Research Article

Epigenetic Methylation of Parathyroid CaR and VDR Promoters in Experimental Secondary Hyperparathyroidism

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Secondary hyperparathyroidism (s-HPT) in uremia is characterized by decreased expression in the parathyroids of calcium sensing (CaR) and vitamin D receptors (VDR). Parathyroid hormone (PTH) is normalized despite low levels of CaR and VDR after experimental reversal of uremia. The expression of CaR in parathyroid cultures decreases rapidly. Methylation of promoter regions is often detected during epigenetic downregulation of gene expression. Therefore, using an experimental rat model, we examined changes in methylation levels of parathyroid CaR and VDR promoters in vivo and in vitro.

Methods. Uremia was induced by 5/6 nephrectomy. Melting temperature profiling of CaR and VDR PCR products after bisulfite treatment of genomic DNA from rat parathyroids was performed. Real-time PCR measured expression of PTH, CaR, VDR, and klotho genes in vitro.

Results. Parathyroids from uremic rats had similar low levels of methylation in vivo and in vitro. In culture, a significant downregulation of CaR, VDR, and klotho within two hours of incubation was observed, while housekeeping genes remained stable for 24 hours.

Conclusion. In uremic s-HPT and in vitro, no overall changes in methylation levels in the promoter regions of parathyroid CaR and VDR genes were found. Thus, epigenetic methylation of these promoters does not explain decreased parathyroid expression of CaR and VDR genes in uremic s-HPT.

1. Introduction

Secondary hyperparathyroidism in uremia (s-HPT)—a disorder caused by progressive loss of kidney function, low levels of active vitamin D (1,25(OH)2D (calcitriol)), increased phosphate retention, and low levels of plasma ionized calcium (Ca2+)—results in the highly elevated synthesis and secretion of parathyroid hormone (PTH) and enlargement of the parathyroid glands in order to maintain normal plasma Ca2+ and phosphate levels.

The calcium-sensing receptor (CaR) plays a key role in maintaining Ca2+ concentrations in extracellular fluids within a narrow range, primarily by modulating the function of the parathyroid glands. The CaR belongs to family C of the superfamily of seven transmembrane G-protein-coupled receptors. It regulates the biosynthesis and secretion of parathyroid hormone (PTH), as well as parathyroid cell proliferation, which is inhibited at high Ca2+ concentrations and stimulated at low Ca2+ concentrations. The effect of low calcium on PTH gene expression is posttranscriptional.

Another important regulator of the PTH gene, calcitriol, decreases PTH gene expression at the transcriptional level. Calcitriol’s action is mediated via binding to the vitamin D receptor (VDR), a steroid hormone receptor. Once bound to calcitriol, the VDR forms a heterodimer with the retinoic X receptor and binds to the vitamin-D-responsive DNA element in the PTH gene promoter. VDR regulates the expression of many genes involved in mineral metabolism, cell proliferation, and differentiation.

In uremic patients, s-HPT may eventually turn into severe nodular hyperplasia, where the parathyroids cease to respond to Ca2+ and vitamin D therapy and continue to secrete significantly elevated amounts of PTH. This indicates a reduced presence or functional ability of CaR or VDR in these cells. Both clinical and experimental s-HPT are characterized by very low expression of the calcium-sensing...
receptor (CaR) and the vitamin D receptor (VDR) genes in the parathyroid glands [1–5]. In a previous study, Lewin et al. designed an experimental rat model in which an isogenic kidney transplantation normalized the glomerular filtration rate (GFR) of severely uremic rats [6, 7]. They demonstrated that the significantly elevated plasma levels of PTH in uremia became normal within one week of the kidney transplantation. This happened despite persistently suppressed gene expression of CaR and VDR one week after the transplantation and despite that normalization of these genes did not occur until four weeks after surgery, as illustrated in Figure 1. These results may indicate the existence of other regulatory pathways which are involved in parathyroid CaR and VDR signalling. In vitro, another situation occurs—the rapid reduction in the expression of parathyroid CaR [3]. The reason for this in vitro reduction is not completely understood at the molecular level. The parathyroid cell in culture loses its phenotype and its responsiveness to changes in extracellular calcium, making it challenging to study changes in the expression of different genes in the parathyroids.

As the parathyroids apparently can normalize the secretion of PTH despite reduced expression of CaR and VDR, the question arises whether this sustained low expression occurs due to epigenetic events, for example, methylation of cytosine nucleotides in CpG islands [8]. The term “epigenetics” describes changes in gene activity in the absence of a change in DNA sequence [9]. Such methylation may distort the transcription factor-binding sites causing transcriptional silencing [10]. Together with histone modifications, these epigenetic events might be reversible in time and tissue [11–13]. Epigenetic events are routinely found in various forms of cancer in tissues like colon, brain, liver, blood, breast, and lung [14–18], but also in chronic kidney disease [8]. CpG islands have been identified in the CaR and VDR genes [19]; methylation of these regions has been detected in different neoplasms [20], and low gene expression due to promoter methylation can be restored by 5-deoxy-3′-azacytidine, an inhibitor of DNA methylation [21]. Research demonstrating the importance of the hypermethylation of CaR and VDR in carcinogenesis makes the CaR and VDR genes interesting candidates for promoter methylation analysis in parathyroid hyperplasia [20, 22].

Further, vitamin D might be linked to epigenetic control of chromatin structure [23–26], since the unresponsiveness of malignant human prostate cells to vitamin D treatment can be reversed by treating the cells with drugs reversing the epigenetic state of the cell (DNA methylation and histone modifications) [25].

We launched the present study to examine whether the low expression of parathyroid CaR and VDR in uremia was associated with changes in methylation levels in vivo or in vitro. Therefore, we examined the methylation levels of parathyroid CaR and VDR genes in both normal and uremic rats. Further, we examined normal parathyroid glands in vitro and analyzed them for aberrant methylation levels. In these experiments, we performed qPCR to validate the expression of CaR and VDR, as well as PTH and klotho, a new hormone of importance in parathyroid physiology [1].

The present study found no signs of methylation in the parathyroids of uremic or normal rats, indicating that changes in methylation levels are not involved in the low expression of parathyroid CaR or VDR genes in uremia, either in vivo or in vitro.

2. Materials and Methods

2.1. Ethics Statement. We performed the experimental studies on rats in accordance with the Danish law on animal experiments; the Animal Experiments Inspectorate at the Ministry of Justice, Denmark approved these studies (permit number 2007/561-1278). Every effort was made to minimize suffering.

2.2. Bioinformatics. We searched for CpG islands in the genes of CaR and VDR using the internet site http://cpgislands.usc.edu/, using default search parameters, as well as http://genome.ucsc.edu/cgi-bin/hgGateway. We
designed PCR primers using the software Methyl Primer Express v1.0, selecting primers that target preferably non-CpG-containing areas. The CpG islands analyzed were located from −250 basepair (bp) to +300 bp from exon 1 of the CaR gene and from −800 bp to +200 bp from exon 1 of the VDR gene (see Figure 2). The analyzed downstream area of VDR near exon 10 is not considered a promoter region, and it only served as a methylation control region to positively demonstrate methylation.

2.3. DNA, PCR, and Melting Curve Analysis. We extracted genomic DNA from parathyroid glands using a QIAamp DNA Mini Kit (cat. no. 51304, Qiagen AB, Sollentuna, Sweden). The bisulfite conversion of the DNA, changing cytosine nucleotides to uracil, was performed using the EZ DNA Methylation Kit (cat. no. D5001, Zymo Research, Irvine, CA, USA). Briefly, 500 ng DNA from each parathyroid gland was treated for 16 hours in the dark at 50°C and eluted in 10 μL elution buffer. We used commercial highly methylated genomic rat DNA (cat. no. 80-8065-RGHM5) and commercial nonmethylated genomic rat DNA (cat. no. 80-8066-RGUM5, EpigenDx, Worcester, MA, USA) as standards. One μL of bisulfite-treated genomic DNA was used for PCR. Table 1 lists the PCR primers used. The PCR products were verified by agarose gel to confirm size and a gene concentration was calculated. We calculated the melting curve analysis, a built-in feature of the Roche Lightcycler 480 II software, calculates the loss of fluorescence with increasing temperature (from 50°C to 94°C) by measuring the slope of the initial raw fluorescence data.

2.4. RNA and qPCR. RNA was extracted using a Trizol extraction kit, (cat. no. T9424, Sigma-Aldrich, St. Louis, MO, USA). We performed cDNA synthesis using 200 ng RNA that was reverse transcribed using an Invitrogen cDNA kit (cat. no. 18080-051, Grand Island, NY, USA) with 125 pmol 15-mer random primer at a 50°C synthesis temperature. We used 5 μL of cDNA (diluted ten times) for qPCR [1]. PCR experiments were performed as follows: we used 15 pmol of each primer in a total volume of 20 μL PCR reaction. The temperature profile was 94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 90 seconds. We calculated CaR, VDR, klotho, and PTH gene expressions, using the second derivative method to calculate the Cp value for each sample against a standard dilution series to incorporate the efficiency of the PCR reaction, and a gene concentration was calculated. We calculated the mean activity of the housekeeping genes EEF1a1, RPL13, and ARBP for each sample and used the results to calculate the gene activity of CaR, VDR, klotho, and PTH (shown in Figures 3(a)–3(d) as gene ratios). In Figures 4(e)–4(g), the gene ratios of one housekeeping gene were calculated using the activity of each of the other housekeeping genes to show healthy, intact parathyroid tissue.

2.5. Animals. Adult male Wistar rats, weighing 225 g (Taconic, Ballerup, Denmark), were kept in a controlled environment with a 12-hour light-dark cycle, a constant temperature (22°C), and a relative humidity of 70 percent. We provided all rats with free access to food and water and fed them either (a) a standard rat chow diet containing 0.9 percent calcium, 0.7 percent phosphorus, and vitamin D (600 IU per kg−1) or (b) a high-phosphorus rat chow diet

Figure 2: Outline of CpG islands in CaR and VDR promoters. The solid horizontal lines labeled CaR#1-2 and VDR#1-4 indicate the locations of the PCR products, and the arrow indicates the exon 1 start. The small vertical lines in the promoter regions indicate individual CpG dinucleotides.
Table 1: PCR primers used for methylation detection by melting temperature profiling.

| Name   | Location | 5′-3′ sequence         | Size $ | Number of CpG |
|--------|----------|------------------------|--------|----------------|
| Promoter                        |           |                        |        |                |
| CaR #1F (−172)        | AGTTTGGAATGGTTAGAGTTTTAT     | 171 bp  | 12             |
| CaR #1R (−2)           | ACTCCCTAAAATCTCTCAAAATCAGAC|         |                |
| CaR #2F (−28)          | AAGGTTGATTTGAGAGTTAGG        | 185 bp  | 9              |
| CaR #2R (+157)         | CTAACCTCCGGCAAAACCTCTTCTTCT|         |                |
| VDR #1F (−58)          | TTTTGTGTTGTTAAAAAGGTGTTG    | 125 bp  | 6              |
| VDR #1R (+67)          | AATCTAATCACTCACCAAAAACCTCT|         |                |
| VDR #2F (−374)         | GTTTATAGTAGATGGTGAGATAATTTA| 175 bp  | 12             |
| VDR #2R (+157)         | CTACTACCTCCCCAAACCTCTCTCTCT|        |                |
| VDR #3F (−576)         | GTTTTTTTGAGTTAATTTAATTAGTG  | 169 bp  | 7              |
| VDR #3R (−389)         | TAATTCCTACCAATCCTCTAAAC     |         |                |
| VDR #4F (−678)         | AGGATTGGGAGGTAGAGTTTTAG      | 188 bp  | 18             |
| VDR #4R (−510)         | CCACTAATTAAATTTAACTCAAC     |         |                |
| Control                     |           |                        |        |                |
| H19 F (−6233)          | GAGGGTAGGATATATGTATTTTATG    | 185 bp  | 13             |
| H19 R (−6049)          | AAAAAAATTCAATCTCAATTACAATCT|         |                |
| VDR DS F (+48639)      | AAGGTTGGAGGTAGAGTTTTAG      | 269 bp  | 12             |
| VDR DS R (+48908)      | AAACAAATACACATCTACTCTCCC    |         |                |

$\text{Relative location of the 5\textquotesingle nucleotide according to exon 1.}$

$\text{Bp denotes DNA base pair.}$

Table 2: Plasma parameters of the sham and uremic group of rats.

|                | PTH pg/ml | Ca$^{2+}$ mM | P mM | Urea mM | Creatinine μM |
|----------------|-----------|--------------|------|---------|---------------|
| Sham, $n = 10$ | 90 ± 39   | 1.29 ± 0.01  | 1.36 ± 0.06 | 6.6 ± 0.5 | 39 ± 1.4      |
| Uremia, $n = 9$| 1851 ± 343| 1.14 ± 0.06  | 2.40 ± 0.26 | 17.6 ± 1.5 | 94 ± 12       |

containing 0.9 percent calcium, 1.4 percent phosphorus, and vitamin D (600 IU per kg$^{-1}$).

We divided the rats into the following experimental groups:

- **group 1: in vivo:** promoter methylation analysis of CaR and VDR in the parathyroid glands of uremic versus sham-operated rats;
- **group 2: in vitro:** promoter methylation analysis of CaR and VDR in sham-operated rat parathyroid glands at time points 0 and 24 hours;
- **group 3: in vitro:** gene expression of CaR, VDR, klotho, and PTH in rat parathyroid glands at time points 0, 1, 2, 3, 5, and 24 hours.

2.6. 5/6 Nephrectomy. We performed one-step 5/6 nephrectomy to induce uremia. In order to induce severe s-HPT, we gave a 5/6 nephrectomized group of nine rats a high phosphorus diet. Ten sham rats were given a standard diet. The duration of uremia was eight weeks. On the day of the nephrectomy, the rats received anaesthesia with Hypnorm/midazolam (Panum Institute, Copenhagen, Denmark). Additional doses were given, when required, to maintain a steady level of anaesthesia, and the rats were given carprofen (Rimadyl, 50 mg/mL, Pfizer, Copenhagen, Denmark) subcutaneously at a dose of 30 μL/rat as pain relief for the following three days. We made every effort to minimize suffering.

2.7. Parathyroidectomy. After eight weeks of uremia, the parathyroid glands were removed, and the glands were snap frozen in liquid nitrogen for subsequent promoter methylation analysis. Table 2 shows the plasma parameters of these rats. For the in vitro experiments, the parathyroid glands were removed and immediately placed in a 37°C incubation medium.

2.8. Culture of Parathyroid Glands In Vitro. Parathyroid glands from normal rats were cultured in vitro for various time intervals: 0, 1, 2, 3, 5, and 24 hours ($n = 3$ at each time point) and cultured in DMEM-HAM’s F12 medium with a calcium concentration of 1.2 mM [27]. The medium was changed after 1, 2, 3, 4, and 23 hours. We assessed gene expression after the incubation by qPCR.

We performed a second set of experiments in order to assess the methylation status of glands grown for 24 hours in vitro. Parathyroids from four rats served as control at time = zero, and we grew the parathyroids from seven rats in vitro for 24 hours.

2.9. Plasma Measurements. We obtained and analyzed blood samples using a Vitros 150 (Ortho-Clinical Diagnostics,
Figure 3: Methylation detection of CaR and VDR promoter regions in parathyroid glands. Analysis of the CaR and VDR promoter regions by PCR melting curve analysis after bisulfite treatment of genomic DNA. CaR is covered by PCR products CaR#1-2, and VDR is covered by PCR products VDR#1-4. Commercial low- and high-methylated rat DNA is analyzed in all of the PCR reactions shown in the “Methylation control” panel in green and red, respectively; it is also included in the “Sham,” “Uremia,” and “in vitro” panels. Control of methylation detection verified high levels of methylation in H19 and VDR downstream gene regions.

Rochester, NY, USA) for plasma phosphate, urea, and creatinine. We measured plasma ionized calcium (p-Ca²⁺) using an ABL 505 (Radiometer, Copenhagen, Denmark). Rat plasma PTH was measured using a rat bioactive intact PTH ELISA assay from Immutopics (San Clemente, CA, USA).

2.10. Statistic Calculations. We used Student’s t-test and presented the data as mean ± standard error of the mean (SEM). We set the statistical significance at P < 0.05.

3. Results

We examined the CpG islands in the CaR and VDR genes, from −250 to +300 base pairs (bp) from exon 1 of the CaR gene and from −800 to +200 bp from exon 1 of the VDR gene, as shown in Figure 2. The analyzed downstream area of VDR near exon 10 is not a promoter region and only served as a positive methylation control region. H19 served as a second control.

We initially performed experiments to ensure that SYBR melting temperature profiling, previously used to detect changes in methylation levels [28–30] would detect both low and high levels of methylation in PCR products. We included positive and negative methylation standards, which clearly showed distinct changes in melting temperature in each of the PCR reactions analyzed, as shown in the upper row of Figure 3. Table 1 presents the primers and the number of CpGs in each PCR product that resulted in changes in the melting temperature profile when methylated.

We validated the bisulfite DNA conversion method in all samples by analyzing the methylation levels of two known methylated gene regions—the imprinted gene H19 [31] and a downstream area of VDR near exon 10 [32], as shown in Figure 3. Two peaks in H19 were found in all samples, as expected, indicating methylation on one strand and not the other. The present study analyzed, in rats, the highly methylated region downstream of VDR near exon 10 also reported in humans. We found high levels of methylation of both DNA strands in every sample, detecting only one peak, which coincided with the high methylation control sample. Thus, the bisulfite conversion reaction performed well, and temperature melting profiling clearly detected the expected changes in the methylation level in these positive control gene regions.

In order to analyze the methylation levels of the CaR and VDR genes in our in vivo experiments, we performed gene-specific endpoint PCR and analyzed the PCR products by melting temperature analysis, as shown in Figure 3. Every peak coincided with the negative methylation standard; thus, no changes were observed in the melting temperature of any of the PCR products from the sham or uremic parathyroid glands.

We grew tissue cultures of parathyroid glands in vitro at various time intervals for up to 24 hours. Figures 4(a)–4(d) outline the gradual reduction of the gene expression of parathyroid CaR, VDR, klotho, and PTH over time. We found the expressions of CaR, VDR, and klotho expressions all significantly downregulated at two hours, whereas we first observed a significant downregulation of PTH at 24 hours. We performed a second set of experiments to examine whether methylation of CpG islands in CaR and VDR coincided with the reduction of parathyroid CaR and
Figure 4: *In vitro* expression of CaR, VDR, klotho, and PTH in parathyroid tissue cultures over time. (a)–(d): gene expression of CaR, VDR, klotho, and PTH over time. (e)–(g): gene expression of housekeeping genes EEF1a1, ARBP, and RPL13 over time. (*) Statistical significance of $P < 0.05$ is indicated, when compared to the zero-hour group. Gene activity is shown as gene ratios.
VDR expression. We compared freshly harvested parathyroid glands to parathyroid glands grown in vitro for 24 hours. We found no aberrant melting curves, indicating that the parathyroid glands in culture had the same low methylation levels at time zero and at 24 hours. Figures 4(e)–4(g) show the stable expression of three housekeeping genes over time, ensuring the viability of the parathyroid glands in vitro.

4. Discussion

Severe uremia is complicated by secondary hyperparathyroidism with hyperplasia of the parathyroid glands and is characterized by low parathyroid gene expression of CaR and VDR [2, 4, 5, 33, 34]. As s-HPT gets worse, the glands become unresponsive to vitamin D and calcium therapy, indicating that the parathyroid cells with low expression of CaR and/or VDR can no longer convey calcium or vitamin D signals. Lewin et al. showed in 2002 that plasma PTH, together with plasma Ca\(^{2+}\), phosphate, creatinine, and urea, normalized after an experimental isogenic kidney transplantation in rats, despite persistently low parathyroid expression of CaR and VDR; only several weeks later did the expression of CaR and VDR become normal in the parathyroids [2, 35, 36]. The delay in the restored expression of CaR and VDR in this model of reversal of uremia by Lewin et al. stresses the importance of a search for not-yet-identified mechanisms that might control CaR and VDR genes in the parathyroids.

The present study examined the methylation status of the CaR and VDR promoter regions. We found no indication of methylation, as the results of all samples coincided with the negative methylation standard, as shown in Figure 3.

No parathyroid cell line exists for culturing, leaving researchers freshly harvested parathyroid tissue/cells to examine. However, parathyroid glands grown in vitro present a significantly reduced expression of the CaR gene within the first 24 hours of culture [3, 34]. In the present study, we assessed the expressions of key parathyroid genes: CaR, VDR, PTH, and klotho. As expected, we observed a significant decline of the expression of CaR within two hours of incubation, reaching a lower steady level after 5 to 24 hours, as shown in Figure 4(a). Similarly, the expression of VDR and klotho genes also declined after two hours of incubation, reaching a lower steady level after 5 to 24 hours, as shown in Figures 4(b)-4(c). The expression of the PTH gene also declined—but slowly, over time—reaching a nadir at 24 hours, as shown in Figure 4(d). In contrast, the expression of the three housekeeping genes was stable during the 0 to 24 hours of culture, as shown in Figures 4(e)–4(g), suggesting the persistent viability of the parathyroid cells.

We studied the methylation levels of the CaR and VDR promoters in vitro at time zero and after 24 hours in order to examine their association to the low expression of these genes. As shown in Figure 3, we detected no changes in methylation levels over time, indicating that the low expression of parathyroid CaR and VDR genes in vitro was not associated with methylation.

The CaR gene is expressed not only in the tissues, where it is primarily involved in calcium homeostasis, such as the parathyroid glands, the C-cells of the thyroid gland, the kidney, and bone, but also in a number of other tissues, where it is implicated in the regulation of multiple cellular functions. It has been proposed that CaR plays an important role in the regulation of intestinal cell proliferation and differentiation. Stimulation of CaR expression in colon epithelial cells was shown to induce an inhibition of proliferation [37, 38]. The loss of expression of CaR was associated with poor differentiation and malignant progression [20, 39]. Recently, epigenetic inactivation of CaR expression by promoter hypermethylation was demonstrated in colorectal carcinogenesis [20].

Activation of CaR is related to the regulation of parathyroid cell proliferation. This was proven indirectly by the observation that the administration of CaR agonists led to the inhibition of parathyroid cell proliferation in uremic rats [40]. However, as our study demonstrates the loss of expression of CaR in parathyroid hyperplasia secondary to uremia is, unlike colonic neoplasms, not associated with hypermethylation of the CaR gene promoter.

VDR has been demonstrated in a broad range of tumors and malignant cell types, and the inhibition of cancer cell growth, angiogenesis, and metastasis by calcitriol has been shown. For colon and breast cancer cells, an inverse relationship between VDR levels and degree of differentiation has been described [41–43]. Recent research has shown the hypermethylation of the VDR gene promoter region in primary breast tumors and its absence in normal breast tissue [44], and the role of the epigenetic silencing of VDR by promoter hypermethylation as the mechanism behind the resistance of breast cancer cells to calcitriol has been proposed. The present results do not, however, support a similar mechanism in parathyroid hyperplasia in uremia. It should, however, be emphasized that experimental secondary hyperparathyroidism does not fully resemble the advanced hyperparathyroidism with clonal transformation which is seen in humans, and it can therefore not be ruled out that CaR or VDR promoter methylation might exist in the human setting.

Some limitations of the present investigation should be stressed. CpG island methylation often goes hand in hand with histone modifications [45–50]. In the present study, we focused only on the methylation status and did not assess the histone modification profile in the CaR and VDR gene areas; thus, histone modifications may still play a role in the delayed CaR and VDR expression profile in the parathyroids. Furthermore, the present results do not exclude epigenetic mechanisms in the upstream signalling pathways that regulate CaR and VDR gene expression or in other areas of the CaR or VDR gene regions. The method used to detect changes in methylation levels can only detect overall changes and will not reveal if one locus becomes methylated in combination with loss of methylation at another locus in the PCR product.

5. Conclusion

In uremia, severe hyperparathyroidism is characterized by low parathyroid expression of CaR and VDR. Disturbances in CaR and VDR gene methylation patterns have been shown
in tissues with rapid growth, such as in various cancer tissues, where these epigenetic changes were responsible for the uncontrolled cell growth. Therefore, we examined the parathyroid glands from uremic rats for changes in the methylation levels of the CaR and VDR genes. We performed the methylation analysis of the CpG islands in the CaR and VDR genes to examine whether uremic parathyroid glands exhibited epigenetic changes. We found no overall changes in the melting temperature curves of any of the PCR products, which we analyzed in this rat model of uremic s-HPT and in rat parathyroid tissue in vitro. We concluded that methylation is not associated with the distorted gene expression of CaR and VDR in experimental uremic secondary hyperparathyroidism or in parathyroid glands grown in vitro.

Conflict of Interests

The authors declare that they have no conflict of interests.

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