results obtained in one healthy control. The graphs represent median with interquartile of the percentage of resorbed area per well, as compared to vehicles that were considered arbitrarily to be at 100%.

**E.** Human osteoclasts were obtained after 7 days of differentiation under standard conditions with M-CSF (20ng/mL) and RANKL (35ng/mL) from 2 healthy controls. Systematic controls were generated without iScript and amplified; qPCR was carried out in duplicate for each data point. CaSR expression was quantified and normalized to corresponding RNA L32 values and expressed as a relative expression using the  $2^{-\Delta\Delta Ct}$  method. CaSR expression was evaluated along the differentiation process at Day 0, Day 3 and Day 7, showing a significant increased expression in healthy donors. Statistical analyses were performed with non-parametric paired tests (Kruskal-Wallis with Dunn's tests).

**Figure 3. In CKD children, there is a progressive resistance to 1.25-D effects on osteoclastic differentiation in advanced CKD**

**A.** The graph represents the individual result of one patient, with median with interquartile range per CKD group (I-II; III, IV-V and dialysis): there is a progressive resistance to 1 nM of 1.25-D when GFR decreases, with a significant decreased inhibition of osteoclastogenesis in CKD IV-V children as compared to CKD stage I-II induced by 1 nM of 1.25-D ( $p=0.008$ ). In dialysis, there is a significant decreased inhibition as compared to stage I-II ( $p=0.032$ ), but no difference in comparison to CKD stage IV-V ( $p=NS$ ). Statistical analyses were performed with non-parametric paired tests (Mann-Whitney).

**B.** The figure represents eGFR ( $\text{mL}/\text{min}/1.73\text{m}^2$ ) on the X-axis, and the percent of inhibition with 1nM of 1.25-D on the Y-axis. The statistical bivariate analysis by Spearman correlation confirms the progressive resistance to 1.25-D when CKD worsens ( $R=0.53$ ;  $p=0.02$ ).

**C.** VDR expression in mature Osteoclasts. The analysis was possible for 11 CKD patients and 2 controls. RNA was obtained after osteoclastogenesis on day 7; qPCR was carried out in triplicate for each data point. VDR expression was quantified and normalized to corresponding RNA L32 values and expressed as a relative expression using the  $2^{(-\Delta\text{Ct})}$  method. The expression of VDR significantly decreases when GFR decreases. Statistical analyses were performed with non-parametric paired tests (Kruskal-Wallis with Dunn's tests).

**Figure 4. In CKD children, KP2326 inhibits osteoclastic differentiation, however to a lesser extent than 1.25-D, with no effect of the degree of renal impairment**

**A.** Each dot on the graph represents the individual result of one patient, with median with interquartile range per CKD group (I-II; III, IV-V and dialysis): although there is a significant inhibition of osteoclastogenesis with KP2326 in patients with CKD (as compared to the

control vehicle PBS), there is no difference between groups when CKD worsens. Statistical analyses were performed with non-parametric paired tests (Kruskal-Wallis with Dunn's tests).

**B.** Analysis of CaSR expression in mature Ocs on day 7 (9 pediatric patients and 2 adult controls were represented). qPCR was carried out in triplicate for each data point. *hCaSR* expression was quantified and normalized to corresponding RNA L32 values and expressed as a relative expression using the  $2^{(-\Delta Ct)}$  method. The expression of CaSR significantly increases when renal function worsens. Statistical analyses were performed with non-parametric paired tests (Kruskal-Wallis with Dunn's tests).

**Figure 5. *Combinatorial treatment inhibits osteoclastogenesis without synergistic effect***

This figure represents the median with interquartile range  $\pm$  min-max of percentage of inhibition of osteoclastogenesis as compared to the respective controls of 1.25-D, KP2326 and 1.25-D+KP2326, namely ethanol (OH), PBS, and OH+PBS, in the different CKD groups. The results of 19 different patients have been pooled in this analysis. Combinatorial treatment has the same effect than 1.25-D in CKD I-II and III, however with a significant difference with KP2326 alone. In CKD IV-V and in dialysis, the combinatorial treatment is not different from 1.25-D or KP2326 alone. Statistical analyses were performed with non-parametric paired tests (Mann-Whitney).



**Table 1: clinical and biological data in the patients**

CKD stage	Age (years)	Primary renal disease	eGFR (mL /min/ 1.73m <sup>2</sup> )	Active D analog	Calcimimetic	% inhibition 1.25-D 1nM	% inhibition KP2326	[KP2326]	% inhibition 1.25-D + KP2326	[KP2326] used for the cotreatment
<b>I-II</b>	12.8	ADPKD	101	No	No	90	48	10 <sup>-6</sup> M	87	10 <sup>-6</sup> M
	6.2	CAKUT	49	No	No	100	31	10 <sup>-8</sup> M	87	10 <sup>-9</sup> M
	13.8	HUS	104	No	No	83	13	10 <sup>-7</sup> M	56	10 <sup>-6</sup> M
	14.3	GN	138	No	No	87	70	10 <sup>-8</sup> M	71	10 <sup>-6</sup> M
	12.7	IgA	68	No	No	92	55	10 <sup>-9</sup> M	96	10 <sup>-9</sup> M
<b>Median (min-max)</b>	<b>12.8 (6.2-14.3)</b>		<b>101 (49-138)</b>			<b>90 (83-100)</b>	<b>48 (13-70)</b>		<b>87 (56-96)</b>	
<b>III</b>	7.5	Dent	45	No	No	37	51	10 <sup>-8</sup> M	63	10 <sup>-8</sup> M
	6.5	CAKUT	50	Yes	No	96	37	10 <sup>-8</sup> M	95	10 <sup>-8</sup> M
	2.2	Bardet Biedel	33	No	No	91	0	10 <sup>-6</sup> M	95	10 <sup>-9</sup> M
	15.1	CAKUT	41	Yes	No	96	33	10 <sup>-7</sup> M	96	10 <sup>-7</sup> M
<b>Median (min-max)</b>	<b>7 (2.2-15.1)</b>		<b>43 (33-41)</b>			<b>93 (37-96)</b>	<b>35 (0-51)</b>		<b>95 (63-96)</b>	
<b>IV-V</b>	6.9	CAKUT	23	Yes	No	55	65	10 <sup>-7</sup> M	56	10 <sup>-7</sup> M
	10.0	CAKUT	17	Yes	No	39	45	10 <sup>-9</sup> M	49	10 <sup>-9</sup> M
	9.9	Nephronoptisis	16	Yes	No	0	50	10 <sup>-7</sup> M	54	10 <sup>-7</sup> M
	14.9	Nephronoptisis	11	Yes	No	41	1	10 <sup>-7</sup> M	27	10 <sup>-9</sup> M
	13.8	CAKUT	21	Yes	No	78	100	10 <sup>-8</sup> M	100	10 <sup>-9</sup> M
<b>Median (min-max)</b>	<b>10 (6.9-14.9)</b>		<b>17 (11-21)</b>			<b>41 (0-78)</b>	<b>50 (1-100)</b>		<b>54 (27-100)</b>	
<b>Dialysis</b>	2.1	CNS	4	Yes	No	82	38	10 <sup>-8</sup> M	67	10 <sup>-7</sup> M
	4.0	CNS	8	Yes	No	0	46	10 <sup>-9</sup> M	26	10 <sup>-7</sup> M
	17.6	Unknown origin	11	Yes	No	74	42	10 <sup>-9</sup> M	72	10 <sup>-7</sup> M
	6.1	CAKUT	9	No	No	87	81	10 <sup>-7</sup> M	78	10 <sup>-6</sup> M
	13.3	Renal vein thrombosis	11	No	No	82	47	10 <sup>-6</sup> M	93	10 <sup>-6</sup> M
<b>Median (min-max)</b>	<b>6.1 (2.1-17.6)</b>		<b>9 (4-11)</b>			<b>82 (0-82)</b>	<b>46 (38-81)</b>		<b>72 (26-93)</b>	

**Abbreviations:** Med: Median; CKD: Chronic Kidney Disease; ADPKD: Autosomal Dominant Polycystic Kidney Disease; CAKUT: Congenital Anomalies of the Kidney and Urinary Tract; HUS: Hemolytic Uremic Syndrome; GN: Glomerulonephritis; IgA: IgA nephropathy; CNS: Congenital Nephrotic Syndrome; eGFR: estimated Glomerular Filtration Rate; PTH: Parathyroid hormone; Ca: Calcium; Ph: phosphate; Ph SD: Phosphate expressed as Standard Deviation for Age; 25.D: 25-OH.vitamin D; ALP: Alkaline Phosphatase ; [KP2326]: concentration of KP2326.