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ORIGINAL ARTICLE

Cinacalcet effect on polymorphonuclear leucocytes of kidney transplant patients

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ABSTRACT

Background Polymorphonuclear leucocytes (PMNLs) play a key role in the nonspecific immune defence. Cinacalcet reduces serum calcium levels in kidney transplant recipients with mineral bone disorder associated with chronic kidney disease. We investigated essential functions of PMNLs of kidney transplant recipients with and without hypercalcaemia and with and without cinacalcet therapy.

Subjects and methods Oxidative burst, phagocytosis, apoptosis and intracellular calcium concentrations of PMNLs from normocalcaemic kidney transplant patients without (KT-NC) or with cinacalcet intake (KT-NC/CI), hypercalcaemic kidney transplant patients (KT-HC) and healthy subjects (HS) were investigated.

Results Stimulation of oxidative burst of PMNLs from KT-HC patients by phorbol-12-myristate-13-acetate or *Escherichia coli* was significantly attenuated compared with PMNLs from KT-NC, KT-NC/CI and HS. Apoptosis of PMNLs from KT-HC patients was significantly decreased compared with cells from KT-NC, KT-NC/CI and HS. Apoptosis correlated significantly with serum calcium concentrations. Intracellular calcium concentrations and phagocytosis of PMNLs did not differ between groups.

Conclusions Our data indicate that stimulation of PMNL oxidative burst and apoptosis is significantly diminished in kidney transplant patients with hypercalcaemia, while kidney transplant patients with serum calcium levels normalized by cinacalcet have normal PMNL functions despite immunosuppressive therapy.

Keywords Apoptosis, cinacalcet, oxidative burst, polymorphonuclear leucocytes, serum calcium.

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Introduction

Polymorphonuclear leucocytes (PMNLs) play a key role in the nonspecific immune defence against bacterial infections. They migrate to the site of infection along a chemotactic gradient, ingest the invading microorganisms by phagocytosis and kill them with proteolytic enzymes and reactive oxygen radicals (ROS) produced during the oxidative burst. Disturbances of one of these essential functions will lead to an increased risk for infections. Whereas the killing of invading microorganisms is crucial for a successful immune response, the coordinated removal of the senescent apoptotic PMNLs by macrophages is important for the resolution of inflammation.

Changes in the concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) mediate the regulation of PMNL functions such as oxidative burst and degranulation as well as PMNL apoptosis [1]. Substances leading to a temporary raise in $[Ca^{2+}]_i$ accelerate the apoptotic cell death of PMNLs [2]. Elevated levels of $[Ca^{2+}]_i$ are associated with cellular dysfunctions found, for example, in diabetes mellitus [3] and chronic kidney disease (CKD) [4].

Cardiovascular disease and infections are the major causes of morbidity and mortality in CKD patients [5,6]. Defective PMNL functions lead to an increased incidence of infections and a state of (micro-)inflammation related to cardiovascular risk in CKD [7]. On the other hand, reports on PMNL functions of kidney transplant recipients are scarce [8].

Mineral bone disorder associated with CKD (CKD-MBD) is further increasing cardiovascular morbidity as well as inflammation. Whether CKD-MBD in kidney transplant patients is associated with PMNL dysfunction has not been investigated. Cinacalcet, a second-generation calcimimetic, is used in the therapeutic management of post-transplant hyperparathyroidism [9]. Cinacalcet reduces serum calcium in kidney transplant recipients with CKD-MBD by decreasing parathyroid hormone (PTH) levels and increasing the urinary calcium excretion [10]. However, it is not known whether cinacalcet affects $[Ca^{2+}]_i$ and/or essential functions of PMNLs in kidney transplant recipients and thereby modulates the immune status of those patients.

Subjects and methods

Patients

We studied 34 stable kidney transplant patients and 17 healthy subjects (HS). Kidney transplant patients were subdivided into three groups: Normocalcaemic kidney transplant patients (KT-NC), hypercalcaemic kidney transplant patients (serum calcium > 2.65 mM) due to persistent post-transplant hyperparathyroidism (KT-HC), kidney transplant patients normocalcaemic under cinacalcet therapy (30–180 mg cinacalcet per day) for > 3 months for the treatment of post-transplant hyperparathyroidism (KT-NC/CI). Immunosuppressive therapy consisted of different immunosuppressive drugs that did not differ between groups (Table 1). The groups were matched with regard to age and gender. Parameters of the study population and relevant serum concentrations in kidney transplant patients are shown in Tables 1 and 2, respectively. Smokers and persons who took medications affecting calcium metabolism other than cinacalcet (e.g. native vitamin D, calcitriol, alphacalcidol or paricalcitol) within 3 months before taking blood samples as well as persons having infection or inflammation were excluded. The study was approved by the local ethic committee (Medical University of Vienna; Nr: 751/2008). Written informed consent was obtained from all participants of the study.

Blood analysis

Serum creatinine and electrolytes were determined by routine laboratory methods. Intact PTH level was measured by an electrochemiluminescent immunoassay, 25-hydroxy vitamin D (25(OH)D) by a chemiluminescent immunoassay and 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) by a radioimmunoassay.

Oxidative burst

The quantitative determination of the PMNL oxidative burst was carried out in heparinized whole blood. The oxidative burst was stimulated by unlabelled opsonized *Escherichia coli* or by phorbol-12-myristate-13-acetate (PMA). After lysis of erythrocytes and fixation, the conversion of dihydrorhodamine 123 to rhodamine 123 by intracellularly produced H_2O_2 was measured using flow cytometry (Epics XL-MCL; Coulter, Hialeah,

Table 1 Epidemiology of the study population

	KT-NC (n = 16)	KT-HC (n = 9)	KT-NC/CI (n = 9)	HS (n = 17)	Statistics
Age (year)	57.4 ± 2.6	54.6 ± 3.6	55.4 ± 2.5	53.5 ± 2.1	n.s.
Gender (female/male)	4/12	3/6	4/5	6/11	n.a.
Native kidney disease					
Glomerulonephritis	5	3	2	n.a.	n.a.
Diabetic nephropathy	1	1	1		
Polycystic kidney disease	2	3	3		
Others	3	1	1		
Unknown	5	1	2		
Time after transplantation (year)	7.3 ± 1.2	2.4 ± 0.5	2.9 ± 0.6	n.a.	*
Immunosuppression					
CsA/Tacrolimus	2/8	3/6	3/7	n.a.	n.a.
MMF/Azathioprine	12/1	8/0	8/0		
Sirolimus/Everolimus	1/1	0/0	0/0		
Leflunomid/Belatacept	0/3	1/0	2/0		
Steroids	15	9	9		
Calcium antagonists	6	6	3	n.a.	n.a.
Angiotensin-converting enzyme inhibitors	5	1	5		

HS, healthy subjects; KT-NC, normocalcaemic kidney transplant patients not receiving cinacalcet; KT-HC, kidney transplant patients with hypercalcaemia; KT-NC/CI, normocalcaemic kidney transplant patients due to cinacalcet therapy. n.a., not applicable; n.s., not significant; CsA, cyclosporine A; MMF, mycophenolate mofetil.

*P < 0.01 KT-NC vs. KT-HC and KT-NC/CI; Mean value ± SEM.

Table 2 Serum concentrations of relevant parameters in kidney transplant patients

	KT-NC (n = 16)	KT-HC (n = 9)	KT-NC/CI (n = 9)	Statistics
Total calcium (mM)	2.45 ± 0.03	2.79 ± 0. 03	2.40 ± 0.05	*
Phosphate (mM)	0.93 ± 0.04	0.87 ± 0.05	0.95 ± 0.06	n.s.
Creatinine (mg/dL)	1.33 ± 0.12	1.61 ± 0.14	1.71 ± 0.29	n.s.
Parathyroid hormone (pg/mL)	145 ± 49	181 ± 28	186 ± 37	n.s.
25(OH)D (nM)	44.9 ± 6.0	58.9 ± 7.0	43.9 ± 5.0	n.s.
$1,25(OH)_2D$ (pg/mL)	45 ± 5	58 ± 4	43 ± 8	n.s.

HS, healthy subjects; KT-NC, normocalcaemic kidney transplant patients not receiving cinacalcet; KT-HC, kidney transplant patients with hypercalcaemia; KT-NC/CI, normocalcaemic kidney transplant patients due to cinacalcet therapy. n.s., not significant; 25(OH)D, 25-hydroxy vitamin D; $1,25(OH)_2D$, 1,25- dihydroxy vitamin D.
*P < 0.0001 KT-HC vs. KT-NC and KT-NC/CI; Mean value ± SEM.

FL, USA). The basal value in the absence of any stimulation was set as 1.

Isolation of polymorphonuclear leucocytes

Polymorphonuclear leucocytes were isolated from heparinized blood of HS and kidney transplant patients. Discontinuous Ficoll-Hypaque density gradient centrifugation and hypotonic lysis of erythrocytes were used. The viability of the PMNLs obtained by this protocol was > 95% as determined under the fluorescence microscope.

Spontaneous polymorphonuclear leucocyte apoptosis

Incubations. Polymorphonuclear leucocytes isolated under sterile conditions were incubated at 37 °C for 20 h in phosphate-buffered saline (PBS, pH 7.4; Gibco, Paisley, UK) at 6×10^6 cells/mL. All samples contained 100 U/mL Penicillin-Streptomycin (Gibco).

Morphological features. Polymorphonuclear leucocytes were examined under the fluorescence microscope after mixing the cell suspension with the fluorescent DNA-binding dyes ethidium bromide (Gibco) and acridine orange (Merck, Darmstadt, Germany) at a final concentration of 5 μg/mL each. Acridine orange binds to DNA and appears green. Ethidium bromide is taken up only by PMNLs with a damaged plasma membrane and stains the DNA orange to a stronger extent. Because the DNA in nonapoptotic cells is structured within the nucleus and the DNA in apoptotic cells is condensed, viable nonapoptotic (green, structured nucleus), apoptotic (green, condensed nucleus) and secondary necrotic (orange, condensed nucleus) cells can be differentiated.

Analysis of the polymorphonuclear leucocyte DNA content by flow cytometry. Apoptotic cells have a lower DNA content as a consequence of DNA cleavage by an activated nuclease. PMNLs (1.2×10^6/200 μL) were centrifuged at 360 *g* for 20 min and washed twice with PBS. 250 μL ice-cold 70% ethanol was added to the pellet.

After 60 min incubation on ice, the PMNLs were centrifuged, washed once with PBS and resuspended in 200 μL PBS containing 250 μg/mL RNAse (type I-A; Sigma, St. Louis, MO, USA) and 50 μg/mL propidium iodide (Sigma). After 15 min incubation at room temperature in the dark, samples were kept on ice in the dark until flow cytometric analysis.

Data presentation of apoptosis. Our data are presented as percentage viable PMNLs. Because apoptotic PMNLs are in a stage between viability and secondary necrosis, and apoptotic PMNLs are readily phagocytozed under *in vivo* conditions, viable PMNLs are most important for the interpretation of our results. We never observed necrotic PMNLs that did not underwent apoptosis first.

Intracellular calcium concentrations of polymorphonuclear leucocytes

$[Ca^{2+}]_i$ was assessed by measuring fura-2 fluorescence in a Luminescence Spectrometer LS50B (Perkin Elmer, Langen, Germany). Four microlitre fura-2AM was added to 10^6 PMNLs in 4 mL Hank's buffer (HBSS; Gibco), incubated at 37 °C for 30 min in the dark and centrifuged at 300 *g* for 10 min at 4 °C. Thereafter, the PMNLs were kept on ice. The cells were taken up in 1 mL Hank's buffer; 0.5×10^6 PMNLs/mL was obtained by addition of Hank's buffer. As described by Grynkiewicz *et al.* [11], the fluorescence was measured at 505 nm using the 340/380 nm dual wavelength excitation spectrofluorometry. The basal $[Ca^{2+}]_i$, set as 100%, and the *N*-formyl-methionine-leucine-phenylalanine (fMLP)-stimulated $[Ca^{2+}]_i$ was determined.

Polymorphonuclear leucocyte phagocytosis

The phagocytotic activity of PMNLs was measured in heparinized whole blood. Flow cytometry (Epics XL-MCL) was used to determine the percentage of PMNLs that had taken up fluorescein isothiocyanate labelled opsonized *E. coli* and the

amount of ingested *E. coli* per PMNL (Phagotest; Opregen Pharma, Heidelberg, Germany).

Statistical analysis

For data analysis of assays testing the PMNL functions, the t-test was used. The correlation between parameters analysed with the program SPSS Statistics 20 (IBM). Data are presented as mean values ± standard error of the mean (SEM).

Results

Oxidative burst activity of polymorphonuclear leucocytes and serum calcium levels of kidney transplant patients

The basal, that is, unstimulated oxidative burst activity of PMNLs from kidney transplant patients correlates significantly with their serum calcium concentration ($P = 0.001$; $R = 0.621$) (Fig. 1a). The basal oxidative burst is increased in the KT-HC group, even though significance is not reached (Fig. 1b). We also observed a correlation between the basal PMNL oxidative burst and the serum calcium concentration in each KT subgroup that was significant in the KT-NC group only (Fig. 1c).

The oxidative burst of PMNLs from KT-NC and KT-NC/CI could be stimulated by PMA (Fig. 2a) to the same extent as of PMNLs from HS (HS: 14.4 ± 2.1-fold; KT-NC: 16.9 ± 2.6-fold; KT-NC/CI: 15.2 ± 2.3-fold). The stimulation of oxidative burst by *E. coli* (Fig. 2b) did also not differ between the three groups (HS: 25.5 ± 4.5-fold; KT-NC: 28.6 ± 4.0-fold; KT-NC/CI: 21.4 ± 3.9-fold). PMNLs from KT-HC patients responded to a significantly lesser degree to both stimuli (PMA: 6.7 ± 1.1-fold, $P < 0.05$; *E. coli*: 12.1 ± 2.5-fold, $P < 0.01$ vs. HS, KT-NC and KT-NC/CI).

Spontaneous apoptosis of polymorphonuclear leucocytes and serum calcium levels of kidney transplant patients

The spontaneous apoptosis of PMNLs from KT-HC patients determined by morphological criteria (Fig. 3a) was significantly decreased and as a consequence, their viability was significantly increased as compared with PMNLs of HS, KT-NC and KT-NC/CI patients (viability: KT-HC: 59 ± 2%; HS: 35 ± 3%; KT-NC: 37 ± 3%; KT-NC/CI: 37 ± 4%; $P < 0.001$). Assessing PMNL apoptosis by measuring the DNA content (Fig. 3c) gave similar results (viability: KT-HC: 60 ± 6%; HS: 34 ± 3%; KT-NC: 35 ± 3%; KT-NC/CI: 40 ± 4%; $P < 0.01$). We found a significant correlation between serum calcium concentration and PMNL apoptosis (Fig. 3b,d). In the individual KT subgroups, however, this correlation was not significant (data not shown).

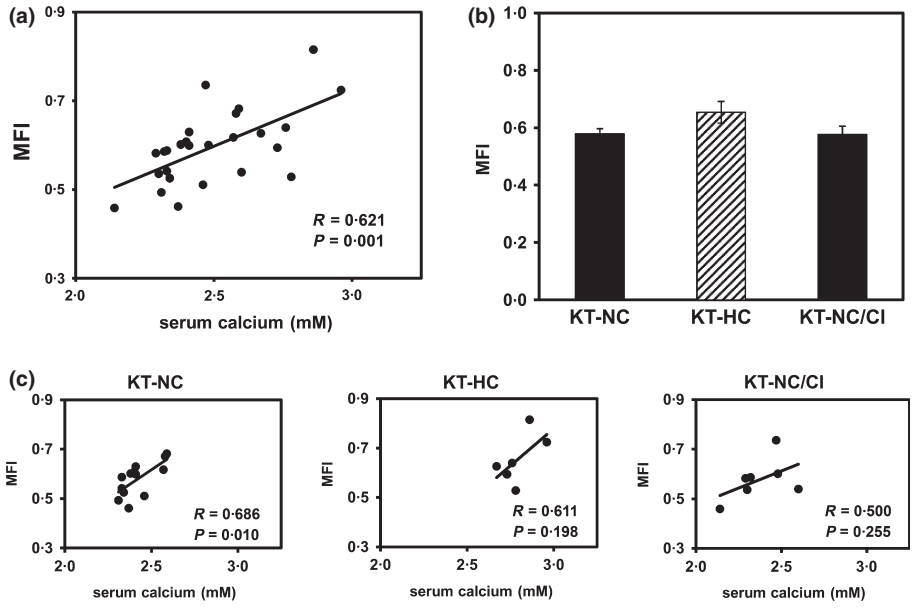

Figure 1 Basal oxidative burst of polymorphonuclear leucocytes from kidney transplant patients correlates with their serum calcium concentration (a). The basal polymorphonuclear leucocyte oxidative burst in heparinized whole blood from 26 kidney transplant patients was measured by flow cytometry and indicated as mean fluorescence intensity (MFI). (b) Basal oxidative burst of normocalcaemic kidney transplant patients not receiving cinacalcet as a control group (KT-NC; $n = 13$), kidney transplant patients with hypercalcaemia (KT-HC; $n = 6$) and normocalcaemic kidney transplant patients due to cinacalcet therapy (KT-NC/CI; $n = 7$). (c) Correlation of the basal oxidative burst with the serum calcium concentration for each KT subgroup.

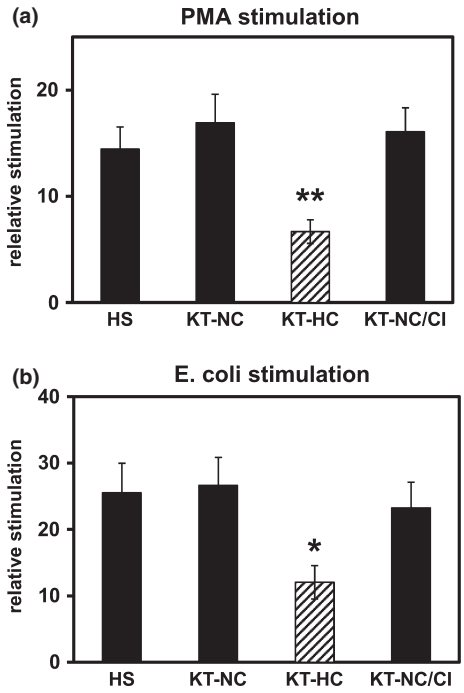

Figure 2 Stimulation of oxidative burst is diminished in polymorphonuclear leucocytes (PMNLs) from hypercalcaemic kidney transplant patients. The phorbol-12-myristate-13-acetate (PMA) (a) and *E. coli* (b) stimulated polymorphonuclear leucocyte oxidative burst in heparinized whole blood from healthy subjects (HS; $n = 17$), normocalcaemic kidney transplant patients not receiving cinacalcet as a control group (KT-NC; $n = 15$), kidney transplant patients with hypercalcaemia (KT-HC; $n = 9$) and normocalcaemic kidney transplant patients due to cinacalcet therapy (KT-NC/CI; $n = 10$) was measured by flow cytometry. The basal value was set as 1. $*P < 0.05$; $**P < 0.01$ vs. HS, KT-NC and KT-NC/CI. Mean values ± SEM.

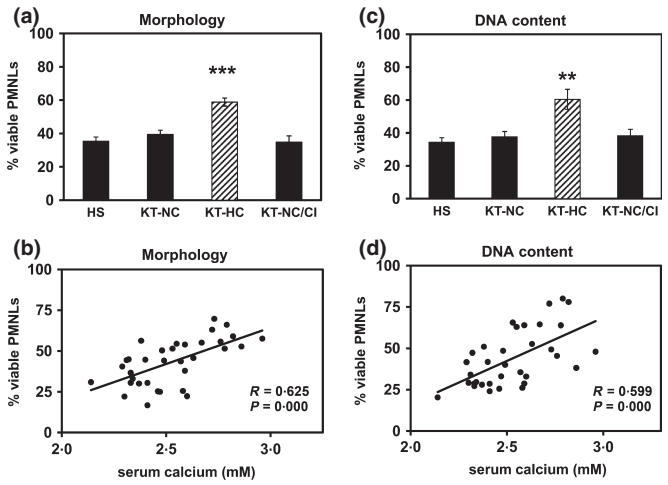

Figure 3 Spontaneous apoptosis and its correlation with serum calcium in polymorphonuclear leucocytes (PMNLs) from hypercalcaemic kidney transplant patients. The spontaneous apoptosis of PMNLs from healthy subjects (HS; $n = 17$), normocalcaemic kidney transplant patients not receiving cinacalcet (KT-NC; $n = 15$), kidney transplant patients with hypercalcaemia (KT-HC; $n = 9$) and normocalcaemic kidney transplant patients due to cinacalcet (KT-NC/CI; $n = 10$) was assessed by morphological criteria under the fluorescence microscope (a and b) and by measuring the DNA content by flow cytometry (c and d). The data are presented as percentage viable PMNLs. $**P < 0.01$; $***P < 0.001$ vs. HS, KT-NC and KT-NC/CI. Mean values ± SEM.

Intracellular calcium concentration of polymorphonuclear leucocytes

There was no significant difference between the basal $[Ca^{2+}]_i$ of PMNLs from HS, KT-HC, KT-NC and KT-NC/CI patients (HS: 88 ± 5 mM; KT-NC: 90 ± 6 mM; KT-HC: 75 ± 9 mM; KT-NC/CI: 73 ± 7 mM). Stimulation with the chemotactic tripeptide fMLP led to the same range of $[Ca^{2+}]_i$ increase in all groups (HS: 382 ± 44 mM; KT-NC: 356 ± 31 mM; KT-HC: 325 ± 31 mM; KT-NC/CI: 328 ± 48 mM). Cinacalcet therapy did not result in reduction of $[Ca^{2+}]_i$ despite normalization of serum calcium.

Phagocytotic capacity of polymorphonuclear leucocytes

Between 95% and 99% of PMNLs of all four groups took up opsonized *E. coli* bacteria in whole blood. The number of *E. coli* ingested per PMNL did not differ among the different groups (data not shown).

Discussion

We investigated essential functions of PMNLs of kidney transplant patients with and without hypercalcaemia and with and without cinacalcet therapy. Whereas ROS production of PMNLs of kidney transplant patients with normal serum calcium levels was the same as of PMNLs from HS, PMNLs from hypercalcaemic kidney transplant patients showed a significantly diminished stimulation of the oxidative burst either by PMA or *E. coli*. In the group of patients whose hypercalcaemia has been successfully corrected by cinacalcet, the response of PMNLs to both stimuli returned into the normal range. The apoptotic cell death of PMNLs from kidney transplant patients with hypercalcaemia was significantly attenuated. Apoptosis of PMNLs from patients whose serum calcium levels were normalized by cinacalcet was in the range of PMNLs obtained from HS despite immunosuppressive therapy.

Cinacalcet is a second-generation calcimimetic drug used to treat primary, secondary or tertiary hyperparathyroidism. It

effectively reduces elevated serum parathyroid hormone and elevated serum calcium levels also in patients with post-transplant hyperparathyroidism [12]. Cinacalcet is a positive allosteric modulator of the calcium-sensing receptor (CaR) [13], which is also involved in a variety of biological processes unrelated to calcium homoeostasis [14]. Whereas the expression of the CaR has been shown in monocyte-differentiated HL-60 cells [15] and in human peripheral blood monocytes [16] on the mRNA and protein level, CaR has not been described in PMNLs.

Polymorphonuclear leucocytes play a crucial role in the innate immune response. Most of their essential functions and features such as the oxidative burst and the apoptotic cell death are compromised in nondialysis patients with CKD and in patients undergoing regular haemodialysis or peritoneal dialysis treatment. While being lifesaving *per se*, dialysis treatment does not correct PMNL abnormalities induced by uraemia. Successful kidney transplantation, however, can normalize several of the uraemia-induced abnormal PMNL functions. For example, transplantation corrects priming of the PMNL oxidative burst [17] and the enhanced oxidative burst activity [8] observed in uraemic patients. Consistent with these reports, we found that oxidative burst activity does not differ between HS and kidney transplant patients with normal serum calcium levels. Hypercalcaemic kidney transplant patients, however, have a diminished response to stimulation of the oxidative burst that may come along with an increased risk for bacterial infections.

We also found a decreased rate of apoptosis in PMNLs of hypercalcaemic kidney transplant patients. ROS are involved in the regulation of PMNL apoptosis [18,19]. The ingestion of *E. coli* results in an increase in cellular ROS production and a corresponding induction of apoptosis. ROS are an important apoptotic signal and drive cells to apoptosis even in the absence of exogenous stimuli. Extracellular calcium can act with the CaR to abrogate apoptosis and indicate a role of calcium ions in regulating cell survival [20]. In the present study, we showed that spontaneous PMNL apoptosis is reduced in hypercalcaemic kidney transplant patients and correlates with the serum calcium concentration. Mild hypercalcaemia (serum calcium between 2.66 and 3.00 mg/dL) did not increase and cinacalcet therapy did not decrease normal $[Ca^{2+}]_i$ in PMNLs of kidney transplant patients. One may ask why cinacalcet therapy does not affect $[Ca^{2+}]_i$ in PMNLs of kidney transplant recipients. First, we have included in this study only patients with mild elevation of serum calcium. Severe hypercalcaemia is also associated with an increase in PMNL $[Ca^{2+}]_i$ [21]. Second, fortunately, normalization of serum calcium by cinacalcet therapy does not result in a decrease of normal $[Ca^{2+}]_i$ which could cause dysfunction of PMNLs. In contrast, elevated $[Ca^{2+}]_i$ decreases after parathyroidectomy [21].

We did not find differences in the serum PTH levels in patients with and without cinacalcet treatment (Table 2). This has already been observed previously [10,22] and does not reflect a lack of therapeutic efficacy. After administration of cinacalcet, PTH levels show a marked decrease within a few hours but increase to nearly initial values after 24 h [12].

An important finding of our study is that cinacalcet therapy in a dose used for kidney transplant patients does neither lower serum calcium level nor PMNL $[Ca^{2+}]_i$ below normal. Cinacalcet therapy *per se* does not alter PMNL apoptosis or oxidative burst. Both parameters are regulated by serum calcium in this patient population. Our data also indicate impairment of essential PMNL functions in kidney transplant patients by increased serum calcium even in the absence of elevated $[Ca^{2+}]_i$. Previous studies from our laboratory have already shown that serum calcium level and PMNL $[Ca^{2+}]_i$ do not correlate after parathyroidectomy in dialysis patients [21]. One may argue that $[Ca^{2+}]_i$ could be influenced by antihypertensive medication such as calcium channel blockers and/or angiotensin-converting enzyme (ACE) inhibitors. The KT subgroups, however, did not significantly differ regarding these drugs (Table 1).

Treatment of post-transplant hyperparathyroidism by cinacalcet normalizes not only elevated serum calcium but also restores PMNL functions. A limitation of our study is the transversal design. To prove the causal relationship between the normalization of calcium levels and the normalization of PMNL activities, a longitudinal study will be necessary, whereby patients with hypercalcemia are evaluated before and after cinacalcet treatment.

In conclusion, we found that the response of oxidative burst to stimuli and apoptosis of PMNLs from hypercalcaemic kidney transplant patients are significantly diminished. PMNLs from patients whose serum calcium levels have been normalized by cinacalcet therapy have normal PMNL functions. Activities of PMNLs from normocalcaemic kidney transplant patients without or with cinacalcet treatment do not differ from those of HS despite immunosuppressive therapy.

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Author contributions

G. C. performed PMNL functional assays, participated in the study's conception and design and in discussion, analysed the data and wrote the manuscript. J. R. performed PMNL functional assays and participated in data analysis. K. B. was responsible for recruiting the patients, participated in the study's conception and design and in discussion and edited the

manuscript. W. H. H. participated in the study's conception, discussion and edited the manuscript.

Conflict of interests

W. H. H. received speaker and consultant fee from Amgen. The other authors of this manuscript have no conflicts of interest to disclose. The manuscript was not prepared or funded by a commercial organization.

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