Growth hormone and Klotho

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Abstract

Acromegaly is characterized by excessively high GH and IGF1 levels. Recent data suggest that soluble Klotho (sKlotho) is also elevated in patients with active acromegaly. sKlotho decreases towards normal following removal of the GH-producing pituitary adenoma. The *Klotho* gene was identified in mice following its accidental disruption by ectopic DNA. It is an ageing suppressor gene of restricted expression (mainly in kidneys, brain, and parathyroid and pituitary glands) encoding a transmembrane protein, mKlotho. mKlotho serves as a co-receptor in fibroblast growth factor 23 (FGF23) signalling. FGF23 promotes urinary phosphate excretion and inhibits the synthesis of calcitriol. The ectodomain of mKlotho is enzymatically released to result in a humoral factor, sKlotho, which exerts systemic effects (on ion channels and signalling pathways), possibly by working as an enzyme that modifies glycans of cell surface glycoproteins. GH enhances renal phosphate reabsorption and calcitriol production, i.e. exerts effects in the proximal tubule opposing those attributed to mKlotho, and attenuates calciuria in the distal tubule similar to sKlotho. sKlotho can be measured in extracellular fluids (serum, urine and cerebrospinal fluid (CSF)) by an ELISA. In line with predominant expression of Klotho in kidneys and choroid plexus, concentrations of sKlotho are particularly high in urine and CSF. Determination of sKlotho in serum and urine (both presumably reflecting GH action on the kidneys) could be used as a supplementary tool in the diagnosis and follow-up of patients with acromegaly. The question arises whether GH exerts selected actions via modifying activities of Klotho.

Introduction

This review on growth hormone (GH) and Klotho covers novel findings on a well-known hormone, the first to be extracted and characterized from the pituitary gland, and a more recently, accidentally discovered multifunctional protein. To enhance comprehension of the relationship between the two, we also discuss insulin-like growth factor 1 (IGF1), the first growth factor isolated and characterized from human serum, and fibroblast growth factor 23 (FGF23), currently the last member of the FGF family to be cloned. IGF1 is important for the apprehension of GH actions, and FGF23 is important for the understanding of the action of Klotho. These two growth factors illustrate the continuum between locally acting growth factors and systemically active hormones. Klotho works both locally and as a circulating humoral factor. Klotho was recently renamed α-Klotho, after the discovery of new Klotho family members; however, β-Klotho will be mentioned only briefly, although GH probably interacts with both α- and β-Klotho endocrine communication lines.

Acromegaly and GH

Acromegaly is a characteristic and disfiguring disease, described as a clinical entity in the late 19th century, which was later found to be caused by an eosinophil pituitary adenoma. GH excess produced by an adenoma of the pituitary gland is the major cause for acromegaly with an incidence of approximately four cases per 1 million persons per year. Clinical features develop insidiously and progressively over many years. It includes changes in the patient's appearance, mainly soft tissue swelling and cartilage and bone growth, resulting in typical acral enlargement and coarse facial features. Thus, with increasing typical signs and symptoms, diagnosis of acromegaly is generally straightforward. However, due to limited awareness of a rare disease, the correct diagnosis is often considerably delayed and is therefore associated with increased mortality even after curative therapy. For patients with acromegaly, (transsphenoidal) surgery remains the most promising therapeutic option, as it offers the best chance for long-term cure and restoration of pituitary function, including normalized GH secretion. Currently, GH and IGF1 (a marker and mediator of GH actions) are the classical biochemical markers of disease activity in acromegaly. Increased levels of GH (i.e. not suppressible to a nadir $<1\,\mu g/l$ during oral glucose tolerance testing, or $<0.3\,\mu g/l$, with more modern assays) and IGF1 on blood testing support the diagnosis. Likewise, to define cure, biochemical assessment is superior to MRI, as tiny tumour remnants are not reliably depicted.

The healthy human pituitary contains several milligrams of GH. Pituitary extracts were found to cause gigantism and pituitary hormones were found to be diabetogenic. GH was first isolated in 1944. In 1957, extraction of human GH from cadaver pituitaries succeeded, and in 1958, use of the extracted hormone to treat pituitary dwarfs was reported. During childhood and adolescence, when the epiphyseal lines have not fused, GH stimulates longitudinal bone growth, and GH excess from adenomas causes gigantism. In 1963, a RIA for GH was developed, and by 1970, Li succeeded with determination of the amino acid sequence and synthesis of GH. In 1985, several cases of Jakob Creutzfeldt disease were reported, and in the same year, recombinant human GH (rhGH) was introduced for therapeutical use in children. GH is effective in promoting growth of children with GH deficiency. However, in the rare condition of GH insensitivity (type Laron-Dwarfism), e.g. due to GH receptor (GHR) deficiency ('GHR-k.o.'), endogenous GH is high, and there is no response to rhGH administration (Godowski *et al*. 1989).

The wide availability of rhGH led to an increasing number of studies in adults with hypopituitarism including GH deficiency, and rhGH was reimbursed in 1996 for use in adulthood. It has never been established that isolated GH deficiency is associated with an increase in morbidity and mortality; however, GH excess is related to an increase in standard mortality rate.

GH is a single-chain polypeptide hormone (with two disulphide bridges) synthesized, stored and secreted by the somatotropic cells of the pituitary gland. GH consists of 191 amino acids, corresponding to the main circulating form, which is 22 kDa in size and mainly bound to a GH-binding protein (GHBP); the latter is derived from the GHR by proteolytic cleavage. GH is secreted in a pulsatile manner, mainly regulated by hypothalamic neuroendocrine mediators such as (inhibitory) somatostatin and GH-releasing hormone, but also by signals from the periphery such as (stimulatory) ghrelin and by feedback inhibition via IGF1. The effects of GH are mediated by the GHR. The human GHR gene was characterized by Wood and colleagues in 1989 (Godowski *et al*. 1989). It is a member of a large cytokine receptor family that also includes erythropoietin and IL6. Activation of the GHR by GH binding results in a conformational change of the receptor as well as in an activation of a JAK system and several downstream pathways, including STAT members and MAPK. The extracellular fragment of the GHR is cleaved from the cell surface and circulates as a GHBP. GHBP prolongs plasma half-life of circulating GH, which is estimated to be 2–5 min in the free form and 15–20 min in the GHBP-bound form. GH is cleared from the circulation via tissues that are also responsive to the hormone, especially liver and kidneys.

Activities of GH and IGF1

GH promotes (longitudinal bone) growth. It is anabolic, increasing protein synthesis and net nitrogen retention, and insulin is required for these effects. Some of the properties of GH have been fairly well characterized, e.g. on liver (increasing IGF1 production, synergistic with insulin), on cartilage (growth) and on adipose tissue (increasing lipolysis, antagonistic to insulin). However, other actions of GH are still poorly understood, such as the action on kidney function and phosphate homoeostasis, or on endothelial function. Some of the effects of GH are direct, e.g. on liver, adipose tissue and striated muscle (i.e. classical insulin target tissues) whereas others are mediated by IGF1 (and possibly other mediators).

Human blood contains several milligrams of IGFs. IGFs were discovered by three independent groups (Salmon & Daughaday 1957, Froesch *et al.* 1963, Pierson & Temin 1972), which were interested in biological activities exerted by serum: i) stimulation of proteoglycan synthesis (sulphate incorporation) in cartilage by a GH-dependent 'sulfation factor' (somatomedin), ii) insulin-like activity (non-suppressible by anti-insulin antibodies) on adipose tissue and iii) mitogenic activity on fibroblasts *in vitro* (multiplication-stimulating activity (MSA)). IGF1 works as a somatomedin in animals and in man (Schoenle *et al.* 1982, Laron *et al.* 1992).

Tests assessing the biological activities of GH and insulin were difficult 60 years ago and required *in vivo* bioassays (measuring the increase in body weight or in epiphyseal width in hypophysectomized rats, or the extent of blood sugar decrease in experimental animals); in search of improved methods (before RIAs were introduced), interesting discoveries were made.

Serum stimulates incorporation of $^{35}SO_4$ into cartilage *in vitro*. In 1957, Salmon and Daughaday reported that serum of hypophysectomized rats lacked such an activity. It could be reconstituted by administration of GH to hypophysectomized rats, but not by addition of GH to cartilage cultured in serum of hypophysectomized rats. These observations led to the concept that GH does not stimulate growth directly, but rather induces formation of factors that mediate action of GH. These factors were first called sulfation factors and later somatomedins; a GH-dependent plasma factor stimulates the incorporation of sulphate into chondroitin sulphate in cartilage (Salmon & Daughaday 1957, Daughaday *et al.* 1972).

Serum contains insulin-like activity. Froesch *et al.* observed that most of the insulin-like activity in serum could not be suppressed by the addition of antiserum neutralizing insulin to the incubation medium. Therefore, 'non-suppressible insulin-like activity (NSILA)' was different from insulin but could mimic its effects on adipose tissue (Froesch *et al.* 1963). Later, molecules (7.6 kDa in size) responsible for this insulin-like activity were purified from serum and characterized (Rinderknecht & Humbel 1978). The designation IGFs appeared justified as IGFs are functionally and structurally closely related to insulin. As the C-domain of IGFs is not removed during prohormone processing, mature IGF peptides are single-chain polypeptides. IGF1 consists of 70 amino acids and displays 43% sequence identity to the insulin A- and B-chain (Rinderknecht & Humbel 1978). IGFs bound to IGF binding proteins (IGFBPs) cannot interact with insulin

receptors; moreover, the affinity of IGFs to insulin receptors is low.

Serum provides components required for optimal growth of most cells in culture, including growth factors. Factors could be extracted from serum with MSA, i.e. factors that stimulate replication of cells in culture (Pierson & Temin 1972). Subsequently, it was found that MSA was also produced by cultured liver cells (Dulak & Temin 1973).

IGFs (IGF1 and IGF2) signal through type 1 IGF receptors. Insulin and IGF have their own distinct cell surface receptors whose structures have been deduced from analysis of isolated cDNA clones. Type 1 IGF and insulin receptor are homologous (Ullrich *et al.* 1986); both consist of $\alpha_2\beta_2$ 'hetero-tetrameric' structures. The α-subunits lie entirely extracellular and contain a ligand-binding domain, while the β-subunits are transmembrane polypeptides that contain a tyrosine kinase domain in their intracellular portion. Signal transduction by type 1 and insulin receptors involves autophosphorylation at tyrosine residues and phosphorylation of other substrates.

Radiolabelled IGFs had been used to characterize specific IGF binding sites (receptors) on cells. In addition, they allowed the discovery of specific IGFBPs in extracellular fluids. IGFBPs in the strict sense are entirely unrelated to IGF receptors and bind IGFs with comparable or even higher affinity than the type 1 IGF receptor. IGFBP3 is by far the most abundant IGFBP in adult human serum, and its production (like that of IGF1) is dependent on GH both *in vivo* and *in vitro*. Intact IGFBP3 prevents access of IGF1 to its receptors, but proteolysis of IGFBP3 activates IGF1 signalling (Schmid *et al.* 1991). GH (but not IGF1) induces an IGFBP3-proteolytic activity (Rutishauser *et al.* 1993).

IGFBPs appear to affect distribution volumes and half-lives of IGFs; they contribute to the large pool of IGFs present in most extracellular spaces, and they modify their biological activity. IGFBP3 serves as the major circulating carrier of IGFs, most of which are associated with a GH-dependent ternary complex of 140 kDa, comprising an acid-labile 85 kDa glycoprotein, IGFBP3, and IGF1 or IGF2. The apparent half-life of IGFs is about 10 min in their free form and 10–15 h when in the ternary complex; the large molecular weight complex accounts for the slow turnover of serum IGFs (Guler *et al.* 1989).

IGF1 is produced continuously, especially in the liver that is estimated to be is responsible for 70–80% of circulating IGF1. Apart from the 'endocrine' IGFs, IGFs are also produced in most tissues where they can act in a paracrine or autocrine manner. Serum IGF1 is a good hepatic marker of GH action. Owing to its long plasma half-life, levels are fairly stable throughout the day,

and timing of blood sampling for diagnostic purposes is not critical.

Until now, GH and especially GH activity, as reflected (in part) by IGF1 concentrations, have been the 'classical' biomarkers for diagnosing and monitoring disease activity during the treatment of patients with acromegaly. Their normalization has been linked to decreased mortality. However, it has been recognized that both parameters entail various shortcomings, both analytical and biological (Clemmons 2011). GH levels are age dependent, and serum levels of IGF1 are influenced not only by GH status but also by age, gender (oestrogens), race, liver function, nutritional status, portal insulin, thyroid hormones and by concomitant inflammatory disease. Unfortunately, measuring IGF1 remains notoriously difficult, especially since IGFBPs interfere with immunoassay analysis. For the classical and time-consuming RIAs, IGF carrier proteins are removed before the samples are incubated with the antibodies (Zapf et al. 1980, 1981). Ideally, IGF1 should normalize and glucose-suppressed GH should be low after surgery for acromegaly. Most often, 1 µg/l (or lower) has been used as a cut-off value (Giustina et al. 2010).

Activities of FGF23

FGF23 may be most easily understood by the recognition of tumours by which it is secreted in excessive amounts. Similar to acromegaly, tumour-induced osteomalacia (TIO) is an acquired condition that can resolve after successful removal of the tumour, the source of excessive FGF23. In 1957, Prader reported the case of an 11½-year-old girl who presented with an acquired disorder of hypophosphataemia, renal phosphate wasting and rickets. He attributed the phenotype of the disease to a circulating factor, a 'rachitogenic substance' secreted by the tumour causing renal phosphate wasting ('phosphate diabetes'), as it disappeared after surgical excision of a benign mixed connective tissue tumour from the rib (Prader et al. 1959). The phenotype described corresponds to what we would nowadays designate a case of FGF23-mediated TIO. FGF23 was later cloned as a causative factor from tumours that result in osteomalacia (Shimada et al. 2001).

Two other groups, independently, had previously cloned FGF23, by homology as an additional FGF family member, preferentially expressed in the ventrolateral thalamic nucleus of the brain, and by genetic analysis of autosomal dominant hypophosphataemic rickets (ADHR; ADHR Consortium 2000, Yamashita et al. 2000).

Shimada reported in 2004 that overexpression of FGF23 in rodents essentially replicated the phenotype of TIO and ADHR. Independent groups confirmed that FGF23 overexpression in transgenic animals or its administration to rodents resulted in renal phosphate loss, hypophosphataemia, impaired production of 1,25-$(OH)_2$D and rickets (Bai et al. 2004, Larsson et al. 2004, Shimada et al. 2004c).

FGF23, a 32 kDa glycoprotein with very weak heparin-binding affinity, is produced mainly in bone by osteoblasts/osteocytes; its production is stimulated by decreased glomerular filtration rate (GFR)/low nephron number as with chronic kidney disease (CKD) and ageing and dietary phosphate overload. The 24 amino acid hydrophobic signal sequence is removed and the protein O-glycosylated by GALNT3 to produce the mature, biologically active $^{25}FGF23^{251}$ form in the circulation, with an estimated half-life of about 50 min (Khosravi et al. 2007). FGF23 acts predominantly on kidneys to decrease sodium (Na)-dependent phosphate transporters NaPi2a and NaPi2c (SLC34a1 and SLC34a3) activity and thereby increase phosphate excretion ('phosphatonin') (Shimada et al. 2004c, Segawa et al. 2007, Gattineni et al. 2009). It inhibits vitamin D 1α-hydroxylase (CYP27B1) and calcitriol generation, and it enhances 24α-hydroxylase (CYP24A1) and calcitriol degradation (Saito et al. 2003, Shimada et al. 2004a). Therefore, excessive FGF23 production (by tumours) or inadequate inactivation (in ADHR) of FGF23 results in hypophosphataemia and rickets (in children) or osteomalacia (in adults). This happens due to renal phosphate loss ('acquired phosphate diabetes'), inhibition of vitamin D 1α-hydroxylase (impaired calcitriol production) and disturbed mineralization. In patients with progressive fatigue, muscle weakness and bone pain, persistent hypophosphataemia due to renal phosphate wasting and an inappropriately low 1,25-$(OH)_2$ D level are the biochemical hallmarks of TIO (Bauer et al. 2010).

To understand the physiological roles of FGF23, mice lacking FGF23 were generated by targeted gene disruption. Defective production results in hyperphosphataemia and activation of vitamin D 1α-hydroxylase, severe calcifications (subcutaneous, periarticular, kidneys, lungs and vessels) and premature ageing. These $Fgf23^{-/-}$ mice have, reminiscent of $Klotho^{-/-}$ mice, hyperphosphataemia and extremely high serum calcitriol levels (Yoshida et al. 2002, Shimada et al. 2004b). It was realized that $Fgf23^{-/-}$ mice (lacking the hormone) and $Klotho^{-/-}$ mice (lacking functioning receptors) had similar phenotypes (see below).

FGFs signal through tyrosine kinase receptors and ERK. Among the 22 FGF members, most act in an

autocrine or paracrine fashion. The FGF19 (endocrine) subfamily includes FGF15 (in mice)/FGF19 (in humans), FGF21 and FGF23 (Itoh & Ornitz 2011). These hormone-like FGFs appear to be vertebrate specific and characteristically lack a heparin-binding domain. Therefore, they are not retained by the nearby extracellular matrix and reach the circulation. Moreover, endocrine FGFs do not require heparan sulphate proteoglycans for efficient FGFR tyrosine kinase activation; instead, they need transmembrane Klotho(s) (mKlotho) as co-receptors (see section Klotho, a transmembrane co-receptor and a multifunctional humoral factor in extracellular spaces). FGF15/FGF19 is mainly produced by the small intestine and predominantly targets the liver (FGFR4 and β-Klotho coexpression) to regulate cholesterol and (suppress) bile acid synthesis. FGF21 is mainly secreted from the liver and predominantly acts on white adipose tissue (FGFR1c and β-Klotho coexpression) to regulate glucose and lipid metabolism. This corresponds to FGF23, which is mainly produced by bone and predominantly targets the kidneys to regulate phosphate and vitamin D metabolism. β-Klotho belongs to the receptor complex of the former two endocrine FGFs and α-Klotho to the receptor complex of FGF23. In the kidneys, FGFR1 may be the most relevant receptor to mediate FGF23 actions (Gattineni *et al.* 2009). An activating mutation of FGF1R has been reported to cause osteoglophonic dysplasia and hypophosphataemia (White *et al.* 2005). Antibodies activating FGFR1 also cause hypophosphataemia (Wu *et al.* 2013).

Klotho, a transmembrane co-receptor and a multifunctional humoral factor in extracellular spaces

While studying the phenotype of transgenic mice over-expressing the rabbit type I sodium proton exchanger, insertion of ectopic DNA happened to occur into the 5′ promoter region of the α-Klotho gene; α-Klotho protein was hardly expressed. The *Klotho* gene was thereby identified serendipitously in this mouse model in 1997 after recognition that its disruption caused a phenotype of accelerated ageing (Kuro-o *et al.* 1997). Later, it was found that ageing suppression and lifespan extension were achievable in mice by overexpression of Klotho; these mice lived about 20% longer than WT mice and were more resistant to oxidative stress (Kurosu *et al.* 2005). The name Klotho stems from the goddess of fate, which is 'spinning the thread of life' in Greek mythology. Mice with deficient *Klotho* expression have a syndrome that resembles human ageing, including shorter lifespan (on average,

only 2 months instead of 2–3 years as for WT mice), premature skin atrophy, osteopenia/osteoporosis, dysfunction of the pituitary gland (GH deficiency), growth retardation, hypogonadotropic hypogonadism (infertility), atrophy of genital organs, thymus and muscle, arteriosclerosis, ectopic (soft tissue and media-) calcifications, pulmonary emphysema and neurodegenerative disorders (hypokinesis, gait disturbances and hearing disorder). Klotho-deficient mice were subsequently characterized in more detail; e.g. the brain phenotype (distinct from the aged human brain with senile plaque deposition) includes memory retention deficits, a reduction in synapses in the hippocampus, disturbed axonal transport and hippocampus degeneration and impaired myelin production in specific areas of the brain (Nagai *et al.* 2003, Li *et al.* 2004, Shiozaki *et al.* 2008, Chen *et al.* 2013a). A recent study suggested that decreased activation of JAK2/STAT3 signalling and cholinergic pathways in hippocampus may play an important role in cognitive impairment observed in Klotho mutant mice (Park *et al.* 2013). Biochemically, Klotho deficiency results in hyperphosphataemia with increased $1,25\text{-}(OH)_2$ vitamin D_3 and high FGF23 in keeping with FGF23 resistance, moreover in hypoglycaemia with low insulin (insulin hypersensitivity).

The *Klotho* gene product was proposed to function as a part of a signalling pathway that regulates ageing *in vivo* and morbidity in age-related diseases. The *Klotho* mouse created by unintentional insertion is not a strictly null strain, but a $Klotho^{-/-}$ mouse was later intentionally generated, and this 'classical' knockout mouse was found to exhibit essentially the same phenotype as the original *Klotho* mouse, including hyperphosphataemia and disturbed vitamin D regulation (Tsujikawa *et al.* 2003). Over the past decade, several aspects reported in the original 1997 paper were described in more detail, including neurodegeneration, hearing loss, sarcopenia, ageing lung/emphysema and arteriosclerosis. However, discussing all of them would be beyond the scope of the current review. Several excellent reviews discuss specific aspects of Klotho in more detail (Nabeshima & Imura 2008, Kuro-o 2010, Manya *et al.* 2010, Martin *et al.* 2012, Hu *et al.* 2013).

The *Klotho* gene encodes a single-pass type I transmembrane protein (1014 amino acids in the mouse and 1012 in humans) with a short cytoplasmic domain and an extracellular domain composed of two β-galactosidase/glycosidase-like tandem repeats (KL1 and KL2) with β-glucuronidase and sialidase activity. It is predominantly expressed in kidneys, brain (choroid plexus (CP), neurons in hippocampus, Purkinje cells and inner ear), the

sinoatrial node of the heart and in several endocrine (pituitary, parathyroid and pancreas) and reproductive organs (gonads and placenta) (Kuro-o *et al.* 1997, Kato *et al.* 2000, Li *et al.* 2004, Takeshita *et al.* 2004, German *et al.* 2012). Although primarily in distal convoluted tubules, Klotho is expressed in all tubular segments along the nephron (Hu *et al.* 2010, Zhou *et al.* 2013). More details on the mouse *Klotho* gene and the identification of the human (96% homology) as well as the rat *Klotho* gene were reported 1 year later (Matsumura *et al.* 1998, Ohyama *et al.* 1998, Shiraki-Iida *et al.* 1998); two distinct transcripts encoding membrane and secreted Klotho protein were characterized. Klotho has been identified not only in the zebrafish, *Danio rerio*, but two *Klotho*-like genes, *klo1* and *klo2*, have also been identified in the nematode *Caenorhabditis elegans* (Polanska *et al.* 2011, Sugano & Lardelli 2011, Mangos *et al.* 2012). The corresponding KLO1 and KLO2 proteins lack a transmembrane domain. The ancestral 'truncated' Klotho forms in nematodes contain only one KL domain and lack the second internal repeat (whereas the full-length transmembrane vertebral Klothos are composed of two KL domains). Therefore, they correspond more closely to the alternatively spliced form for the shorter, directly secreted isoform in men (Polanska *et al.* 2011).

The function of mKlotho protein remained an enigma for years. It was first recognized that FGF23 deficiency and Klotho deficiency (with FGF23 resistance) result in closely resembling phenotypes, namely with hyperphosphataemia by up-regulated NaPi cotransporters, grossly elevated 1,25-$(OH)_2$ D_3, due to up-regulated 1α-vitamin D hydroxylase, vascular calcification and premature ageing (Yoshida *et al.* 2002). In 2006, two independent groups reported that mKlotho served as a co-receptor for FGF23 (Kurosu *et al.* 2006, Urakawa *et al.* 2006). mKlotho forms a constitutive binary complex with the FGFR1c to create a *de novo* high-affinity binding site for FGF23. This Klotho–FGFR complex binds to FGF23 with much higher affinity than FGFR or Klotho alone; Klotho greatly enhances the ability of FGF23 to induce phosphorylation of FGFR substrate and ERK and is thus considered an essential cofactor for activation of FGF23 signalling by FGFR23. FGF23 elicits ERK phosphorylation and up-regulation of the expression of early growth-responsive (Egr) mRNA in Klotho-expressing tissues and cells, in the kidneys, parathyroids and pituitary, but not in liver, heart, bone and arterial walls (Kurosu *et al.* 2006, Urakawa *et al.* 2006, Ben-Dov *et al.* 2007, Krajisnik *et al.* 2007, Andrukhova *et al.* 2012, Lindberg *et al.* 2013). These (renal) FGF23 activity-enhancing properties of mKlotho are

particularly important as reducing phosphate (by depleting the diet in vitamin D or phosphate or by depleting NaPi cotransporters) and abolishing vitamin D activities rescue most phenotypes of *Klotho*$^{-/-}$ mice (Morishita *et al.* 2001, Tsujikawa *et al.* 2003, Hesse *et al.* 2007, Nakatani *et al.* 2009, Ohnishi *et al.* 2009).

Klotho protein also exists in a soluble form, which can arise either from a distinct transcript or from ectodomain shedding of mKlotho (Fig. 1). The large extracellular domain of the membrane-bound form can be enzymatically cleaved (by α- and β-secretases) and released as 'secreted' Klotho into blood, urine and cerebrospinal fluid (CSF). The cleaved 130 kDa extracellular domain may be the preferably detected Klotho form in extracellular fluids, as indicated (by the green bars) in Figs 1 and 2 (Imura *et al.* 2004, Yamazaki *et al.* 2010). Soluble Klotho (sKlotho) released into the extracellular spaces can reach and affect a number of target tissues and processes, including regulation of hormone and growth factor signalling (e.g. inhibition of insulin/IGF1) (Kurosu *et al.* 2005, Wolf *et al.* 2008), and the regulation of plasma membrane amount and activity of ion channels and transporters, e.g. to
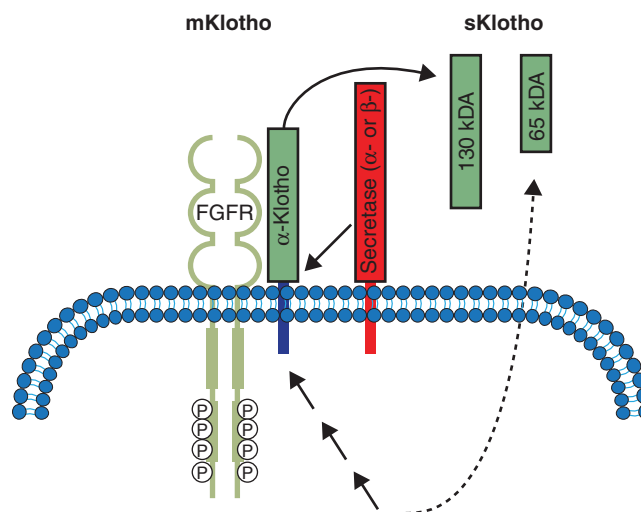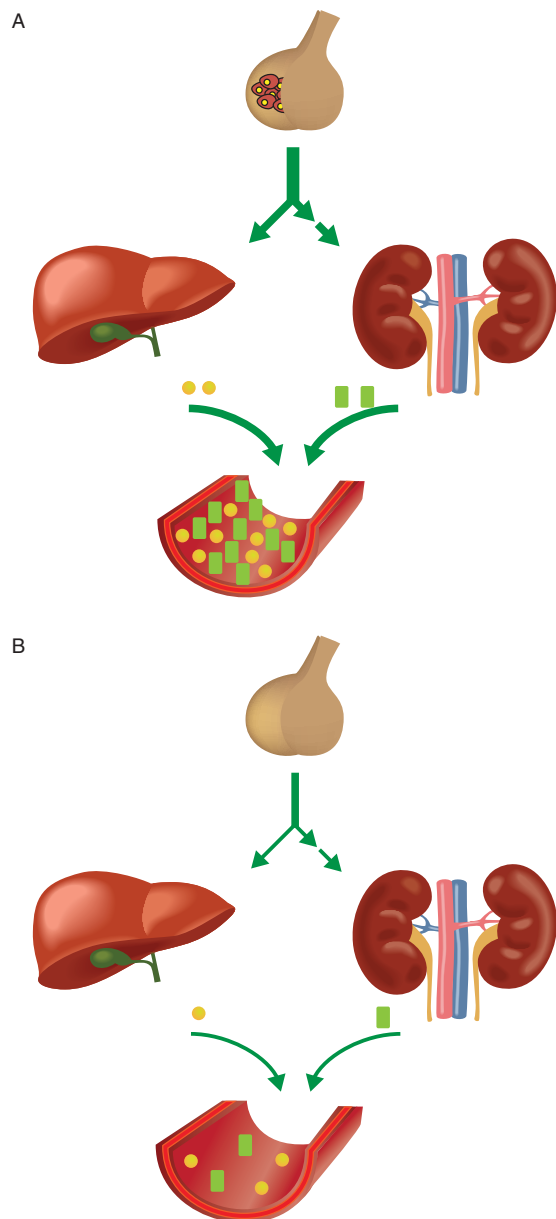

Figure 1

Transmembrane and soluble α-Klotho. A plasma cell membrane is shown into which mKlotho (single-pass transmembrane, very short cytoplasmic tail) is inserted. mKlotho associates with an FGFR; the latter can transmit (FGF23-induced) signals through its tyrosine kinase domains and ERK activation. sKotho can be derived from mKlotho when its large extracellular part (130 kDa) is clipped off by enzymes; apart from this large sKlotho form, there may be a smaller, directly secreted sKlotho form (65 kDa), resulting from differential splicing. mKlotho (cofactor for FGF23 binding to FGFR) expression is restricted to a limited number of tissues while sKlotho is present in most extracellular spaces and can regulate many processes throughout the body, including calcium handling, nitric oxide synthesis, insulin and (e.g. insulin-like) growth factor signalling, as discussed in the text.

Figure 2

Effect of GH-secreting pituitary adenomas on circulating levels of IGF1 and sKlotho. (A) In the preoperative condition, there is a GH-secreting pituitary tumour in the pituitary gland. Excessive GH directly acts on the liver (predominant source of IGF1) and the increased production of IGF1 results in markedly increased serum IGF1 levels (mean ± s.e.m., 0.58 ± 0.03 mg/l). Excessive GH also results in markedly increased serum sKlotho levels (4.1 ± 0.4 ng/ml); sKlotho is most likely of renal origin, but the mechanism (direct/indirect) by which GH excess results in increased release of kidney-derived sKlotho has not yet been elucidated. sKlotho particles as detected by the Yamazaki–ELISA (most likely the 130 kDa form) possibly represent only a tiny fraction of the total amount of sKlotho in the serum. The number of both IGF1 particles (small yellow circles) and sKlotho particles (green bars, as in Fig. 1) is dramatically increased, by factors of about 3 and 5 respectively. (B) Postoperatively, the GH-secreting adenoma has been removed, and GH action is normal; IGF1 (mean ± s.e.m., 0.20 ± 0.01 mg/l) and sKlotho (0.8 ± 0.1 ng/ml) levels returned towards normal (data from Kohler *et al*. 2013).

attenuate calciuria. There is an ongoing debate on whether FGF23 can exert physiologically relevant effects on cells that do not express functional mKlotho, or do so only to a level below detection limits for the protein. Experimental data have suggested that at high doses, FGF23 and 130 kDa sKlotho, when combined, could allow FGF23 signalling, e.g. in chondrocytes and osteoblastic cells (Shalhoub *et al*. 2011, Kawai *et al*. 2013). Concerning vascular function and calcification, more recent data in mice and humans indicate that arterial Klotho expression was low or absent and did not mediate vascular FGF23 signalling (Lindberg *et al*. 2013, Scialla *et al*. 2013). Currently, sKlotho should not be labelled as a hormone as cognate receptors have not been identified so far, in contrast to those known for GH, IGF1, FGFs and for erythropoietin, 1,25-(OH)$_2$ D and angiotensin II. In this regard, sKlotho (like renin) could be considered a renal enzyme rather than a hormone. Circulating sKlotho can regulate biological processes, e.g. by (enzymatic) glycan modification. Illustrating an action on the nephron, sKlotho modifies *N*-glycan chains of the epithelial Ca^{2+} channel transient receptor potential cation channel, subfamily V, member 5 (TRPV5) in the distal tubule. As a consequence, TRPV5 is retained on the cell surface and the activity of the ion channel is increased, and renal calcium loss is decreased (Chang *et al*. 2005, Cha *et al*. 2008, Alexander *et al*. 2009, Huang 2012, Olauson *et al*. 2012). α-Klotho has been found to be a central player in calcium (particularly sKlotho) and phosphate (predominantly mKlotho, FGF23 dependent) homoeostasis, mainly by acting on the kidneys and the parathyroids (Imura *et al*. 2007, Nabeshima & Imura 2008, Kuro-o 2010). FGF23 suppresses production of PTH via mKlotho/FGFR–ERK1/2 (Ben-Dov *et al*. 2007, Krajisnik *et al*. 2007). Considering its effects on renal phosphate and calcium handling, α-Klotho shares phosphaturic effects (proximal tubule) and calcium-retaining effects (distal tubule) with PTH; however, their effects on generation of calcitriol are opposite to each other (stimulation by PTH, inhibition by Klotho). In the proximal tubule, FGF23 inhibits phosphate reabsorption by activating FGFR1 in a mKlotho-dependent fashion. sKlotho could also act as an enzyme (β-glucuronidase-like) to induce phosphaturia in an FGF23-independent manner; however, the direct enzyme target (potentially NaPi2a and NaPi2c) has been less well defined than in the case of TRPV5 (Hu *et al*. 2010). Disruption of the Klotho gene not only causes growth arrest, hyperphosphataemia and high calcitriol but also causes low glucose and insulin levels; however, whether loss of Klotho directly improves insulin sensitivity is still unclear (Utsugi *et al*. 2000, Hesse *et al*. 2007, Lorenzi *et al*. 2010). The mice are lean and have

almost no white adipose tissue (Klotho promotes adipocyte differentiation), lower body temperature and less energy expenditure (Mori *et al.* 2000, Chihara *et al.* 2006, Razzaque 2012). Another important role for Klotho, possibly related to inhibition of insulin/IGF1 signalling, is increased resistance to oxidative stress, which may contribute to the anti-ageing properties of Klotho (Yamamoto *et al.* 2005). Along this line, Klotho protein exerts protective effects, e.g. Klotho protein treatment attenuated cisplatin-induced reactive oxygen species generation. Thereby, it possibly helped to prevent reduction in cell viability in response to this ototoxic drug in an auditory cell line (Park *et al.* 2012). On the other hand, Klotho sensitized lung cancer cells to cisplatin, apparently via the PI3K/PKB pathway (Wang *et al.* 2013).

CP epithelial cells, besides renal tubular cells, express the highest levels of Klotho but they have been studied less often than kidney cells. Imura *et al.* (2007) were the first to address potential roles of Klotho in this tissue and reported on the action of α-Klotho on Na^+/K^+ ATPase activity and transcellular calcium transport. It appears likely that Klotho in CP cells contributes to regulation of composition and homoeostasis of CSF similarly as the kidneys contribute to the homoeostasis and regulation of plasma composition; therefore, CP has been considered as the 'kidney' of the brain. Recently, a molecular characterization employing a combination of transcriptomics and high-resolution tandem mass spectroscopy-based proteomics methods was carried out (in rats) to gain insight into CP function (Sathyanesan *et al.* 2012). A validation of the findings at the mRNA and protein level was done by *in situ* hybridization and immunohistochemistry. The close relationship between the transcriptome and proteome profiles from the CP and the kidney tubules was a main finding; physiological parallels between CP and renal tubuar cells include high energy (and high blood flow) requirement and highly regulated pumps and transporter activities in both tissues. In addition, strong expression of Klotho was confirmed, and co-localization with transthyretin (an excellent CP marker) in CP was demonstrated (Kuro-o *et al.* 1997, Li *et al.* 2004, Sathyanesan *et al.* 2012). Chronic unpredictable stress reduced expression of Klotho in CP of their rats, as Ohyama found that acute inflammatory stress decreased renal Klotho expression (Ohyama *et al.* 1998, Sathyanesan *et al.* 2012).

Soon after discovery of the Klotho-deficient mouse, it was found that Klotho protein protects against endothelial dysfunction (Saito *et al.* 1998, 2000). Klotho contributes to maintenance of endothelial function and NO production via cAMP and calcium channel activation, upregulation of the mitogen-activated kinase pathway, and increases stress tolerance, decreases oxidative damage, apoptosis and senescence of endothelial cells (Ikushima *et al.* 2006, Rakugi *et al.* 2007, Kusaba *et al.* 2010, Maekawa *et al.* 2011, Carracedo *et al.* 2012).

Germ line mutations or polymorphisms in *Klotho* do not obviously predispose to the development of neoplasms, and there is no evidence for an increased incidence of malignancies in Klotho-deficient mice. Nevertheless, Klotho can be viewed as a potential tumour suppressor, e.g. in breast, pancreatic, lung and renal cancer (Wolf *et al.* 2008, Chen *et al.* 2010, Abramovitz *et al.* 2011, Zhu *et al.* 2013). Intratumoral Klotho decline correlates with cancer progression, tumour size, TNM stage and nuclear grade in renal cell carcinoma, and with enhanced activation of the PI3K/PKB pathway (Zhu *et al.* 2013). Functional loss of Klotho due to epigenetic silencing was found to promote tumour progression in a variety of cancers.

Inhibition of Wnt signalling may play a role in the senescence-suppressing and renal fibrosis inhibitory effects of Klotho (Liu *et al.* 2007, Doi *et al.* 2011, Satoh *et al.* 2012, Sugiura *et al.* 2012, Zhou *et al.* 2013); TGFβ appears to activate β-catenin via suppression of Klotho. sKlotho via its Kl1 domain blocks Wnt-triggered activation and nuclear translocation of β-catenin and thereby protects the kidneys from developing fibrosis in response to injury (Zhou *et al.* 2013). sKlotho attenuates IGF1 signals in the compensatory renal hypertrophy following uninephrectomy (Nagasu *et al.* 2011). Decreased serum sKlotho was associated with enhanced cyst growth in patients with autosomal dominant polycystic kidney disease (Pavik *et al.* 2012). As described for IGF1/PI3K/PKB signalling, sKlotho may also contribute to apoptosis induction and cell growth inhibition by decreasing the activity of the Wnt- β-catenin signalling pathway (Chen *et al.* 2012).

Gene delivery of Klotho could influence several pathophysiological phenotypes of Klotho mice, including improvement of vascular calcification (Shiraki-Iida *et al.* 2000), protection against endothelial dysfunction (Saito *et al.* 2000) and preventing progression of angiotensin II-induced or hypertensive renal damage (Mitani *et al.* 2002, Wang & Sun 2009). More recently, administration of recombinant sKlotho protein to *Klotho*$^{-/-}$ mice was found to suppress accelerated ageing (Chen *et al.* 2013*b*).

Human pathologies due to deficient or excessive Klotho?

Years before immunoassays became available, genetic studies were performed. Arking *et al.* (2002) reported an association of human ageing with a functional variant of

Klotho. Humans with the Klotho-VS polymorphism were found to have reduced cognitive abilities (Deary *et al.* 2005). More recently, a functional variant of *Klotho* was found to be associated with early-onset ischaemic stroke (Majumdar *et al.* 2010). A 13-year-old girl with a remarkable phenotype of hyperphosphataemia, and soft tissue and vascular calcification, 'tumoral' calcinosis, was found to harbour a homozygous missense (H193R) mutation in the Klotho gene. Hyperphosphataemia was consistent with renal FGF23 resistance, as Klotho mutations are expected to prevent complex formation with FGF23 (functionally defective receptors), and lead to decreased FGF23 signalling: FGF23 was indeed markedly elevated (familial 'tumoral' calcinosis was a known phenotype caused by inactivating mutations in FGF23 or GALNT3, i.e. diseases caused by defective ligand had been described before). Hyperparathyroidism was proposed to be caused by a lack of an α-Klotho-dependent inhibitory effect of FGF23 on PTH production, although it should be noted that α-Klotho could also affect PTH secretion directly (without FGF23), by increasing cell surface Na^+–K^+-ATPase activity (Ichikawa *et al.* 2007, Imura *et al.* 2007). Another exceptional case was reported, with a translocation adjacent to α-Klotho gene, causing markedly increased circulating α-Klotho (and FGF23) level, resulting in hypophosphataemia (with rickets) and hyperparathyroidism (Brownstein *et al.* 2008). In animals, circulating α-Klotho *per se* may induce FGF23 production and phosphaturia (Smith *et al.* 2012), and so may antibodies activating FGFR1 (Wu *et al.* 2013).

sKlotho is released into the circulation, and its plasma half-life has been estimated to be about 20–30 min by Imura. sKlotho can be detected by a recently developed and commercially available sandwich ELISA (Yamazaki *et al.* 2010; Table 1). Levels of sKlotho decrease with age in healthy subjects and are inversely related to mortality in the elderly (Semba *et al.* 2011). High levels of sKlotho were found in human umbilical cord blood (Ohata *et al.* 2011, Siahanidou *et al.* 2012). The placenta is known to produce sKlotho and may account for these high levels. However, except for the neonatal period, the kidneys appear to be the major source of sKlotho in sera of human subjects. Accordingly, serum sKlotho decreased by about 40% following nephrectomy in living donors (Akimoto *et al.* 2013). So far, there is no well-established reference range for serum sKlotho. In our Zürich area, the median value of healthy adult subjects is around 0.6 ng/ml and, therefore, somewhat lower than previously reported by Japanese and Italian authors using the same assay (Yamazaki *et al.* 2010, Semba *et al.* 2011, Akimoto *et al.*

2012, Neidert *et al.* 2013, Pavik *et al.* 2013). Interestingly, the Italian authors found that low circulating sKlotho levels in elderly adults are not only associated with increased mortality but also associated with poor muscle strength and disability in activities of daily living (Semba *et al.* 2011, 2012, Crasto *et al.* 2012).

It should be mentioned that many uncertainties still exist when assessing sKlotho levels, as the identity of the detected molecules is not always clear (see also Figs 1 and 2). In fact, in 2004, Xiao *et al.* reported on a polyclonal antibody raised in rabbits against the C-terminal of human secreted Klotho protein (amino acids 290–549). It could be used for western blotting to detect mainly a 60 kDa protein and for an ELISA to measure sKlotho in human serum; sKlotho was found to decrease with age with this particular assay (Xiao *et al.* 2004).

The ImmunoBiological Laboratories (IBL, Fujioka, Japan) ELISA as developed by Yamazaki and the time-resolved fluorescence immunoassay (Cusabio, Wuhan, China) assays have recently been directly compared in a Danish study where considerable differences became evident (Pedersen *et al.* 2013). The IBL ELISA utilizes two monoclonal antibodies that may detect and measure one larger form of ('intact', full-length shedded) Klotho. On the other hand, the Cusabio assay (using mouse mono-clonal antibody-precoated plates and goat polyclonal antibodies of unknown epitope specificity for detection) may recognize additional, shorter forms (65 kDa and possibly other forms) that may be much more abundant (Matsumura *et al.* 1998). As mentioned, the IBL assay has been used quite often lately (Table 1), whereas the Cusabio–ELISA has been validated just recently in studies performed in the USA and Denmark (Devaraj *et al.* 2012, Pedersen *et al.* 2013). In their review, Hu *et al.* (2013) estimated plasma concentrations of sKlotho in the range of 10–50 nM, which would be much higher than what is measured by the Yamazaki assay (Yamazaki *et al.* 2010).

CKD can be viewed as a state of Klotho deficiency. As the kidney is the primary source and a major target of Klotho, the focus of Klotho research has been on nephrology, apart from basic and ageing research. Although renal expression of the *Klotho* gene and urinary Klotho levels have been found to be decreased in renal disease (Koh *et al.* 2001, Hu *et al.* 2011), it currently remains controversial whether plasma sKlotho is a useful biomarker for kidney disease (Sugiura *et al.* 2011, Pavik *et al.* 2012, 2013, Kim *et al.* 2013, Seiler *et al.* 2013). FGF23 may give a much better readout than sKlotho, as the hormone released by bone in response to kidney disease appears to quite reliably reflect renal resistance to FGF23.

Table 1 Soluble serum Klotho levels in healthy human subjects as assessed by IBL ELISA

Author/year	n	Subjects	Age (years)	Sex (m/f)	Klotho levels (pg/ml)	Comments
Akimoto et al. (2013)	10	Living kidney donors	64±9	4/6	910 (median); 755–1132 (IQR)	The kidneys seem to be the major source of serum sKlotho
Amitani et al. (2013)	11	Healthy	21±1 (S.E.M.)	0/11	1392±145 (S.E.M.±S.E.)	Klotho levels are lower in patients with anorexia nervosa and patients with obesity than in normal weight controls
Crasto et al. (2012)	802	Elderly	>65	357/445	689 (mean); 238 (S.D.)	Low serum Klotho is associated with ADL disability
Neidert et al. (2013)	26	Healthy	39±8	15/11	596 (median); 506–734 (IQR)	Serum Klotho is excessively elevated in patients with untreated acromegaly
Pedersen et al. (2013)	120	Healthy	40 (19–66) median (range)	58/62	472±137 (mean±S.D.)	Different immunoassays recognize distinct forms of sKlotho
Pavik et al. (2012)	20	Healthy	32±6	12/8	1200±385 (mean±S.D.)	Low serum sKlotho is associated with cyst growth and low phosphaturic activity of FGF23 in ADPKD
Pavik et al. (2013)	21	Healthy	48±8	9/12	600 (median); 429–862 (IQR)	Serum Klotho decreases in advanced CKD
Semba et al. (2011)	1023	Elderly	73 (69–79) median (IQR)	564/459	676 (median); 530–819 (IQR)	Klotho is inversely related to CVD risk
Semba et al. (2011)	804	Elderly	>65	354/450	697±325 (mean±S.D.)	Serum sKlotho is suggested as an independent predictor of all-cause mortality
Sugiura et al. (2011)	10	Healthy	20–44	NA	404±87 (mean±S.D.)	Increased serum sKlotho is suggested as a renoprotective factor in CKD
Yamazaki et al. (2010)	181	142 healthy adults (39 children)	61±19 (7±8)	66/76 (23/16)	562±146 (mean±S.D.) 952±282 (mean±S.D.)	Serum Klotho is inversely related to age and creatinine

ADL, activities of daily living; ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FGF23, fibroblast growth factor 23; IBL, ImmunoBiological Laboratories Co. Ltd.; IQR, interquartile range.

In this context, it may be worth mentioning potential off (classical) target effects of hormones adapting to a resistance induced by Klotho deficiency: FGF23 excess may (not result in hypophosphataemia but) induce left ventricular hypertrophy in a Klotho-independent manner (Faul et al. 2011).

As reported by Imura et al. (2004), sKlotho can be found not only in serum and urine but also in CSF. Interestingly, levels of sKlotho are quite high in CSF and in urine; CP and kidney as Klotho-expressing sites may account for these findings. CSF Klotho levels as measured by immunoassay have not been reported in the literature, but data for urine are available and suggest that urine Klotho is linked to renal function (Akimoto et al. 2012). Over the past 6 months (January–June 2013), we have also started to assess CSF and urine samples for sKlotho. According to these preliminary data, concentrations of sKlotho are similar or slightly higher in CSF than in serum: sKlotho was 888 (595–1319) pg/ml (median (range)) in CSF (n=9) vs 557 (255–1017) pg/ml in the corresponding sera. This is remarkable, given that the total protein concentration is two orders of magnitude lower in CSF than in plasma and serum. Despite samples obtained from quite distinct patients (none of them, with acromegaly) for CSF analysis, we found a narrow concentration range in CSF when compared with serum. This could suggest that Klotho levels are maintained within a certain limit within this compartment. Klotho may be biologically active and play an important role in brain and regulating functions of CSF (Imura et al. 2004, 2007). It is currently unknown whether changes in CSF Klotho reflect and/or contribute to neurological and neurosurgical disorders.

As mentioned earlier, urinary Klotho concentrations have been measured in patients with renal disease. We had

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a special interest to compare serum and urine sKlotho levels in patients with acromegaly.

Urinary Klotho concentrations remained below the detection limit in unconcentrated spot urine samples of nine patients cured from acromegaly and of six patients with non-functioning pituitary adenomas but detectable in nine patients with newly diagnosed acromegaly, which suggests that increased urinary sKlotho could have a high positive predictive value for the diagnosis of the disease (Fig. 3). However, technical issues need to be addressed and the sensitivity of the analysis refined before urinary Klotho can be considered a useful marker for estimating GH excess in individual patients.

Based on sequence similarity with the *Klotho* gene, *β-Klotho* was identified, and the former (the founder of the family) occasionally renamed *α-Klotho* (Ito *et al.* 2000). γ-Klotho was identified as another, functionally more remote member of the family, which is expressed particularly in kidneys, adipose tissue and eyes (Ito *et al.* 2002). α-Klotho protein shares 41% amino acid sequence identity with β-Klotho. *β-Klotho* is expressed predominantly in the liver and white adipose tissue, i.e. in tissues that are targets of FGF19 and FGF21 (endocrine FGFs, like

Figure 3

Concentrations of soluble Klotho (sKlotho) in serum and urine of patients with acromegaly. Concentrations of Klotho were assessed by ELISA in serum and urine samples (spots, obtained the same time point as blood) from patients with pituitary adenomas (between 1/2013 and 6/2013). Each point represents an individual; filled circles, active (newly diagnosed, untreated) acromegaly (nine patients, three females); empty circles, successfully (by transsphenoidal surgery) treated acromegaly (as confirmed by normal IGF1 and GH suppressible to <1 ng/ml during oGTT; nine patients, three females) and filled triangles, clinically non-functioning pituitary adenoma (six patients, three females). Note that both the x-axis (serum sKlotho (pg/ml)) and y-axis (urine Klotho, expressed in pg sKlotho/mmol creatinine) are drawn on logarithmic scales. Patients with active acromegaly have higher serum and urinary sKlotho levels than patients without active acromegaly; in urine, sKlotho was readily detectable (concentration above the detection limit of the ELISA) only in samples from patients with acromegaly.

FGF23), and β-Klotho was found to work as co-receptor for FGF19 and FGF21 signalling. In mammals, Klothos (α and β) are type 1 transmembrane proteins that function as obligatory cofactors for signalling of all known, vertebrate specific, hormone-like ('endocrine') FGFs: FGF15/FGF19 (enterocyte), 21 (hepatocyte) and 23 (osteocyte). The (FGF) ligand, the (FGF) receptor and its (Klotho) co-receptor usually define the specificity of the endocrine actions (Tomiyama *et al.* 2010); however, in artificial experimental settings, promiscuity of signalling is observed, for instance by FGF23 mutants with an ability to activate FGFR signalling through both α- and β-Klotho (Wu *et al.* 2012), or with antibodies activating FGFR1, which may act in a FGF21-like manner (requiring β-Klotho on target cells) or in a FGF23-like manner (requiring α-Klotho on target cells), to induce hypophosphataemia (Wu *et al.* 2013).

Role for α-Klotho in acromegaly?

Luft and colleagues recognized that acromegaly had a marked impact on renal function (Ikkos *et al.* 1956). Administration of GH increased renal GFR and tubular phosphate reabsorption (Corvilain *et al.* 1962, Bianda *et al.* 1997). The latter effect of GH cannot be explained by a deficiency of known classical phosphaturic hormones such as PTH and FGF23. Reviewing old data on GH, IGF1 and phosphate transport, it is important to realize that stimulatory effects of GH on renal phosphate reabsorption in adult humans have been repeatedly found *in vivo*. A stimulatory effect of IGF1 on phosphate uptake can be documented in most cultured cells, but the finding of phosphate uptake stimulation by IGF1 *in vitro* has not been shared by GH, and the claim that IGF1 is mediating this effect of GH may have been premature (Quigley & Baum 1991, Zoidis *et al.* 2004). Maximal tubular phosphate reabsorption per GFR was raised by GH but not by IGF1 compared with saline; thus, IGF1 does not mimic (and possibly not mediate) the TmP/GFR raising effect of GH on renal phosphate reabsorption despite comparable effects on GFR (Bianda *et al.* 1997, Schmid & Meili 2000).

Acromegaly has unique biochemical and endocrine features; it leads to high serum phosphate and FGF23 levels despite enhanced GFR, as well as to insulin resistance (IR) and hyperglycaemia (Table 2). These characteristics are distinct from those observed in more common conditions such as renal failure and type 2 diabetes: high phosphate levels cannot be attributed to decreased GFR, and IR and hyperglycaemia are not caused by increased visceral fat. Although levels of phosphate and FGF23 are high in
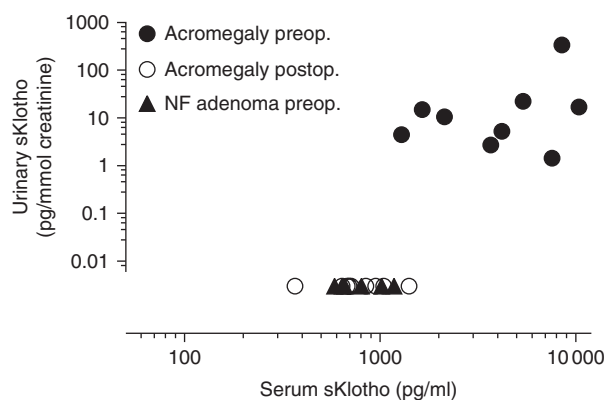
Table 2 Parameters and phenotypes characterizing GH excess, Klotho deficiency and CKD

Parameter	GH excess	Klotho deficiency	CKD
Growth	↑↑	↓↓	↓
Lifespan	↓	↓↓↓	↓↓
Energy expenditure	↑	↓	↔↓
Glucose, IR	↑↑	↓↓	↔↑
Creatinine	↓	↑	↑↑↑
eGFR	↑	↓	↓↓↓
1,25-(OH)$_2$ vitamin D	↑↑	↑↑↑	↓↓
Phosphate	↑	↑↑↑	↑↑
FGF23	↑	↑↑↑	↑↑↑
Calcium	↔↑	↑↑	↔↓
PTH	↔	↔↓	↑↑

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; IR, insulin resistance.

acromegaly, calcitriol is also increased in this disease. FGF23 (usually inhibiting renal phosphate reabsorption and calcitriol production) and phosphate tend to be high despite increased GFR, in keeping with a condition of renal FGF23 resistance (Table 2; Ito *et al.* 2007).

Longer lasting excessive secretion of GH by a pituitary adenoma results in premature death, mainly due to an increased risk for cardiovascular disease. In human adults, GH (but not IGF1) increases the set points of plasma glucose and phosphate, and both IR and elevated serum phosphate are associated with increased mortality in the general population.

As Klotho levels may relate to FGF23 resistance and IR (or high phosphate and glucose respectively) in acromegaly, we measured sKlotho in sera of patients before and after transsphenoidal surgery. Removal of the pituitary adenoma led to the expected decrease in GH and IGF1. sKlotho was markedly increased in relation to GH excess and declined towards normal levels after surgery (Sze *et al.* 2012); differences in sKlotho before and after surgery were at least as pronounced as IGF1 (Sze *et al.* 2012, Kohler *et al.* 2013; Fig. 2). While sKlotho was elevated in sera of patients with active acromegaly, this did not hold true for patients with clinically non-functioning pituitary adenomas (Neidert *et al.* 2013). In the latter study, it was shown that sKlotho fell at least as quickly as IGF1 after surgery. Moreover, the pituitary adenomas were analysed for Klotho by immunohistochemical staining. It was found that the rise in serum α-Klotho is not explained by increased pituitary (adenoma) Klotho expression, but rather by increased pituitary GH secretion and possibly due to a systemic action of GH. However, renal biopsies to check for changes in amounts of mKlotho were not performed.

As sKlotho may reflect activity of GH-producing adenomas, it could serve as an additional serum marker in the long-term follow-up of acromegalic patients. Overall, when assessing patients with GH-producing adenomas, we observed concomitant and parallel changes in serum IGF1 and sKlotho over time in a given individual patient, and levels of sKlotho and IGF1 appeared to be similarly dependent on GH (Sze *et al.* 2012, Kohler *et al.* 2013). A remarkable difference was found with regard to gender; for a given GH (excess) status, IGF1 tended to be higher in males whereas sKlotho levels were higher in females; more detailed analysis revealed that oestrogens accounted for lower IGF1 in (premenopausal) women, but not for the higher sKlotho; overall, GH was by far the strongest predictor of both sKlotho and IGF1 (L Sze, MC Neidert, RL Bernays & C Schmid, 2013, unpublished observations).

In human adults, GH deficiency results in a more or less characteristic but much less obvious phenotype than in children (dwarfism); hypoglycaemia and hypophosphataemia are not obvious features. In the study by Neidert, sKlotho did not appear to be particularly low in patients with GH deficiency. It remains to be tested whether sKlotho levels are decreased in patients with GH deficiency, and whether GH treatment results in increasing serum sKlotho levels. Considering sKlotho as a GH-dependent serum protein, a response to GH replacement might be anticipated, but in view of kidney function and phosphate homoeostasis, differences in sKlotho are possibly smaller between healthy adults and patients with GH deficiency when compared with those with GH excess.

GH and Klotho

As mentioned, *Klotho* mutant (*Klotho*$^{-/-}$) mice exhibit growth retardation after weaning and premature death. Electron microscopic examination of GH-producing cells in pituitary glands revealed a reduction in GH granules. Growth retardation was described in the original 1997 paper (Kuro-o *et al.* 1997). As it has not been known whether growth retardation in *Klotho* mutant mice is related to loss of GH function, Kashimada *et al.* examined whether treatment with GH could rescue retardation of growth. At the end of 3 weeks of treatment with human GH, body weight was not increased in *Klotho* mutant (*Klotho*$^{-/-}$) mice in contrast to WT mice. Growth retardation in *Klotho* mutant mice is therefore resistant against GH treatment, and GH deficiency is not the cause of growth arrest in *Klotho*$^{-/-}$ mice (Kashimada *et al.* 2002).

There are data suggesting a negative impact on lifespan by an excess in the anabolic peptide hormones GH, IGF1 and insulin. These data are difficult to accept not only by people abusing such hormones but also for the medical community, including endocrinologists. With regard to life expectancy, apparent contradictions concerning the action of GH should be considered. GH has generally been associated with positive attributes of adolescence and youth, such as growth, health, muscle mass and well-being, yet the bulk of scientific evidence suggests that signalling through GH, IGF1 and insulin receptors is related to a shortened lifespan in adults (Berryman *et al.* 2008, Junnila *et al.* 2013). It is currently difficult to estimate whether high sKlotho levels can attenuate insulin and IGF1 receptor signalling *in vivo*, e.g. in patients with acromegaly. In their overviews, Kopchick and colleagues have underlined the importance of GH, IGF1 and insulin signalling in regulating lifespan; according to our view, not only the GH–IGF1 axis and IR but also FGF23 resistance and hyperphosphataemia in the context of the GH–Klotho axis deserve more attention.

Concerning the renal effects of GH, several aspects of Klotho can be considered (Table 2). Apparently, GH clearly opposes the effects attributed to mKlotho and its essential role in mediating FGF23-induced renal phosphate loss and FGF23-mediated inhibition of calcitriol production (Bianda *et al.* 1997). Hypervitaminosis D due to over-expression of CYP27B1 is central to the phenotypes of α-mKlotho and FGF23 deficiency, and hypovitaminosis D due to suppression of CYP27B1 is a central phenotype of FGF23 transgenic mice. With regard to vitamin D metabolism, GH increases vitamin D activation in humans and experimental animals and can, therefore, be considered as an antagonist of FGF23 action mediated via the FGFR/α–mKlotho complex (Bianda *et al.* 1997, 1998, Zoidis *et al.* 2002). GH stimulates 25-hydroxy-vitamin D 1α-hydroxylase (CYP27B1) mRNA and markedly down regulates 24-hydroxylase (CYP24A1) mRNA expression; the former enzyme favours activation and the latter degradation of active vitamin D (Zoidis *et al.* 2002). As mentioned in the previous section, the high levels of phosphate and calcitriol together with increased FGF23 suggest a state of renal FGF23 resistance in patients with acromegaly (Ito *et al.* 2007, Sze *et al.* 2012). It is, therefore, conceivable that GH induces functional impairment of the FGF23–mKlotho signalling pathway in proximal tubular cells.

On the other hand, GH shares the effects of sKlotho on the distal convoluted tubule to attenuate calciuria. However, as calcium, calcitriol and GFR tend to be increased in patients with acromegaly, increased calcium reabsorption in the distal convoluted tubule may be masked (acromegalic patients tend to have fasting hypercalciuria). sKlotho may not only account for an increased calcium loss when deficient (as in diabetic nephropathy) but also for attenuation of renal calcium loss or when present in excess (as in acromegaly) (Asai *et al.* 2012, Kamenicky *et al.* 2012).

Concerning parathyroid function, findings reported from patients with acromegaly have been variable, especially in studies of drug-treated patients (White *et al.* 2006, Kamenicky *et al.* 2012). More consistent data from patients with adenectomy-treated acromegaly suggest that PTH levels remain essentially unchanged when the preoperative condition is compared with the postoperative condition (Lund *et al.* 1981, Takamoto *et al.* 1985). Likewise, high-dose short-term GH treatment in a more controlled setting of healthy volunteers or GH-deficient patients did not alter PTH levels, despite significant increases in $1,25\text{-}(OH)_2 D$ and a trend for increased calcium levels, as observed in acromegaly (Lund *et al.* 1981, Takamoto *et al.* 1985, Bianda *et al.* 1997, Bianda *et al.* 1998). Given the fact that in acromegalic patients, consistently calcium tends to be high in parallel with increased calcitriol and FGF23 values, but in the presence of unchanged PTH, it is likely that inappropriate feedback inhibition of PTH occurs (Table 2). One potential explanation would be that FGF23 action on PTH secretion is impaired possibly by a functional mKlotho deficiency (as proposed for kidneys; Ben-Dov *et al.* 2007, Krajisnik *et al.* 2007).

Considering effects of GH on the endothelium, it appears that some are potentially favourable for cardio-vascular health whereas others such as IR, hyperglycaemia and hyperphosphataemia negatively impact on endothelial function. Ageing is associated with a decrease in GH, endothelial function (nitric oxide synthase activity and abundance of eNOS), cardiovascular and renal health. GH deficiency has been associated with impaired endothelial function, and GH replacement could improve endothelial function (Boger *et al.* 1996, Evans *et al.* 2000). As mentioned earlier, Klotho protein protects against endothelial dysfunction and exerts several beneficial (anti-senescence) effects on endothelial cells, including some mediated via the MAPK pathway (Saito *et al.* 1998, 2000, Ikushima *et al.* 2006, Kusaba *et al.* 2010, Maekawa *et al.* 2011, Carracedo *et al.* 2012). However, it is currently unknown whether Klotho is involved (a downstream effector or mediator) in the effects of GH on the endothelium.

In the context of acromegaly, it has been well recognized that hypertension and hyperglycaemia/diabetes contribute to the increased cardiovascular disease risk in these patients. Phosphate has been considered less as a risk factor, although more recent data suggest that hyperphosphataemia and possibly phosphate intake are associated with increased cardiovascular risk, even in the absence of established renal disease (Tonelli *et al.* 2005, Dhingra *et al.* 2007, Foley 2009, Shuto *et al.* 2009, Ellam & Chico 2012, Osuka & Razzaque 2012, Yamamoto *et al.* 2013). Excessive phosphate not merely favours ectopic (including media) calcification but also impairs endothelial function (e.g. NO synthesis, annexin II regulation, etc.) (Lau *et al.* 2011, Di Marco *et al.* 2013). In acromegaly, it may be that potentially beneficial effects of GH and Klotho on endothelial function and NO synthesis are outweighed by IR and hyperphosphataemia, so that there is a net increase in cardiovascular risk. GH-induced high phosphate levels may make sense in the context of growth, but it appears likely that they are harmful in adulthood.

The findings of remarkably high serum sKlotho levels in patients with active acromegaly are difficult to interpret, and additional data are required. The biological meaning remains unclear, and currently, it is uncertain whether changes in Klotho turnover account for the high threshold of renal phosphate reabsorption and FGF23 resistance with GH excess; moreover, the mechanisms by which acromegaly leads to excess sKlotho remain to be elucidated. One can speculate about several mechanisms; GH may increase Klotho gene expression in kidneys (and other sites). Soluble forms of α-Klotho could be generated by alternative splicing of its transcript but they could also result from proteolytic cleavage of the transmembrane form by secretases into various body fluids. Two members of the 'A Disintegrin and Metalloproteinase' (ADAM) family, ADAM10 and ADAM17 (α-secretases), and β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) (β-secretase), have been suggested as responsible enzymes (Chen *et al.* 2007, Bloch *et al.* 2009, Saftig & Reiss 2011). The high sKlotho levels in acromegalic patients do not necessarily reflect a high level of cell mKlotho (in the kidneys, the parathyroids and elsewhere). A discrepancy between renal mKlotho and sKlotho has been discussed for experimental settings, and it was considered that increased sKlotho could be caused by accelerated shedding of Klotho without corresponding changes in mKlotho expression (Lau *et al.* 2012). In acromegaly, FGF23 resistance cannot be explained by high sKlotho *per se*, unless sKlotho is elevated at the expense of mKlotho, which

serves as a co-receptor for FGF23. Formally, resistance to FGF23 may be caused by decreased expression of the Klotho–FGFR1 complex. This should be investigated at the level of the kidneys and the parathyroids, and this has indeed been addressed in patients with CKD where parathyroids were studied to find the basis for parathyroid resistance to FGF23 (Galitzer *et al.* 2010, Komaba *et al.* 2010, Krajisnik *et al.* 2010). Similar investigations have not been performed with tissues from patients with acromegaly. According to our hypothesis, the activity of secretases (shedding the ectodomain from the integral mKlotho) may be increased in acromegaly, either directly by GH or indirectly by factors or by proteolytic activity induced by GH. As previously mentioned, GH has a proteolytic activity on other systems, as it induced an IGFBP3-proteolytic activity *in vivo* in rats; moreover, GH stimulates the mRNA expression not only of the cytochrome P450 enzyme CYP27B1 involved in the production of active vitamin D (calcitriol) but also the phex mRNA that encodes for an endopeptidase important in phosphate homoeostasis (Rutishauser *et al.* 1993, Zoidis *et al.* 2002). Increased mKlotho-proteolytic activity could result in deficient mKlotho and impaired FGF23 signalling, thereby explaining FGF23 resistance; the enhanced enzymatic clipping of the extracellular Klotho domain would lead to an excess in sKlotho contributing to channel and transporter regulation, to attenuation of calciuria, growth factor signalling and IR.

Thus far, it has not been studied whether GH stimulates the production of Klotho. It will be of interest to see whether GH increases serum levels of sKlotho in GH-deficient subjects and to see whether GH influences expression of Klotho at the mRNA level and whether it regulates the abundance of mKlotho in tissues and sKlotho in extracellular spaces. Such studies have been performed in experimental animals with calcium- and phosphate-regulating hormones (PTH, calcitriol, FGF23; increasing renal expression and serum levels in rats) but not with GH (Takenaka *et al.* 2013).

As mentioned earlier, β-Klotho is important as a co-receptor for FGF19 and FGF21 signalling. GH induces hepatic production of FGF21, apparently through a mechanism dependent on lipolysis in adipocytes. In this context, it is interesting to know that GH stimulates lipolysis especially in the state of fasting (when insulin is low). FGF21, in turn, inhibits GH action on chondrocytes directly at the growth plate; FGF21 may also mediate GH resistance in anorexia nervosa as illustrated by high GH and low IGF1. Taken together, there are not only multiple interacting targets between GH and α-Klotho in calcium

and phosphate mineral handling but it is likely that GH and β-Klotho pathways are closely linked in order to regulate fuel metabolism, at both afferent and efferent parts of endocrine loops. So far, due to lack of appropriate assays to measure sβ-Klotho and sα-Klotho, data lag far behind basic research in this field.

Conclusions

There is increasing interest in the field of GH and Klotho and their mutual interaction. Many questions can be addressed by both basic and clinical studies. Both IGF1 and sKlotho are relatively abundant in the circulation; both of them are regulated by GH (Sze *et al.* 2012, Hu *et al.* 2013, Neidert *et al.* 2013). In the case of IGF1, there is fairly good evidence that it mediates some actions of GH and that there is a negative feedback at the pituitary level. α-Klotho expression can also be documented in the pituitary gland (Kuro-o *et al.* 1997, Li *et al.* 2004, Neidert *et al.* 2013), and FGF23 administration to rats increased early growth response-1 (*Egr1*) mRNA levels in the pituitary as it did in the kidney and parathyroids (Urakawa *et al.* 2006); however, a feedback action of FGF23 and Klotho on the pituitary GH secretion has not been described so far. There are no data available that show how FGF23 or Klotho may mediate GH actions.

In acromegaly, IGF1 (predominantly liver-derived), a marker and mediator of GH action, is increased, as is sKlotho (predominantly kidney-derived), a marker of GH-producing pituitary adenomas. Further observations and experiments should allow more insight, e.g. as to whether generation of sKlotho could mediate selected actions of GH, causing FGF23 resistance, hyperphosphataemia, possibly contributing to IR and hyperglycaemia, contributing to improved endothelial function, increased energy expenditure and decreased life expectancy.

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