Parathyroid Klotho and FGF-receptor 1 expression decline with renal function in hyperparathyroid patients with chronic kidney disease and kidney transplant recipients

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Type I membrane-bound α-Klotho (Klotho) has a critical role in extracellular calcium regulation and is expressed at high levels in parathyroid glands and kidneys.1,2 It is essential for the release of parathyroid hormone (PTH) at hypocalcemic conditions. In a setting of low extracellular calcium, Klotho recruits Na+/K+/ATPases to the parathyroid cell membrane and leads to enhanced PTH secretion.3 Klotho further stimulates tubular calcium reabsorption through glycosylation and anchoring of the calcium-transporting channel, transient receptor potential ion V5, to the cell surface.4

Klotho is also a cofactor that converts fibroblast growth factor-receptor 1 (FGFR1) into a specific receptor for FGF23,5,6 a bone-derived circulating peptide that regulates serum inorganic phosphate (Pi) and 1,25-dihydroxy-vitamin D3 (1,25(OH)2D3) levels.7–9 We along with others, previously showed that FGF23 directly suppresses PTH mRNA level and protein secretion in vitro10 and in vivo.11

The diminished renal capacity of chronic kidney disease (CKD) patients to produce 1,25(OH)2D3, and the presence of hyperphosphatemia due to decreased Pi clearance, are major causatives of PTH hypersecretion and parathyroid gland hyperplasia.12 Importantly, CKD patients inevitably develop secondary hyperparathyroidism (sHPT) despite concomitantly high circulatory FGF23 levels.13 The postponement of hyperphosphatemia by FGF23 presumably occurs at the expense of accentuated 1,25(OH)2D3 deficiency,14 however, the markedly elevated FGF23 levels also indicate parathyroid and possibly other end-organ15 resistance. A possible mechanism could be downregulation of the receptors mediating FGF23 signaling.15 Herein, we evaluated the parathyroid expression of FGF-receptors Klotho and FGFR1 in hyperparathyroid CKD patients and kidney transplant recipients.
RESULTS

Glandular Klotho mRNA levels
Klotho mRNA expression level in 88 secondary hyperplastic parathyroid glands showed a large interglandular variation (Figure 1). We found no association between Klotho expression and glandular weight (data not shown), or any difference in Klotho mRNA levels because of the treatment with active vitamin D₃ derivatives (Figure 1). Klotho levels did not differ between transplanted and non-transplanted patients, but were generally decreased compared with that in normal parathyroid tissue (Figure 1).

Nodularity and protein levels of Klotho, Ki67, and vitamin D₃ receptor (VDR)
The majority (67%) of all analyzed glands were of nodular type. Immunohistochemical (IHC) analysis revealed varying intensity and heterogeneous intraglandular distribution of Klotho (Figure 2a, A–B). Only a weak tendency toward lower Klotho expression in nodular, as compared with diffuse, hyperplasias was observed with whole-section scoring. Occasionally, Klotho expression was equal both outside and within a nodule (Figure 2a, G–H). Thus, there is no compelling evidence of lower Klotho protein expression in nodules. Klotho mRNA and protein levels tended to correlate positively ($n = 42$; Figure 2b). We observed a trend of higher Ki67 scores in nodular glands (data not shown). Repeated analysis of whole sections showed no conclusive relation between Klotho and Ki67 protein levels, nor was statistical significance reached when applying correlation analysis to the IHC scores. Figure 2a, C–D illustrates the typically small number of Ki67-expressing cells. Protein levels of VDR mainly correlated to Klotho levels (Figure 2a, E, K–L).

Klotho in relation to renal function
Glomerular filtration rate (GFR) was higher in transplanted patients than in non-transplanted patients (30.2 ± 17.7 versus 9.64 ± 3.80, $P < 0.01$). Mean Klotho levels correlated positively with log GFR ($n = 31$; $r = 0.42$; $P < 0.05$; Figure 3a) and the correlation was stronger in the transplanted group (data not shown). Accordingly, the glandular Klotho mRNA levels decreased with increasing CKD stages ($P < 0.05$; Figure 3b).

Klotho in relation to serum biochemistry
Patients’ serum biochemistry parameters are shown in Table 1. According to the defined cutoffs of 2.5 and 1.5 mmol/l, 27 patients suffered from hypercalcemia (2.76 ± 0.16 mmol/l), and 13 from hyperphosphatemia (2.14 ± 0.79 mmol/l), respectively. Univariate correlation analysis revealed a negative association between serum P, and mean Klotho mRNA levels ($n = 29$, $r = -0.44$, $P < 0.05$; Figure 3c). The correlation was somewhat stronger in transplanted patients ($n = 20$, $r = -0.57$, $P < 0.01$). No association was observed between mean Klotho mRNA and serum calcium or PTH levels, not even after stratification for kidney transplantation (data not shown).

FGFR1 in relation to renal function, Klotho, and serum biochemistry
Mean FGFR1 mRNA levels did not differ between transplanted and non-transplanted patients (data not shown). A positive correlation was observed between mean FGFR1 and log GFR levels (Figure 4a left), and FGFR1 expression decreased across CKD stages ($P < 0.01$; Figure 4a middle). FGFR1 and Klotho mRNA levels correlated positively (Figure 4a right). Mean FGFR1 correlated negatively with serum P, (Figure 4b left), and log serum PTH levels (Figure 4b right). No association existed between mean FGFR1 and serum calcium levels (data not shown).

Regulation of Klotho expression in cultured bovine parathyroid cells
To further explore the regulation of parathyroid Klotho expression, we performed cell culture experiments using isolated bovine parathyroid cells. We previously showed that calcium treatment suppressed parathyroid Klotho mRNA level. Treatment with up to $10^{-7}$ mol/l of 1,25(OH)₂D₃ or with the vitamin D₃ analog EB1089 dose dependently increased the Klotho mRNA level at 24 h ($P < 0.01$, Figure 5a). On the contrary, FGF23(R176Q) treatment decreased the Klotho level ($P < 0.01$; Figure 5b). Cotreatment with EB1089 and FGF23(R176Q) resulted not only in blunting of the EB1089 effect ($P < 0.001$), but also in a decreased Klotho expression in compared with control-treated cells ($P < 0.001$) (Figure 5c). Addition of EB1089, however, did not mitigate the FGF23(R176Q)-induced suppression of Klotho ($P > 0.05$; Figure 5c). Cotreatment with FGF23(R176Q) and calcium caused a further suppression of Klotho mRNA levels as compared with the FGF23(R176Q) treatment alone ($P < 0.01$; Figure 5c). There was no additive effect on Klotho mRNA suppression after treatment with FGF23(R176Q) and calcium, compared with calcium alone ($P > 0.05$; Figure 5c). Combination treatment with FGF23(R176Q), calcium, and EB1089 resulted in a net

![Distribution of 88 glandular Klotho mRNA levels.](image)

Klotho mRNA levels in hyperplastic glands from 31 patients and 7 normal parathyroid tissues are depicted. Patients are categorized on the basis of kidney transplantation and active vitamin D₃ compound treatment (+D). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LL, lower left; LR, lower right; Norm, normal; UL, upper left; UK, unknown glandular localization; UR, upper right.
Klotho mRNA suppression of magnitude similar to that with FGF23(R176Q) and calcium (Figure 5c). Finally, we observed no effect on Klotho transcript level after treatment with either P\textsubscript{i} or PTH (data not shown).

Regulation of Klotho expression in cultured hyperplastic parathyroid cells from a CKD patient
Calcium was the only single agent that exerted an inhibitory effect on the Klotho transcript level after 24 h treatment.

Figure 2 | Klotho, Ki67, and vitamin D\textsubscript{3} receptor (VDR) protein levels. (a) Representative tumor sections from two patients are depicted (A-E and G-L). Immunostaining of Klotho/Ki67/VDR is presented in the left/middle/right columns, respectively, and the rows represent the different magnifications (row 1/2/3/4: 20 ×/40 ×/10 ×/20 ×). Heterogeneous staining of Klotho protein in the two nodules of the first tumor (A–B) indicates large intraglandular differences. The staining of the second tumor confirms that, regardless of nodular borders, the overall expression of Klotho is irregular (G–H). There was no association between Ki67 and Klotho expression exemplified by the nodular Ki67 staining (I–J), and the more diffuse Klotho expression (G–H). VDR levels mainly parallel the levels of Klotho (E, K–L). Negative control is shown in F (× 20 magnification). (b) Klotho mRNA and protein levels tended to correlate positively (n = 42). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry.
Klotho mRNA levels over chronic kidney disease (CKD) stages

parathyroid Klotho R mRNA levels and log GFR were observed (n=31; r=0.42; P<0.05). (b) This was supported by a decline in glandular Klotho mRNA levels over chronic kidney disease (CKD) stages (n=3/23/14/39; ***P<0.001 versus stage 3). Error bars represent the 5th and 95th percentiles, and outliers are represented by dots.
(c) Mean Klotho mRNA levels correlated negatively with serum inorganic phosphate (Pi) (n=29; r=-0.44; P<0.05). Gray dots represent the four patients from whom only one gland was available (if these are excluded: n=25; r=-0.54; P<0.01). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3 | Klotho expression in relation to renal function and serum biochemistry. (a) Glomerular filtration rate (GFR) values were calculated according to the Cockcroft-Gault formula: GFR (ml/min per 1.73 m²) = (140 – age (years)) × (weight (kg)) × (0.85; if female)/(72 × creatinine (mg/dl)). A positive correlation between mean parathyroid Klotho mRNA levels and log GFR was observed (n=31; r=0.42; P<0.05). (b) This was supported by a decline in glandular Klotho mRNA levels over chronic kidney disease (CKD) stages (n=3/23/14/39; ***P<0.001 versus stage 3). Error bars represent the 5th and 95th percentiles, and outliers are represented by dots. (c) Mean Klotho mRNA levels correlated negatively with serum inorganic phosphate (Pi) (n=29; r=-0.44; P<0.05). Gray dots represent the four patients from whom only one gland was available (if these are excluded: n=25; r=-0.54; P<0.01). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 1 | Patient characteristics

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<th>sHPT</th>
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<td>No. of patients; glands</td>
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<td>No. of glands removed</td>
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<td>CKD stage (no. of patients; glands)</td>
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<td>Age (years)</td>
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<td>Gender (F; M)</td>
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<td>Weight (kg)</td>
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<td>Glend weight (mg)</td>
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<td>Pi (mmol/l)</td>
<td>1.00 ± 0.27 (hypo-normal (n=16)); 2.14 ± 0.79 (hyper (n=13))</td>
</tr>
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<td>Ca (mmol/l)</td>
<td>2.45 ± 0.05 (normal (n=4)); 2.76 ± 0.16 (hyper (n=27))</td>
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<td>PTH (pmol/l)</td>
<td>81.9 ± 59.8 (n=29)</td>
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<tr>
<td>Creatinine (µmol/l)</td>
<td>310 ± 179 (F); 412 ± 298 (M)</td>
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<td>ALP (µkat/l)</td>
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</tr>
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<td>Etiology</td>
<td>GN (n=11); PCKD (n=5); DN (n=4); HNS (n=2); PN (n=2); V (n=1); other (n=6)</td>
</tr>
</tbody>
</table>

**Abbreviations:** ALP, alkaline phosphatase; CKD, chronic kidney disease; DN, diabetic nephropathy; F, female; GN, glomerulonephritis; HNS, hypertensive nephrosclerosis; M, male; PCKD, polycystic kidney disease; Pi, inorganic phosphate; PN, pyelonephritis; PTH, parathyroid hormone; sHPT, secondary hyperparathyroidism; V, vasculitis. The values are presented as arithmetic mean ± s.d., except for the tumor weight (geometric mean ± multiplicative s.d.). The glomerular filtration rate values were calculated according to the four-variable Modification of Diet in Renal Disease Study equation: Glomerular filtration rate (ml/min per 1.73 m²)=175 × [C2 creatinine (mg/dl)] × [C2 calcium (mmol/l)] × [C2 age (years)] × [C2 weight (kg)] 0.44, P<0.001 versus stage 3. Error bars represent the 5th and 95th percentiles, and outliers are represented by dots. (c) Mean Klotho mRNA levels correlated negatively with serum inorganic phosphate (Pi) (n=29; r=-0.44; P<0.05). Gray dots represent the four patients from whom only one gland was available (if these are excluded: n=25; r=-0.54; P<0.01). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The inhibition by FGF23(R176Q) treatment did not reach the threshold of statistical significance (Figure 6a), however, the presence of FGF23(R176Q) mitigated the calcium-induced suppression of Klotho (P<0.05; Figure 6a). Neither EB1089, 1,25(OH)2D3, and Pi (data not shown) had any regulative effects on Klotho expression (P>0.05). Treatment with a combination of calcium, EB1089, and FGF23(R176Q) was the only treatment besides calcium that had a suppressive effect on Klotho as compared with control treatment (P<0.05; Figure 6a), and the resulting suppression was slightly stronger than the one induced by combined calcium and FGF23(R176Q) treatment (P<0.05), or EB1089 and FGF23(R176Q) treatment (P<0.01) (Figure 6a).

Regulation of FGFR1 expression in cultured hyperplastic parathyroid cells from a CKD patient

FGFR1 mRNA level was stimulated by 24 h treatment with FGF23(R176Q), 1,25(OH)2D3, and Pi (P<0.05), whereas calcium and EB1089 had no significant effects (P>0.05) (Figure 6b). Addition of either calcium (P<0.05) or EB1089 (P<0.001) to FGF23(R176Q)-containing treatment media
Figure 4 | Fibroblast growth factor receptor 1 (FGFR1) expression in relation to renal function and Klotho. (a) Left: mean parathyroid FGFR1 mRNA levels were positively associated with log glomerular filtration rate (GFR) \((n = 31, r = 0.50, P < 0.01)\); transplanted \(n = 21, r = 0.53, P < 0.05\). Gray dots represent the four patients from whom only one gland was available (if these are excluded: \(n = 27, r = 0.52, P < 0.01\)). Middle: a decline in glandular FGFR1 mRNA levels was evident when comparing between chronic kidney disease (CKD) stages \((n = 3/32/14/39, **P < 0.01, ***P < 0.001\) versus stage 5). Error bars represent the 5th and 95th percentiles, and dots represent outliers. Right: log glandular levels of FGFR1 correlated induced further suppression of Klotho as compared with combined EB1089 and FGF23(R176Q) as well \((P < 0.001)\). The effect of the same combination did not differ from the effect of FGF23(R176Q) alone \((P = 0.98)\), but also suppressed Klotho mRNA level as \((P < 0.001)\) in comparison with FGF23(R176Q) alone \((P < 0.001)\). However, the same combination induced a slightly stronger suppression \((P < 0.001)\) in comparison with FGF23(R176Q) alone \((P < 0.001)\). Addition of calcium induced further suppression of Klotho as compared with combined EB1089 and FGF23(R176Q) as well \((P < 0.001)\). The concentrations used were \(4\) mmol/l calcium, \(2000\) pg/ml FGF23(R176Q), and \(10^{-7}\) mol/l EB1089. All graphs show effects observed after 24 h treatment of bovine parathyroid cells in vitro \((mean \pm s.e.m., n = 3–4)\). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 5 | Klotho regulation by calcium, active vitamin D\(_3\) compounds, and fibroblast growth factor 23 (FGF23(R176Q)) in cultured bovine parathyroid cells. (a) Vitamin D\(_3\) analog EB1089 (left) and 1,25-dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\); right) dose-dependently stimulated Klotho mRNA level, whereas \(b\) FGF23(R176Q) treatment decreased Klotho expression at 200 pg/ml. (c) Treatment with FGF23(R176Q) in combination with EB1089 not only blunted \((P < 0.001)\) the stimulating effect of the latter \((P < 0.01)\), but also suppressed Klotho mRNA level as compared with control (Ctrl)-treated cells \((P < 0.001)\). The effect of the same combination did not differ from the effect of FGF23(R176Q) alone \((P = 0.25)\). FGF23(R176Q) and calcium did not suppress Klotho further \((P < 0.001)\) than calcium treatment alone \((P < 0.001)\). However, the same combination induced a slightly stronger suppression \((P < 0.001)\) in comparison with FGF23(R176Q) alone \((P < 0.001)\). Additon of calcium induced further suppression of Klotho as compared with combined EB1089 and FGF23(R176Q) as well \((P < 0.001)\). The concentrations used were \(4\) mmol/l calcium, \(2000\) pg/ml FGF23(R176Q), and \(10^{-7}\) mol/l EB1089. All graphs show effects observed after 24 h treatment of bovine parathyroid cells in vitro \((mean \pm s.e.m., n = 3–4)\). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
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of treatment of secondary hyperplastic parathyroid cells

Figure 6 | Klotho and fibroblast growth factor receptor 1 (FGFR1) regulation by calcium (Ca), active vitamin D3 compounds, FGF23(R176Q) (F), and inorganic phosphate (Pi) in cultured hyperplastic parathyroid cells from a chronic kidney disease patient. (a) Klotho mRNA level was mainly unaffected after 24 h of treatment with various agents at the
given concentrations. Ca was the only single agent that had an
inhibitory effect on Klotho compared with control (Ctrl) treatment (P < 0.05). Treatment with FGF23(R176Q), EB1089 (EB), and 1,25-
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comparison with control treatment (P < 0.05). (b) Single treatment with FGF23(R176Q), 1,25-dihydroxyvitamin D3, and Pi increased
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of treatment of secondary hyperplastic parathyroid cells in vitro
are shown (mean ± s.e.m., n = 3). GAPDH, glyceraldehyde
3-phosphate dehydrogenase.

DISCUSSION
An overall decreased expression of Klotho and FGFR1 in
parathyroid glands of CKD patients has been described recently.17–19 In our cohort despite of varying expression in
the analyzed secondary hyperplastic parathyroid glands, both
Klotho and FGFR1 expression levels were found to be positively correlated to renal function and significantly
decreased over CKD stages.

The decrease in parathyroid Klotho/FGFR1 level after
progression of CKD may be due to several causes. It may in part reflect an intrinsic glandular defect, as the development
of parathyroid hyperplasia parallels the decline in renal
function and is more likely to occur in late CKD. This would
be analogs to the reduced parathyroid expression of VDR and
calcium-sensing receptor, which is observed at later stages
of CKD in parallel with glandular nodularity and reduced
sensitivity to the suppressive effects of 1,25(OH)2D3 and
calcium.20–22 A reduction in Klotho/FGFR1 expression could
be a temporal event that coincides with hyperplasia
development. It is however important to emphasize that
these two pathways, and especially the calcium/calcium-
sensing receptor pathway, are of major importance in the
rapid and (normally) very effective PTH regulation,23,24 and
that the FGF23/FGFR1/Klotho pathway may have a smaller,
yet unexplored and contributing role in sHPT.

Alternatively, the reduction in Klotho levels may rather be
a consequence of dynamic biochemical changes occurring in
CKD. This is supported by our bovine in vitro data, showing
that Klotho was suppressed by FGF23 and calcium, but
stimulated by active vitamin D3 compounds. Indeed, a
majority of our patients were hypercalcemic. In concert, CKD
patients suffer from gradually increasing serum FGF23 and
decreasing 1,25(OH)2D3 levels in parallel with declining renal
function.14,25 Unfortunately, because of lack of serum, we
were unable to determine the 1,25(OH)2D3 and FGF23 levels
of our patients. This would have been enlightening, especially
because of the fact that serum Pi inversely correlated with
Klotho expression, although Pi did not regulate Klotho
expression in our cell models. Thus, in this context serum Pi
may just be a marker of higher FGF23 or lower 1,25(OH)2D3
levels. The lack of response in our bovine cell model may be
explained by the fact that intact parathyroid tissue
architecture is required in order for extracellular Pi to exert
its effects,26 or for a possible post-transcriptional regula-
tion.27 Maintenance of the tissue architecture may not be as
necessary in hyperplastic cells, as Pi did regulate the
expression of FGFR1 in the cultured parathyroid cells of a
uramic patient.

Pathophysiologically of hyperparathyroidism might be differ-
ent in kidney transplant recipients compared with CKD
patients undergoing dialysis. Although not statistically
significant, there was a tendency for Klotho and a somewhat
stronger tendency for FGFR1 mRNA expression to be higher
in transplanted patients. This suggests that the levels of the
receptor and its coreceptor may improve by an increase in
GFR that may accompany a kidney transplant. The issue

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of treatment of secondary hyperplastic parathyroid cells in vitro
are shown (mean ± s.e.m., n = 3). GAPDH, glyceraldehyde
3-phosphate dehydrogenase.

further stimulated FGFR1 expression (Figure 6b). Similarly,
FGF23(R176Q) and EB1089 combined had a stronger stimulatory effect than EB1089 alone (P < 0.001; Figure 6b).
Finally, calcium and FGF23(R176Q) together stimulated the
FGFR1 expression less effectively in the presence of EB1089
(P < 0.01; Figure 6b).

...
would need to be investigated in a larger cohort. The effect of treatment by immunosuppressive agents on parathyroid function should be investigated further.

Because Klotho is a specific FGF-receptor co-factor for FGFR2,
5 it is likely that parathyroid glands with a reduced Klotho level do not adequately respond to the inhibitory action of FGFR2 on PTH secretion. 13,28 This is in agreement with what is observed in vivo, that is, high FGFR2 levels are paralleled by high PTH levels. 14 Although we did not previously observe any major effects of FGFR2 on parathyroid cell proliferation or apoptosis in short-term in vitro culturing, 10 we cannot rule out the possibility that FGFR2 may be implicated in cell growth and division in the long-term in vivo through unknown mechanisms. Therefore, reduced Klotho expression could impair the ability of FGFR2 to control cell growth and thereby contribute to the development of hyperplasia. On the other hand, the declining parathyroid Klotho expression and the accelerating PTH levels that occur in progressing CKD, again indicate that PTH secretion can occur through a Klotho-independent mechanism and despite hypercalcemia. 16 Indeed, FGFR2 may start to lose its inhibitory influence on PTH even in moderate CKD with adequate Klotho expression.

In this study, in vitro data provide mechanistic insights into the regulation of Klotho and may have several implications for parathyroid FGFR2 action in the uremic milieu. We showed that active vitamin D3 compounds stimulate Klotho expression in cultured normal (bovine) parathyroid cells, as has been observed in the kidney. 28 Thus, treatment with active vitamin D3 derivatives may not only be beneficial in preventing not only sHPT through direct VDR signaling, but also by enhancing the FGFR2/FGFR1/Klotho-induced suppression. Nevertheless, the stimulatory effect of EB1089 was blunted in the presence of high calcium or FGFR2, suggesting that administration of active vitamin D3 compounds in CKD might not induce parathyroid Klotho expression in such settings, not even in the presence of normal VDR levels. In fact, EB1089 had no effect on either Klotho or FGFR1 expression in cultured hyperplastic (human) parathyroid cells, indicating altered sensitivity and Klotho regulation of the latter. Active vitamin D3 analogs administered to CKD patients may even interfere with the FGFR1 stimulation induced by calcium and FGFR2, as suggested by our human in vitro data.

Another important observation from our bovine cell model was that the degree of Klotho suppression by FGFR2 and calcium was similar to that of high calcium alone. High calcium interfering with FGFR2 signaling might translate into weakened PTH suppression because of emerging hypercalcemia later in sHPT.

It is worth mentioning that our normal cell model originated from 15–20 bovine glands, whereas the uremic cell model came from three glands of one CKD individual. Repeated similar experiments, optimally on a mixture of hyperplastic parathyroid cells from several uremic individuals, would be valuable. Interpreting and interpolating data from cell models with such different initial biology should be carried out with precaution.

We previously discovered that the Klotho expression in parathyroid adenomas from patients with primary HPT inversely correlated to serum calcium. 16 In this investigation, we did not find evidence for such a relationship, indicating that the regulation of parathyroid Klotho differs between primary HPT and shPT and involves factors other than calcium. This is not surprising given the different etiology, tissue heterogeneity, and the more prominent derangements in mineral metabolism in sHPT related to CKD.

In summary, parathyroid Klotho and FGFR1 levels decrease with declining renal function in CKD patients. This may be related to an intrinsic glandular defect, although our data suggest biochemical changes related to CKD, such as hypercalcemia, low 1,25(OH)2D3 and high FGFR2 levels, to be a probable cause. This may explain the co-occurrence of high circulatory FGFR2 and PTH levels, and the failure of FGFR2 to prevent PTH hypersecretion in late CKD.

MATERIALS AND METHODS

Patients

The patients included had undergone surgical parathyroidectomy between 1998 and 2008. Inclusion criteria were access to at least one frozen parathyroid tissue sample and a GFR <90 ml/min per 1.73 m2. A total of 21 patients were kidney recipients (4 of them repeated the operation once or twice), and parathyroidectomy was performed at least 1 year after the kidney transplantation. One patient underwent partial surgical parathyroidectomy twice with a 3.5-years delay and was regarded as two distinct patients with different biochemistries. In all, 15 patients were on phosphate binders, 14 were treated with active vitamin D3 derivatives (Etalpha (n = 13) or Rocaltrol (n = 1)) and two patients received daily calcimimetics (Mimpara) daily before surgery. Serum calcium, albumin, PTH, creatinine, and alkaline phosphatase were measured according to standard techniques at the Department of Clinical Chemistry, Uppsala University Hospital (Table 1). Informed consent was obtained from all participants and the procedure was in accordance with the institutional ethical committee at Uppsala University.

Bovine parathyroid cells in vitro

Primary bovine parathyroid cells were isolated and cultured according to previous descriptions. Conditioned serum-free FGFR2(R176Q)-containing Dulbecco’s modified Eagle’s medium medium was produced as described earlier. Cells were treated in triplicates/quadruplicates (if not stated otherwise) with: control medium, 10 also used as diluent, FGFR2 (R176Q), calcium, PTH, creatinine, and alkaline phosphatase were measured according to standard techniques at the Department of Clinical Chemistry, Uppsala University Hospital (Table 1). Informed consent was obtained from all participants and the procedure was in accordance with the institutional ethical committee at Uppsala University.
### Human hyperplastic parathyroid cells in vitro

Three glands from a female patient with GFR at 6.32 ml/min per 1.73 m² were excised, collagenase treated and subjected to treatments as described above. The biochemical status of the patient at the time of surgery was: 2.36 mmol/l (albumin-corrected) calcium, 2.20 mmol/l P₉, 112 pmol/l PTH, 26400 pg/ml intact FGF23 measured by enzyme-linked immuno-sorbent assay from Kainos Laboratories, Tokyo, Japan and 588 pmol/l creatinine. The patient was a renal transplant recipient who had returned to dialysis because of allograft rejection.

### Quantitative real-time polymerase chain reaction

RNA extraction and complementary DNA synthesis was carried out as described before. Exonic-specific primers were designed and the amplicons were confirmed by sequencing analysis. Semiquantitative reverse transcriptase-polymerase chain reaction using SYBR Green was carried out on IHC scores, wherein non-parametric Spearman’s correlation analyses were used instead. Values are presented as arithmetic means ± s.e.m. in the figures, if not stated otherwise. A P-value of <0.05 was considered statistically significant.

### Immunohistochemistry

Protein levels were analyzed by IHC according to standard frozen tissue protocols. The antibodies used were polyclonal goat anti-Klotho antibody (Santa Cruz Biotechnology Inc., CA, USA; cat. no. sc-22220; 1:7 dilution); monoclonal mouse anti-Ki67 antibody (Dako Denmark A/S, Glostrup, Denmark; cat. no. M7240; 1:100 dilution) and monoclonal mouse anti-VDR antibody (Santa Cruz; cat. no. sc-13133; 1:650 dilution). A consecutive section from one gland was used each time as a negative control (without primary antibody). The sections were analyzed twice and each obtained an IHC score (0.0/0.5/1.0/1.5/2.0/2.5/3.0) as a representative of the whole section. The intensity of the different protein levels was compared both metrically by using the IHC scores, as well as by studying diffuse tissue/nodule boundaries of consecutive series of individual sections. One section per gland (a total of 42 sections) was used for microscopic determination of glandular nodularity. Sections were counterstained with Mayer’s hematoxylin for visualization of the nuclei.

### Statistical analyses

GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) and Statistica 8 (StatSoft Inc., Tulsa, OK, USA) softwares were used for statistical data analyses. Normality testing of patient biochemistry data by the Shapiro–Wilk test, the χ²-square test, and quantile-quantile and probability-probability plots revealed a normal distribution of mean Klotho and mean FGFRII values, calcium, P₉, and age. All other parameters were logged. Unpaired t-test or analysis of variance was used for comparison between independent groups. Univariate, parametric correlation (Pearson) analysis was applied, except in correlation analyses carried out on IHC scores, wherein non-parametric Spearman’s correlation analyses were used instead. Values are presented as arithmetic means ± s.d. in the text and in Table 1 and as arithmetic means ± s.e.m. in the figures, if not stated otherwise. A P-value of <0.05 was considered statistically significant.

### ACKNOWLEDGMENTS

We thank Birgitta Bondeson for technical assistance. This work was supported by the Swedish Research Council, the Novo Nordisk Foundation, the Swedish Kidney Foundation, the Swedish Society of Medicine, as well as by Magn Bergvalls Stiftelse, Åke Wibergs Stiftelse, Selanders Stiftelse, Harald Jeanssons Stiftelse, and Greta Jeanssons Stiftelse.

### DISCLOSURE

All the authors declared no competing interests.

### REFERENCES


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**Table 2 | Primer sequences**

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**Abbreviations:** GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FGFR1, fibroblast growth factor receptor 1; Klotho, membrane-bound alpha-Klotho (used as internal control). *GenBank accession number.


