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Inflammation induces osteoclast differentiation from peripheral mononuclear cells in chronic kidney disease patients: crosstalk between the immune and bone systems

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ORIGINAL ARTICLE

ABSTRACT

Background. Inflammation and immune system alterations contribute to bone damage in many pathologies by inducing the differentiation of osteoclasts (OCs), the bone resorbing cells. This link is largely unexplored in chronic kidney disease (CKD) and haemodialysis (HD) patients, in which reduced renal function is accompanied by an increased inflammatory state and skeletal abnormality.

Methods. We used *ex vivo* culture experiments to investigate the osteoclastogenic potential of peripheral blood mononuclear cells (PBMCs) of CKD and HD patients, focusing on immune cell subsets and inflammatory cytokines such as LIGHT and receptor activator of nuclear factor κB ligand (RANKL).

Results. We observed spontaneous osteoclastogenesis with a significant increase in OC formation and bone resorbing activity in late-stage CKD and HD patients when compared with early-stage CKD patients and healthy donors, likely due to an increased expression of RANKL and LIGHT (homologous to Lymphotoxins exhibiting Inducible expression and competing with herpes simplex virus Glycoprotein D for herpes virus entry mediator [HVEM], a receptor expressed by T lymphocytes) in PBMCs. Specific inhibition of these cytokines in PBMCs isolated from CKD stages 3b–5 and HD patients induced the reduction of OC formation *in vitro*. The phenotypic characterization of peripheral blood cells revealed a significant increase of OC precursors ($CD14^+CD11b^+CD51/61^+$) and $CD14^+CD16^+$ monocytes in advanced CKD and HD patients compared with the control group.

Conclusions. Our results suggest that circulating inflammatory monocytes from advanced CKD or HD patients trans differentiate into OCs *in vitro* and play a relevant role in mineral bone disorders and that LIGHT and RANKL represent new potential therapeutic targets in these settings.

Keywords: chronic kidney disease, haemodialysis, LIGHT, osteoimmunology, RANKL

INTRODUCTION

Chronic kidney disease (CKD) is a progressive impairment of renal functions that often degenerates to a uraemic state that requires dialysis or kidney transplantation [1]. During the progression of renal damage, CKD patients show considerable metabolic changes: enhancement of inflammation/oxidative stress [2], production of pro-inflammatory cytokines [3] and immune system alterations [4]. It is well established that the uraemic milieu induces a dysfunction of the immune system and chronic inflammation is frequently noted in end-stage renal disease [5]. It is well known that these patients present a high serum level of inflammatory cytokines that is often correlated with cardiovascular and bone disease [6]. Under physiological conditions, a balance is maintained between bone formation by osteoblasts and bone resorption by osteoclasts (OCs) for skeletal homeostasis [7]. Canonical OC formation requires both macrophage colony-stimulating factor (M-CSF) and receptor activator factor of nuclear factor κB ligand (RANKL), which act on cells of the monocyte–macrophage lineage (i.e. pre-osteoclasts), inducing their fusion to form polynucleated active resorbing cells [8]. RANKL is a tumour necrosis factor (TNF) ligand superfamily member that is essential for the formation, activation and function of OCs [9]. RANKL acts via its receptor, RANK, and is inhibited by the soluble decoy receptor osteoprotegerin (OPG) [10]. In pathological states such as rheumatoid arthritis (RA),

Paget's disease, osteoporosis, osteoarthritis, multiple myeloma and metastatic bone tumours, inflammatory cytokines and activated immune cells disrupt this balance in favour of osteoclast-mediated bone resorption [11]. Over the last two decades, studies have shown that skeletal homeostasis is dynamically influenced by the immune system and that lymphocyte- or macrophage-derived cytokines are among the most potent mediators of osteoimmunological regulation [12]. A large number of inflammatory cytokines, including TNF-α, interleukin-1β (IL-1β) and IL-6, directly or indirectly regulate OC formation and function [13, 14]. In addition, recent studies have reported the involvement of a new pro-osteoclastogenic cytokine known as LIGHT in bone erosions in patients with RA and multiple myeloma [15–17]. Some of these inflammatory cytokines are produced by $CD14^+CD16^+$ monocytes, a particular subset that differs from classical $CD14^+CD16^-$ monocytes and that has been recently shown to be expanded in acute or chronic inflammatory diseases [18]. This subset also represents a potential precursor of OCs in inflammatory arthritis [19]. The literature subdivides $CD14^+CD16^+$ monocytes into two smaller subsets: the intermediate subpopulation ($CD14^{++}CD16^+$) and non-classical monocytes ($CD14^+CD16^{++}$) [18]. Both subsets increase during inflammatory diseases such as RA [20], cardiovascular events [21] and also in CKD and uraemic patients [22], where their increase is associated with cardiovascular events [23]. Another subset of monocyte cells, the OC precursors (OCp; $CD14^+CD11b^+CD51/61^+$), is particularly committed to the differentiation of mature OCs in inflammatory bone diseases and is a target for anti-inflammatory therapy [24]. It has been reported that altered bone turnover is associated with a high release of circulating pro-inflammatory cytokines in CKD and haemodialysis (HD) patients [25], but the mechanism underlying the involvement of immune cells and bone disease in these settings has yet to be demonstrated. Here, we determined that inflammatory monocyte subsets, as well as RANKL and LIGHT, may contribute to the development of bone disease in CKD and HD patients through increased OC formation.

MATERIALS AND METHODS

Patients

The study involved 50 patients, including 12 CKD patients in Kidney Disease Outcomes Quality Initiative (KDOQI) stages 1–3a (three patients in stage 1, five patients in stage 2 and four patients in stage 3a), 20 in CKD stages 3b–5 (five patients in stage 3b, eight patients in stage 4 and seven patients for stage 5), 18 HD patients and 10 healthy donors. The demographic and clinical parameters of patients and controls included in the study are summarized in Table 1 and the pharmacological treatment is reported in Table 2. HD patients were being treated thrice weekly with standard bicarbonate dialysis (Na 138 mmol/L,

Table 1. Demographic and clinical parameters of patients and controls

	HD	CKD stage 3b–5	CKD stage 1–3a	Donors
Baseline characteristic				
Number	18	20	12	10
Age, years	60.8 ± 14.7	60.4 ± 11.9	59.7 ± 7.8	55 ± 11.2
Female/male	7/11	6/14	4/8	5/5
Biochemical parameters				
Phosphate (mg/dL)	4.4 ± 1.4	4.7 ± 1.1	3.5 ± 0.3	3 ± 0.7
Calcium (mg/dL)	8.7 ± 0.7	8.9 ± 0.9	8.5 ± 1.4	7 ± 0.8
25-OH vitamin D (ng/mL)	17.1 ± 7.7	16.9 ± 6.3	27.7 ± 12.8	31.3 ± 3.6
PTH (pg/mL)	155.6 ± 71.1	156.1 ± 73.6	87.9 ± 37.1	13.3 ± 6.3

Data are expressed as mean ± SD.
Phosphate (mg/dL) $P < 0.05$ HD, CKD stage 3b–5 and CKD stage 1–3a versus donors; CKD stage 3b–5 versus CKD stage 1–3a.
Calcium (mg/dL) $P < 0.05$ HD, CKD stage 3b–5 and CKD stage 1–3a versus donors.
25-OH vitamin D (ng/mL) $P < 0.05$ HD, CKD stage 3b–5 versus CKD stage 1–3a and donors; $P < 0.05$ HD, CKD stage 3b–5 versus CKD stage 1–3a and donors.
PTH (pg/mL) $P < 0.05$ HD, CKD stage 3b–5 versus CKD stage 1–3a and donors.

Table 2. Pharmacological treatment of the patients enrolled in the study

Medication use	HD	CKD stage 3b–5	CKD stage 1–3a	Donors
Calcium-based phosphate binders				
Percentage of treated patients	11	25	0	0
Average daily dosage (mg)	2000	1700		
Calcium-free phosphate binders (sevelamer)				
Percentage of treated patients	55	20	0	0
Average daily dosage (mg)	4160	3200		
Vitamin D3 (per os)				
Percentage of treated patients	67	85	33	0
Average daily dosage (μg)	11.6	14.4	11.2	
Paricalcitol				
Percentage of treated patients	33	0	0	0
Average weekly dosage (mg)	15			

HCO_3 35 mmol/L, K 1.5 mmol/L, Ca 1.5 mmol/L and Mg 0.75 mmol/L) and 1.6–1.8 m^2 dialysers. HD patients were dialysed for at least 6 months with synthetic membranes [polysulphon FX60, ultrafiltration coefficient (UFC) 38 mL/h/mmHg; FX80 UFC 53 mL/h/mmHg; Fresenius, Bad Homburg, Germany). Patients with systemic autoimmune disorders, infectious diseases, neoplasm or inflammatory diseases and patients receiving antibiotics, corticosteroids, calcimimetics, glucocorticoids, bisphosphonates or non-steroidal anti-inflammatory agents were excluded. Our population was selected excluding the patients being treated with drugs that may interfere with spontaneous osteoclastogenesis. According to the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, patients suffering from altered bone mineral disease were treated with phosphate binders, calcitriol or a vitamin D analogue to maintain serum calcium and phosphorus in the normal range [26].

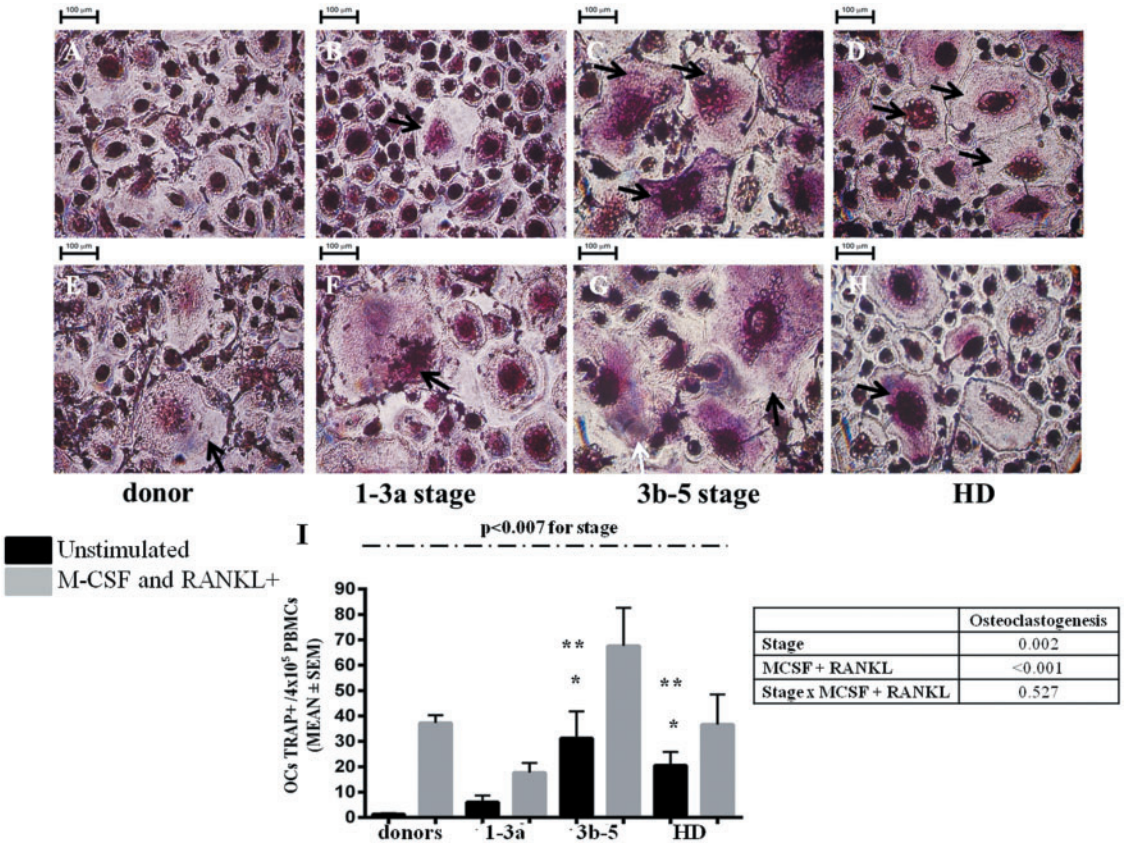
Cell cultures

Peripheral blood mononuclear cells (PBMCs), isolated as previously reported [15], were cultured in 96-well plates (4×10^5 cells/well) with α-minimal essential medium (α-MEM; Gibco, Uxbridge, UK) supplemented with 10% foetal bovine serum in the absence or presence of recombinant human M-CSF (25 ng/mL) and RANKL (30 ng/mL) (both from R&D Systems, Minneapolis, MN, USA) in quintuplicate for each condition. In some experiments, PBMCs were cultured in the presence of increasing concentrations of RANK-Fc (20–100 ng/mL) and human anti-LIGHT (0.005–500 ng/mL) (both from R&D Systems). At the end of the culture period (∼21 days), mature OCs were stained by tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St Louis, MO, USA) and identified as TRAP-positive multinucleated cells containing eight or more nuclei. The culture medium pH was measured at day 10 and at the termination of an experiment using a blood gas analyser (ABL 330 or ABL 705, Radiometer, Copenhagen, Denmark).

To study the resorbing activity of OCs, PBMCs were plated on Osteo Assay Surface well plates (Corning, Union City, CA, USA).

Flow cytometry

Fresh ethylenediaminetetraacetic acid peripheral blood from controls and patients was stained with conjugated antibody:



	Osteoclastogenesis
Stage	0.002
MCSF + RANKL	<0.001
Stage x MCSF + RANKL	0.527

FIGURE 1: OC differentiation from PBMCs of HD, CKD stage 3b–5, CKD stage 1–3a patients and healthy donors. OCs were obtained from unfractionated PBMCs of healthy donors (**A** and **E**), CKD stage 1–3a patients (**B** and **F**), CKD stage 3b–5 (**C** and **G**) and HD patients (**D** and **H**) and were cultured for 21 days in the absence (A–D) or presence (E–H) of rh-M-CSF and rh-RANKL. After this period, the cultures stained with TRAP showed multinucleated OCs (purple colour, original magnification ×10, scale bar = 100 µm). The graph (**I**) represents the mean ± SEM of the OC number for each group of patients (10 healthy donors, 12 CKD stage 1–3a, 20 CKD stage 3b–5 and 18 HD patients) obtained after culture with (grey histograms) or without (black histograms) rh-M-CSF and rh-RANKL. P = 0.007, for comparison of spontaneous osteoclastogenesis from all groups (one-way ANOVA on ranks) and *P < 0.05 versus controls, **P < 0.05 versus CKD stage 1–3a patients (Dunn's *post hoc* test). Results reported are for two-way ANOVA on treated and untreated cultures for controls and patients. The photomicrographs were obtained using a Zeiss IM35 microscope fitted with a Coolpix 990 digital camera (Nikon, Calenzano, Italy).

Pc5.5-CD8, FITC-CD4, Pc5.5-CD14, PE-CD16, PE-CD11b, PE-CD16 (all Beckmann Coulter, Milan, Italy), PE-CD51/61 (BD Pharmingen, San Diego, CA, USA), PE-RANKL or PE-LIGHT (R&D Systems). Data were acquired using an FC500 flow cytometer and analysed using CXP software (both Beckmann Coulter). The area of positivity was determined using an isotype-matched monoclonal antibody (mAb).

Enzyme-linked immunosorbent assay (ELISA)

C-terminal telopeptides of type I collagen (CTX), LIGHT and soluble RANKL were measured with a Serum Cross Laps ELISA kit (Immunodiagnostic Systems, Boldon, UK), human LIGHT/TNFSF14 ELISA kit (R&D Systems) and a FREE soluble RANKL High Sensitivity ELISA (third-generation; Biomedica Immunoassays, Vienna, Austria), respectively, according to the manufacturer's instructions.

Statistical analyses

Experimental data were compared using an analysis of variance (ANOVA) test. P-values <0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Parameters with normal distribution linear correlations were calculated with Pearson's correlation coefficient, whereas parameters with skewed distribution were calculated using Spearman's correlation coefficient.

RESULTS

PBMCs from HD and CKD stage 3b–5 patients spontaneously differentiate into mature OCs

First, to evaluate the capacity of PBMCs from patients and controls to differentiate into mature OCs, we cultured them with or without recombinant human RANKL (rh-RANKL) and rh-M-CSF, which are known to promote osteoclastogenesis. OCs are usually considered as multinucleated cells with more than three nuclei; however, in our study we focused on cells with eight or more nuclei since they were predominant in the cultures from patients. Rare OCs were observed in the PBMC cultures from healthy donors (Figure 1A) and CKD stage 1–3a patients (Figure 1B), while the addition of rh-M-CSF and rh-RANKL in the same cultures induced osteoclastogenesis (Figure 1E and F, respectively). In contrast, in the cultures from CKD stage 3b–5 and HD patients, we observed a high number of OCs that were very large and multinucleated both in the

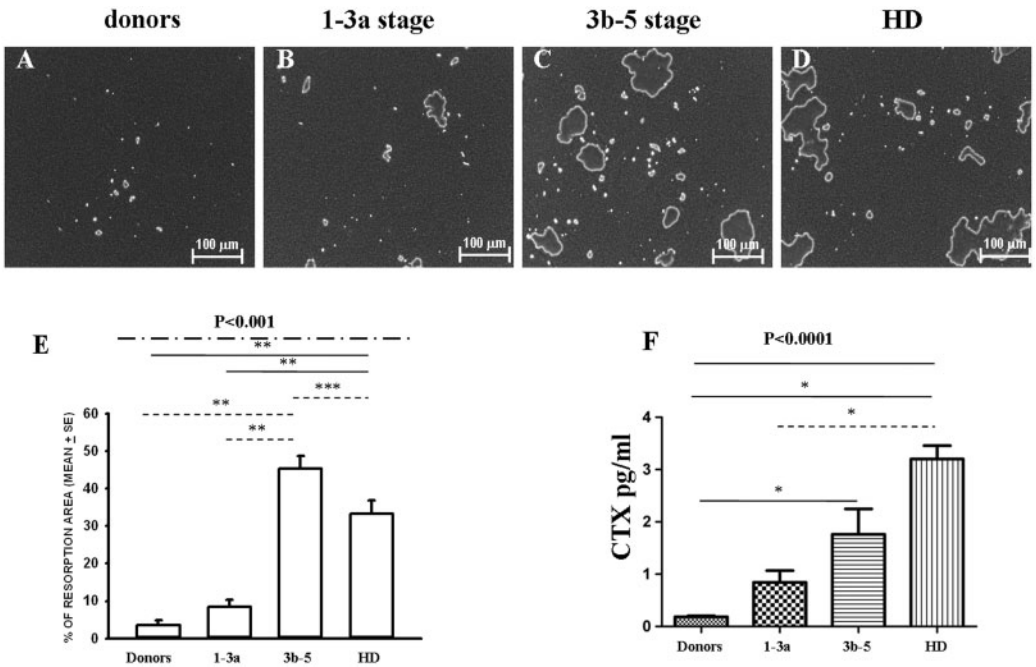


FIGURE 2: Bone resorption assay as functional evidence of OC differentiation. PBMCs were cultured on specific 24-well plates coated with a specific degradable matrix for OCs for 25–30 days. Resorbing areas were microphotographed in cultures from **(A)** healthy donors and **(B)** CKD stage 1–3a, **(C)** CKD stage 3b–5 and **(D)** HD patients. The figures are representative of three independent experiments for each group of patients (HD, CKD stage 3b–5 and CKD stage 1–3a) and healthy donors (original magnification $\times 10$, scale bar $= 100$ µm) and the results are expressed as mean $\pm$ SE of the percentage of the resorption area. **(E)** $P < 0.001$, for comparison of all groups (one-way ANOVA) and $^{**}P < 0.001$, $^{***}P < 0.02$ (Bonferroni *post hoc* test). The photomicrographs of resorption pits were obtained using a Nikon Ellipse E400 microscope equipped with a Nikon Plan Fluor 10×/0.30 dicl. The microscope was connected to a Nikon digital camera D × M 1200; the acquisition software was Lucia G version 4.61 (build 0.64) from Nikon. The percentage of mineral surface resorbed by the OCs was quantified with the image analyser Lucia G. Concentrations of soluble CTX in 10 healthy donors and 12 CKD stage 1–3a, 20 CKD stage 3b–5 and 18 HD patients are shown. We found that CTX was significantly increased in CKD stage 1–3a (0.84 $\pm$ 0.22 pg/mL), CKD stage 3b–5 (1.9 $\pm$ 0.33 pg/mL) and HD (3.20 $\pm$ 0.25 pg/mL) patients as compared with healthy donors (0.18 $\pm$ 0.02 pg/mL; $P < 0.05$). Furthermore, a significant increase in circulating CTX levels was observed in HD patients compared with CKD stage 1–3a patients ($P < 0.05$). **(F)** $P < 0.0001$, for comparison of all groups (one-way ANOVA on ranks) and $^{*}P < 0.05$ (Dunn's *post hoc* test).

unstimulated PBMCs (Figure 1C and D) and in the cultures with rh-M-CSF and rh-RANKL (Figure 1G and H). We divided CKD stage 3 patients into two groups, 3a and 3b patients, because we observed the first significant modification of osteoclastogenesis in CKD stage 3b patients. In detail, slight spontaneous osteoclastogenesis was observed in CKD stage 1–3a patients (6 ± 2 OCs per well), but it did not reach statistical significance when compared with healthy donors. Instead, spontaneous osteoclastogenesis increased with the progression of renal failure and was significantly higher in CKD stage 3b–5 (31.4 ± 10.9 OCs) and HD (20.5 ± 5 OCs) patients when compared with healthy donors (1.3 ± 0.4 OCs; $P < 0.007$, one-way ANOVA on ranks) (Figure 1I).

The addition of M-CSF and RANKL to PBMC cultures from healthy donors and patients significantly increased the number of OCs compared with the same cultures without stimuli (two-way ANOVA). The results of the two-way ANOVA are reported in Figure 1 and show no significant interaction between stage and growth factor, indicating that cells from patients and healthy controls react to stimulation with M-CSF/RANKL in the same way.

Interestingly, the morphology and size of OCs were similar in the healthy controls and CKD stage 1–3a patients when compared with the CKD stage 3b–5 and HD patients. OCs from CKD stage 3b–5 patients appeared larger than those from HD patients. Although it is known that pH could affect osteoclastogenesis, no significant differences were detected between the media pH of patients and controls (data not shown).

OC erosive activity increased in advanced CKD and HD patients

To evaluate the resorption activity of OCs derived from PBMCs of each group of patients, we cultured PBMCs on specific cell culture plates coated with a calcium phosphate membrane. We found that OCs derived from PBMCs of healthy donors and CKD stage 1–3a patients (Figure 2A and B) generated very few and small resorption areas, contrary to OCs derived from PBMCs of CKD stage 3b–5 and HD patients, which generated strikingly large resorption areas (Figure 2C and D). The differences between CKD stage 3b–5 and HD patients and healthy donors and CKD stage 1–3a patients were statistically significant (Figure 2E; $P < 0.001$).

To further establish the importance of active osteoclastogenesis from PBMCs of HD and advanced CKD patients, we evaluated the erosive activity of *in vivo* OCs by measuring CTX, a specific serum marker of bone resorption. CTX levels were significantly higher in patients compared with the controls ($P < 0.0001$), as shown in Figure 2F.

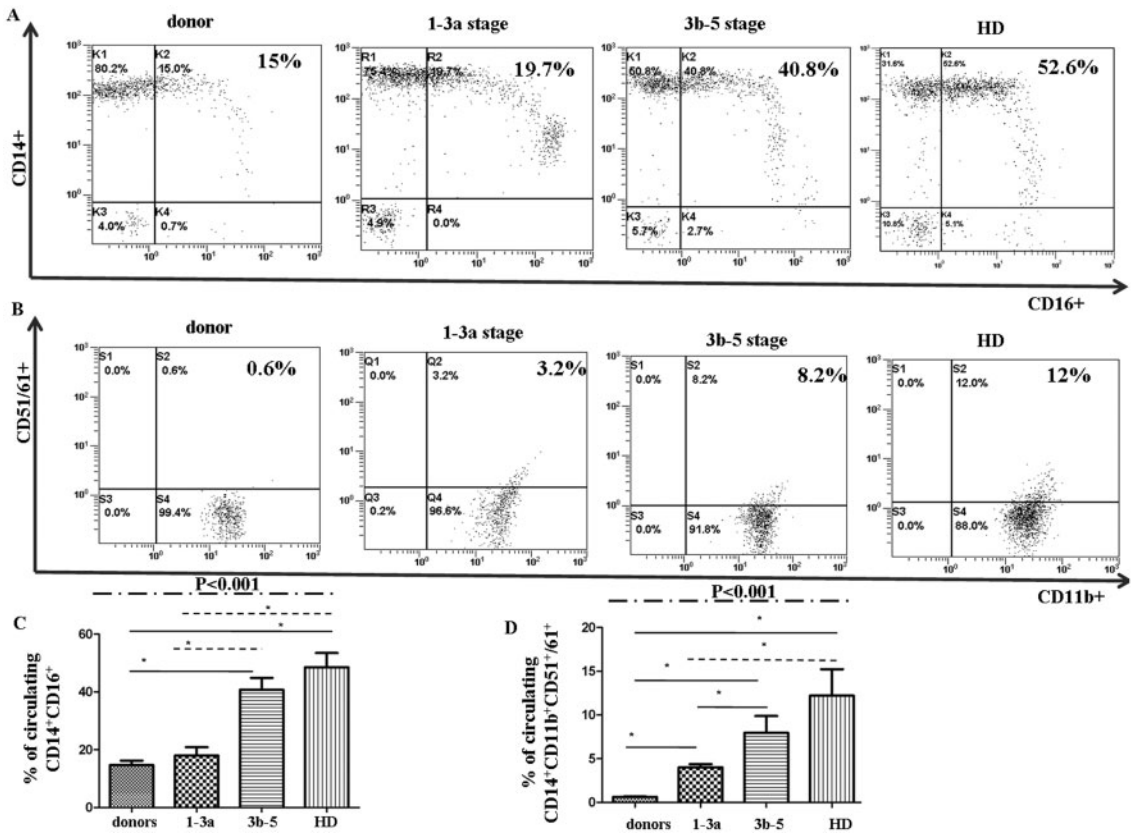


FIGURE 3: $CD14^+CD16^+$ and $CD14^+CD11b^+CD51\text{-}61$ monocytes are increased in CKD and HD patients. PBCMs isolated from healthy donors, or CKD stage 1–3a, stage 3b–5 or HD patients were analysed by flow cytometry to evaluate the proportions of the (**A**) different monocyte or (**B**) OC precursor subsets. Dot plots from one representative experiment are shown in (A) and (B). The percentage of circulating (**C**) $CD14^+CD16^+$ and (**D**) $CD14^+CD11b^+CD51/CD61^+$ monocytes are shown (mean ± SEM). Mean results of values obtained from 10 healthy donors and 12 CKD stage 1–3, 20 CKD stage 3b–5 and 18 HD patients. $P < 0.001$, for comparison of all groups (one-way ANOVA on ranks), and $^*P < 0.05$ (Dunn's *post hoc* test).

$CD14^+CD16^+$ and $CD14^+CD11b^+CD51/61^+$ monocytes increased with progression of renal disease

Some monocyte subsets may contribute to osteoclastogenesis, differentiating into mature OCs or releasing pro-osteoclastogenic cytokines, hence we evaluated the percentage of circulating $CD14^+CD16^+$ and $CD14^+CD11b^+CD51/61^+$ monocyte subsets. As shown in Figure 3A and C, $CD14^+CD16^+$ monocytes were significantly higher ($P < 0.05$) in CKD stage 3b–5 ($40.7\% \pm 4$) and HD ($49.5\% \pm 5$) patients as compared with healthy donors ($14.7\% \pm 1$; $P < 0.05$) and CKD stage 1–3a patients ($18\% \pm 3$; $P < 0.05$). All the results were significant with $P < 0.001$. Similarly, $CD14^+CD11b^+CD51/61^+$ monocytes progressively increased in CKD stage 1–3a ($4\% \pm 0.4$), CKD stage 3b–5 ($7.9\% \pm 2$) and HD ($12.2\% \pm 3$) patients when compared with healthy donors ($0.6\% \pm 0.04$; $P < 0.001$). A higher percentage of $CD14^+CD11b^+CD51/61^+$ monocytes was observed in HD patients as compared with CKD stage 1–3a patients ($P < 0.05$) (Figure 3B–D). Both pro-inflammatory monocyte subsets increased with the impairment of renal function and probably contribute to osteoclastogenesis.

RANKL and LIGHT mediate osteoclastogenesis in HD and CKD stage 3b–5 patients

We assessed the expression of both the RANKL and LIGHT cytokines on lymphomonocytes. We found that RANKL-expressing cells significantly increased with disease progression in CKD stage 1–3a ($2\% \pm 0.2$), CKD stage 3b–5 ($6.4\% \pm 0.7$) and HD patients ($8.6\% \pm 0.9$) as compared with healthy donors ($0.9\% \pm 0.3$; $P < 0.001$). At the different stages of renal disease, a significant increase in RANK-expressing cells was observed in

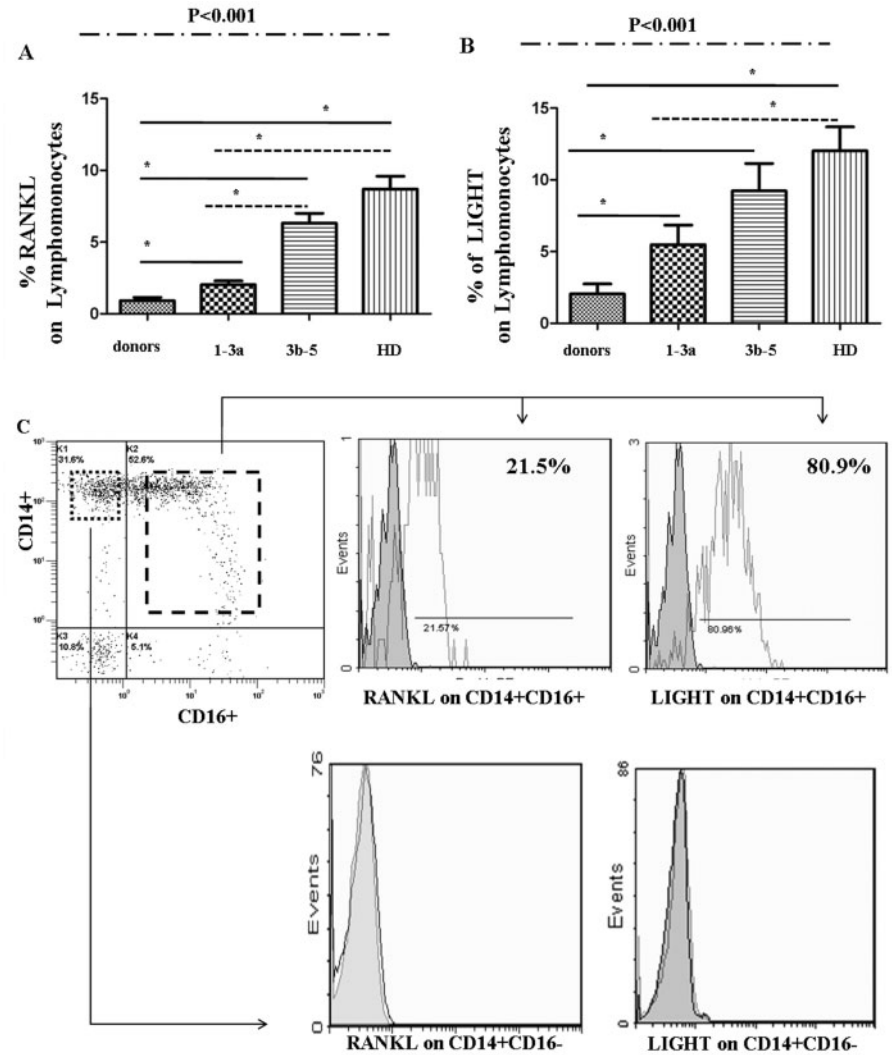


FIGURE 4: Expression of RANKL and LIGHT on lymphomonocytes is increased with progression of renal failure. PBMCs isolated from healthy donors and CKD stage 1–3a, CKD stage 3b–5 or HD patients were analysed by flow cytometry to evaluate the surface expression of two cytokines, RANKL and LIGHT, on lymphomonocytes. The histograms represent the mean ± SEM expression of (**A**) RANKL and (**B**) LIGHT on total lymphomonocytes, with values obtained from 10 healthy donors and 12 CKD stage 1–3a, 20 CKD stage 3b–5 and 18 HD patients. (**C**) Data from one representative experiment showing the expression of RANKL (middle panels) and LIGHT (right panels) on $CD14^+CD16^+$ (upper panels) and $CD14^+CD16^-$ monocytes (lower panels). The gating for the identification of monocyte subsets is shown in the dot plot (upper left panel). $P < 0.001$ for comparison of all groups (one-way ANOVA on ranks) and $^*P < 0.05$ (Dunn's *post hoc* test).

CKD stage 3b–5 and HD patients compared with CKD stage 1–3a patients ($P < 0.05$; Figure 4A). Interestingly, RANKL was slightly expressed on the T cell surface and highly expressed on monocytes and within monocyte subsets; RANKL was mostly expressed on the $CD14^{+}CD16^{+}$ monocyte population and almost absent on classical $CD14^{+}CD16^{-}$ monocytes (Figure 4C).

Similarly, LIGHT expression progressively increased with renal failure: expression in CKD stage 1–3a (5.4% ± 1.3), CKD stage 3b–5 (9.2% ± 1.9) and HD patients (12% ± 1.6) was significantly higher than in healthy donors (2% ± 0.7; $P < 0.001$). HD patients expressed significantly higher LIGHT levels when compared with CKD stage 1–3a patients ($P < 0.05$; Figure 4B). Also, this cytokine was mainly expressed on the $CD14^{+}CD16^{+}$ monocyte subset (Figure 4C) and only weakly on $CD14^{+}CD16^{-}$ monocytes.

Measurement of serum levels of RANKL and LIGHT revealed a slight but non-significant increase of RANKL serum levels in CKD stage 3b–5 and HD patients compared with CKD stage 1–3a and healthy donors (Figure 5A). In contrast, LIGHT levels significantly increased in CKD stage 3b–5 (159.8 ± 24.63 pg/mL) and HD (280 ± 38 pg/mL) patients as compared with CKD stage 1–3a patients (77.7 ± 13.7 pg/mL; $P < 0.05$) and healthy donors (83.3 ± 13 pg/mL; $P < 0.05$). All results were significant ($P < 0.001$; Figure 5B). These data show that RANKL and LIGHT production increases with the progression of renal disease and corroborates our results on OC formation and function in the same patients.

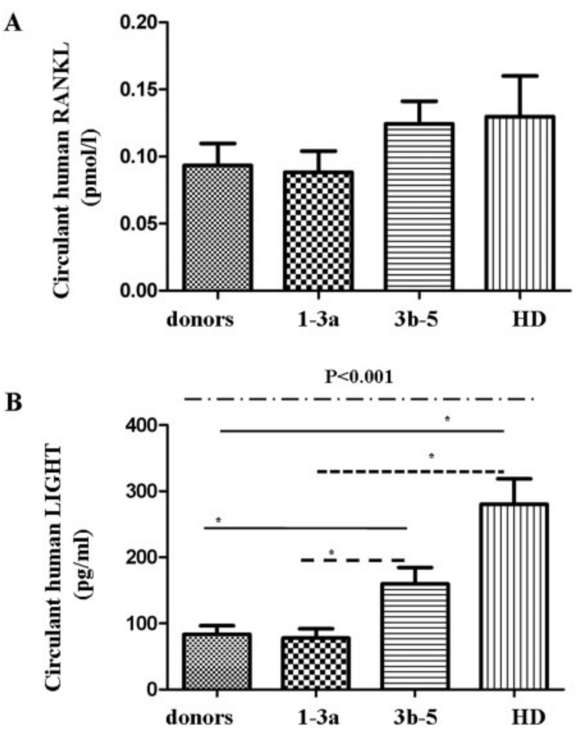


FIGURE 5: Serum LIGHT and RANKL are increased in late-stage CKD and HD patients. Serum levels of **(A)** RANKL and **(B)** LIGHT in healthy donors, CKD and HD patients were measured by ELISA. Results are shown as mean ± SEM of sera from 10 healthy donors and 12 CKD stage 1–3a, 20 CKD stage 3b–5 and 18 HD patients. $P < 0.001$ for comparison of all groups (one-way ANOVA on ranks) and $^{*}P < 0.05$ (Dunn's *post hoc* test).

Inhibition of RANKL and LIGHT mediates OC formation in PBMCs of HD and CKD stage 3b–5 patients

To confirm that RANKL and LIGHT are involved in the osteoclastogenic process in these patients, we cultured PBMCs from HD or CKD stage 3b–5 patients, in separate individual cultures, with their specific inhibitors (RANK-Fc and h-anti-LIGHT mAb). As shown in Figure 6A, we found that increasing doses of RANK-Fc led to a progressive reduction of OC number and size as compared with the culture without the inhibitor. The histogram in Figure 6C shows the reduction of osteoclastogenesis in the presence of 20 ng/mL RANK-Fc (18.5 ± 6), 50 ng/mL RANK-Fc (11.7 ± 3) and 100 ng/mL RANK-Fc (9.7 ± 2.4) as compared with culture in the absence of inhibitor (29.5 ± 8) (at 50 and 100 ng/mL, $P < 0.01$ and $P < 0.001$, respectively).

Accordingly, LIGHT inhibition resulted in a reduction of OC number in a dose-dependent manner (Figure 6B). The histogram in Figure 6D showed the reduction of osteoclastogenesis at 0.005 ng/mL (15 ± 3), at 100 ng/mL (10.5 ± 3) and at 500 ng/mL (6.7 ± 2) of h-anti-LIGHT compared with culture in the absence of inhibitor (24.5 ± 4) (at 100 and 500 ng/mL, $P < 0.01$ and $P < 0.001$, respectively). The inhibition of osteoclastogenesis could be observed at the lowest dose (0.005 ng/mL) of the anti-LIGHT mAb and was increased at 500 ng/mL.

Correlation between osteoclastogenic potential and the number of OCs with clinical and biochemical parameters

The number of OCs (TRAP-positive cells) significantly correlated with CKD stage, CTX level, OCp subsets, RANKL and LIGHT expression on lymphomonocytes, serum LIGHT and parathyroid hormone (PTH). LIGHT expression on lymphomonocytes significantly correlated with its serum level, whereas serum RANKL did not reach any statistical significance. OCp subsets also significantly correlated with stage, OC number, RANKL lymphomonocyte expression and serum LIGHT (Table 3).

DISCUSSION

We demonstrated here, for the first time, the spontaneous osteoclastogenic capacity of PBMCs from CKD stage 3b–5 and HD patients to differentiate into mature OCs upon the release of the inflammatory cytokines RANKL and LIGHT from activated immune cells. In contrast, osteoclastogenic stimuli, such as M-CSF and RANKL, were required to trigger this process in healthy donors and CKD stage 1–3a patients. We showed that these mature OCs presented erosive activity through significantly increased CTX serum levels, especially in advanced CKD and HD patients as compared with early CKD patients and healthy donors. Although we observed a slight reduction of resorption area from CKD stage 3b–5 to HD patients, there was

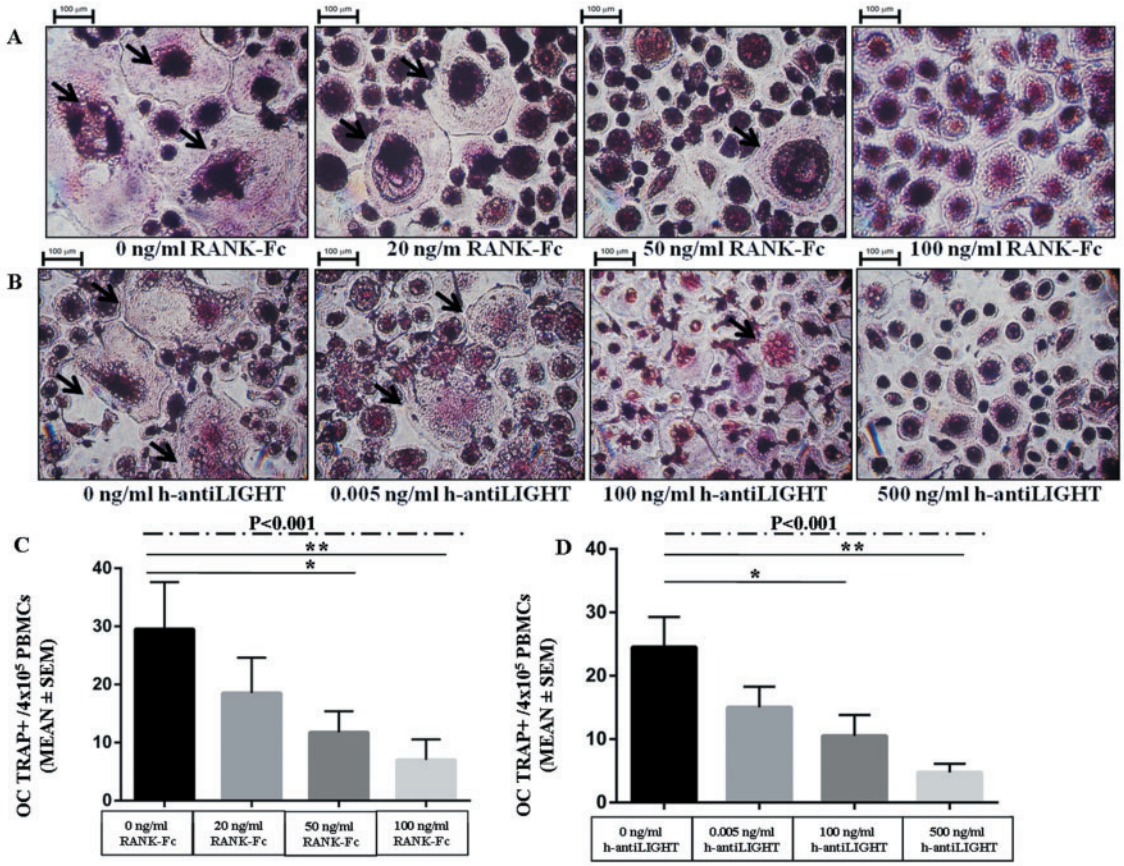


FIGURE 6: RANKL and LIGHT were relevant to promote osteoclastogenesis. Unfractionated PBMCs from CKD stage 3b–5 or HD patients were cultured without and with their specific inhibitors (RANK-Fc and h-anti-LIGHT mAb). (**A**) The panel shows the dose-dependent inhibitory effects exerted by RANK-Fc (at 20, 50 and 100 ng/mL) on OC formation from PBMCs of a representative CKD stage 5 patient as compared with the same cultures in the absence of RANK-Fc. (**B**) The panel shows the dose-dependent inhibitory effects exerted by h-anti-LIGHT (at 0.005, 100 and 500 ng/mL) in unfractionated PBMCs from a representative CKD stage 5 patient compared with PBMCs of the same patient in the absence of inhibitor. (**C** and **D**) The panels represent, respectively, the mean ± SEM of OC numbers with or without RANK-Fc and h-anti-LIGHT at the different concentrations. The graphs are representative of three independent experiments for each group of patients (HD and CKD stage 3b–5). $P < 0.001$ for comparison of all groups (one-way ANOVA), $^{*}P < 0.01$ and $^{**}P < 0.001$ (with Bonferroni *post hoc* test). Original magnification ×10, scale bar = 100 µm. The photomicrographs were obtained using a Zeiss IM35 microscope fitted with a Coolpix 990 digital camera (Nikon).

a gradual, continuous increase in CTX serum levels from CKD stage 3b–5 to HD patients. This may be explained by a reduced clearance of this peptide in HD patients.

We investigated which immune cell subsets and inflammatory cytokines are involved in the osteoclastogenic process in renal failure. It is already well known that the state of immune dysfunction in HD and CKD patients is due to an altered balance between immunoactivation and immunodepression. We found an increased percentage of $CD14^{+}CD16^{+}$ monocytes in HD and CKD stage 3b–5 patients, in which a spontaneous osteoclastogenesis was observed, compared with controls. This subpopulation is involved in the development of cardiovascular pathology [21] and inflammatory or autoimmune diseases [20], representing a potential marker of OCp in psoriatic arthritis [19]. These data and our findings suggest a potential role of $CD14^{++}CD16^{+}$ in the bone alterations observed in CKD and HD patients. Furthermore, another monocyte subset, $CD14^{+}/CD11b^{+}/CD51\text{-}61^{+}$, which is increased in advanced CKD and HD patients compared with CKD stage 1–3a patients and healthy donors, is particularly committed to the generation of OCs and increases in autoimmune disease patients [27]. Our data are in agreement with previous findings showing the crucial role of crosstalk between T cells and monocytes in osteoclastogenesis [28]. Elevated levels of RANKL and LIGHT were expressed on $CD14^{+}CD16^{+}$ monocytes of CKD stage 3b–5 and HD patients. Consequently, to prove our hypothesis that immune cells release osteoclastogenic factors, we inhibited RANKL and LIGHT in PBMCs cultured from CKD stage 3b–5 and HD patients and found that osteoclastogenesis was significantly decreased. This previously unsuspected implication of these monocyte subsets in bone resorption in advanced CKD patients is very interesting and deserves further investigation. Thus, in CKD stage 3b–5 and HD patients, the commitment of OCps and the presence of high levels of pro-osteoclastogenic cytokines sustain spontaneous osteoclastogenesis in culture, even if complete OC formation takes ∼21 days. These

Table 3. Statistical correlation between clinical parameters

	TRAP-positive OCs	CTX	$CD14^+$ $CD16^+$	$CD14^+CD16^+$ $CD14^+CD11b^+$ $CD51/61^+$	RANKL lymphomonocytes	LIGHT lymphomonocytes	Serum LIGHT	Serum RANKL	PTH	25-OH vitamin D
Stage	$r = 0.689$ $P = 0.0001$	$r = 0.652$ $P = 0.001$	$r = 0.591$ $P = 0.0001$	$r = 0.326$ $P = 0.03$	$r = 0.675$ $P = 0.0001$	$r = 0.437$ $P = 0.001$	$r = 0.566$ $P = 0.0001$	$r = 0.124$ $P = 0.392$	$r = 0328$ $P = 0.02$	$r = -0.331$ $P = 0.02$
TRAP-positive OCs		$r = 0.715$ $P = 0.0001$	$r = 0.573$ $P < 0.0001$	$r = 0.477$ $P = 0.002$	$r = 0.770$ $P < 0.0001$	$r = 0.535$ $P = 0.0002$	$r = 0.345$ $P = 0.02$	$r = 0.089$ $P = 0.576$	$r = 0.404$ $P = 0.02$	$r = -0.355$ $P = 0.059$
CTX			$r = 0.695$ $P < 0.0001$	$r = 0.588$ $P = 0.001$	$r = 0.580$ $P = 0.0008$	$r = 0.579$ $P = 0.0008$	$r = 0.484$ $P < 0.006$	$r = 0.200$ $P = 0.289$	$r = 0.605$ $P = 0.005$	$r = -0.372$ $P = 0.106$
$CD14^+CD16^+$				$r = 0.262$ $P = 0.053$	$r = 0.608$ $P = 0.0001$	$r = 0.682$ $P = 0.0001$	$r = 0.533$ $P = 0.0001$	$r = 0.213$ $P = 0.102$	$r = 0.110$ $P = 0.463$	$r = -0.217$ $P = 0.143$
$CD14^+CD11b^+$ $CD51/61^+$ RANKL Lymphomonocytes					$r = 0.396$ $P = 0.003$	$r = 0.230$ $P = 0.092$	$r = 0.381$ $P = 0.004$	$r = 0.017$ $P = 0.900$	$r = 0.135$ $P = 0.392$	$r = -0.087$ $P = 0.585$
LIGHT Lymphomonocytes						$r = 0.551$ $P = 0.0001$	$r = 0.395$ $P = 0.002$	$r = 0.017$ $P = 0.898$	$r = 0.242$ $P = 0.101$	$r = -0.260$ $P = 0.07$
Serum LIGHT							$r = 0.460$ $P = 0.0002$	$r = 0.187$ $P = 0.152$	$r = 0.288$ $P = 0.04$	$r = -0.161$ $P = 0.279$
Serum RANKL								$r = 0.142$ $P = 0.180$	$r = 0.191$ $P = 0.198$	$r = -0.311$ $P = 0.03$
PTH									$r = 0.053$ $P = 0.725$	$r = -0.284$ $P = 0.053$
										$r = -0.276$ $P = 0.0605$

settings have been also demonstrated in other types of bone disease [29].

We also assessed the presence of serum LIGHT and RANKL levels and found increased levels of LIGHT in CKD stage 3b–5 and HD patients compared with CKD stage 1–3a patients and healthy donors. We observed a discrepancy between the RANKL measurement in serum and that expressed on lymphomonocytes. Indeed, the concentration of the serum cytokine was not significantly modulated with renal failure progression. This could be due to the increase of circulating OPG levels in CKD patients, which is known to act as a decoy receptor for RANKL and is responsible for its sequestration [30]. Taken together, our data clearly show that the immune system influences bone homeostasis in HD and CKD stage 3b–5 patients with increased systemic inflammation. Thus bone alteration in advanced CKD stages and HD patients does not exclusively depend on biochemical parameters but also on immune and inflammatory alterations.

The functional connection between the immune and skeletal systems and how this crosstalk alters the structure of the bones is well understood [31]. Bone disorders are also observed in patients with renal disease. Indeed, CKD–mineral bone disorder is a common complication for patients and increases the risk of bone fractures as well as all-cause and cardiovascular mortality among CKD and HD patients, placing an enormous financial burden on health care systems [32]. Although deranged parathyroid function is currently considered to be the fundamental alteration responsible for bone disease in CKD patients [33], over the last two decades, pro-inflammatory cytokines have fully emerged as major players in bone remodelling, alongside PTH and other major hormonal regulators of bone metabolism. To date, the causes of bone disorder in CKD patients have been mainly attributed to biochemical abnormalities such as alterations in calcium, phosphorus and PTH or the vitamin D–fibroblast growth factor 23–Klotho axis [34]. These parameters, together with dual-energy X-ray absorptiometry or, more rarely, bone biopsy, represent the factors that are most often considered for the diagnosis of bone disorder in CKD patients; as a consequence, current therapies are mainly formulated to restore these biochemical alterations [35]. The efficacy of drugs interfering with the RANKL pathway has been demonstrated in osteoporosis treatment in elderly women [36], including CKD stage 2–4 patients [37]. Inflammation is a feature of advanced CKD; indeed, TNF-α is associated with incident fractures in CKD stage 5D patients, supporting the hypothesis that systemic inflammation might contribute to the increased bone fracture risk in these patients [38]. The issue is of relevance because bone fractures in CKD patients not only engender disabling orthopaedic problems but are also associated with increased mortality [39]. RANKL is the therapeutic target of denosumab, a human mAb, and recent studies have reported its treatment benefit for preventing bone loss in patients with rheumatoid arthritis and postmenopausal women [40, 41]. In contrast, no drug is currently available that targets LIGHT. These promising findings will hopefully lead to the identification of new therapeutic targets and need to be confirmed in larger cohorts of CKD patients to enable the gathering of a substantially greater number of bone events.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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