The Effects of Lithium on FGF23

Die Wirkung von Lithium auf FGF23

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To My Family

For their love, support and encouragement throughout my life.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>ADHR</td>
<td>Autosomal dominant hypophosphatemic rickets</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>AQP2</td>
<td>Aquaporin 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBM</td>
<td>Brush boarder membrane</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX2</td>
<td>Cytochrome c oxidase subunit 2</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DMP-1</td>
<td>Dentin matrix acidic phosphoprotein 1</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinases</td>
</tr>
<tr>
<td>FGF23</td>
<td>Fibroblast growth factor 23</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>IMPase</td>
<td>Inositol monophosphatase</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>Magnesium</td>
</tr>
<tr>
<td>Mg/dl</td>
<td>Milligrams per decilitre</td>
</tr>
<tr>
<td>MEPE</td>
<td>Matrix extracellular phosphoglycoprotein</td>
</tr>
<tr>
<td>mEq/L</td>
<td>Milliequivalents per liter</td>
</tr>
<tr>
<td>mmol/L</td>
<td>Millimol per liter</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na/H</td>
<td>Sodium–hydrogen exchanger</td>
</tr>
<tr>
<td>NaPi</td>
<td>Sodium-dependent phosphate co-transporters</td>
</tr>
<tr>
<td>NDI</td>
<td>Nephrogenic diabetes insipidus</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NKCC2</td>
<td>Sodium potassium chloride co-transporter 2</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>P53</td>
<td>Tumor proteins p53</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHEX</td>
<td>Phosphate regulating endopeptidase homolog, X-linked</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositid-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal protein S6 kinase alpha</td>
</tr>
<tr>
<td>TrKB</td>
<td>Tropomyosin receptor kinase B</td>
</tr>
<tr>
<td>VD</td>
<td>Vitamin D</td>
</tr>
<tr>
<td>VD$_2$</td>
<td>Vitamin D$_2$</td>
</tr>
<tr>
<td>VD$_3$</td>
<td>Cholecalciferol (Vitamin D$_3$)</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>V2R</td>
<td>Vasopressin receptor antagonist</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Lithium

1.1.1 A historical and clinical approach

Lithium is a soft, silver white metal, which belongs to the group of alkali metals together with sodium and potassium. Normally, all tissues and body fluids contain lithium in trace amounts (<0.2 mEq/L) (1). Dietary sources of lithium salt include: vegetables, plant-derived foods and drinking water. It is also found in almost all igneous rocks. Lithium salt has a long history of human consumption beginning in the 1800s (2). Furthermore, lithium has been used in treatment of many diseases including epilepsy, diabetes, gout and insomnia. In 1949, lithium was introduced for the first time into the modern psychiatrics. In 1974, it has been approved by the US Food and Drugs as a treatment for bipolar disorder. Until now, lithium treatment has remained the "gold standard" therapy for patients with mood disorders. Lithium has also been used as an effective augmenting therapy for other antidepressant drugs, especially the refractory depression when the patients respond ineffectively to adequate usual dose (3, 4). Lithium is the only drug that consistently reduces the suicidal rate in patients with neuropsychiatric disorders (5, 6). Moreover, lithium has been utilized as prophylaxis for cluster headaches (7, 8).

In the last years, lithium has been demonstrated to be an effective neuroprotective agent in acute brain injuries and chronic neurodegenerative disorders (9, 10), since it is the only agent that stimulates the anti-apoptotic pathways in the brain. These observations made lithium more interesting to the medical field. However, one of the major limitations of lithium is its narrow therapeutic index (2, 11). Recent studies have demonstrated that lithium could promote longevity in humans (12, 13). In spite of its extensive clinical applications, the underlying mechanism of its therapeutic action remains to be incompletely investigated (14).
1.1.2 Pharmacokinetics

Lithium is rapidly and completely absorbed from the gastrointestinal tract (GIT), reaching the peak plasma concentration within 1 to 2 hours following oral intake. Its half-life is about 12 to 27 hours. It can be increased up to 58 hours in elderly and chronic kidney disease (CKD) patients. Lithium is excreted without metabolism by the kidney; around 95% of absorbed lithium is excreted in the urine and less than 1% in the faeces. Lithium is filtered freely by the glomerulus; 80% of the filtered lithium is reabsorbed in the proximal tubule, whereas 20% is reabsorbed in the thick ascending limb, connecting tubule and cortical collecting duct (1).

1.1.3 Dose and route of administration

In humans, lithium dose must be adjusted individually according to serum lithium concentration, and clinical response of the patient. The initial recommended dose is usually 450 - 900 mg per day, divided into 2 to 3 doses, targeting lithium serum concentration between 0.6 – 1.2 mmol/L. Lithium is generally administered orally as lithium carbonate (capsule) or lithium citrate (Syrup) (1). In animals, lithium can be administered through drinking water, food or directly by subcutaneous or intraperitoneal injection. Studies on rats have demonstrated that lithium carbonate in drinking water and intraperitoneal injection of lithium chloride have similar effects (15).

1.1.4 Side effects

Lithium has a narrow therapeutic index 0.6 – 1.0 mmol/L, therefore the lithium serum concentrations have to be closely monitored in order to prevent toxic side effects, which are observed when serum lithium concentrations are above 1.5 mmol/L. Short term side effects of lithium include headache, nausea, vomiting, diarrhoea and muscle weakness. Long term side effects on the other hand include nephrogenic diabetes insipidus (NDI), hand tremor, weight gain, hypothyrodisism, hyperthyroidism, hyperparathyroidism and hypercalcemia (16-19).
1.1.5 The mechanisms of action

Lithium exerts its neuroprotective effects through multiple intersecting pathways, but the precise mechanisms of its action are still under investigation. Many studies have demonstrated that lithium prolongs cell survival through activating brain-derived neurotrophic factor (BDNF) and thereby stimulating anti-apoptotic pathways. In addition, lithium inhibits a large number of pro-apoptotic factors through inhibiting glycogen synthase kinase-3 (GSK-3) and N-methyl-D-aspartate (NMDA)-receptor-mediated calcium influx. All these changes together shift the Bcl-2 family member ratio towards the anti-apoptotic members, preventing the cytochrome c release, and inhibiting the caspase activation. Therefore, lithium prolongs cell survival through the stimulation of the anti-apoptotic and the inhibition of the pro-apoptotic pathways. It also up-regulates anti-inflammatory cytokines and down-regulates the pro-inflammatory cytokines through suppression of NF-κB (Fig. 1) (20, 21).

Fig. 1. Neuroprotective effects of lithium.

Lines with solid arrows represent stimulatory connections; lines with flattened ends represent inhibitory connections. Dashed lines represent pathways with reduced activity as a result of lithium treatment.
1.2 Lithium and GSK-3

The discovery that lithium inhibits GSK-3 raised the possibility that impaired inhibition of GSK-3 is associated with mood disorders. GSK-3 activity is found to be elevated in postmortem brains of depressed suicide victims. This observation suggests that GSK-3 inhibition may contribute to anti-suicidal effects of lithium. Moreover, varieties of pharmacological and molecular studies have demonstrated that inhibition of GSK-3 reduces both depression and manic-like behaviors (22, 23).

1.2.1 GSK-3

GSK-3 is a multifunctional serine/threonine protein kinase, which is ubiquitously expressed in the body with particularly high levels in the brain. GSK-3 has two isoforms α and β, both of which are ~98% identical in their catalytic cores, but differ in the N- and C-terminals. Both isoforms are encoded by different genes (GSK-3α, -51KDa and GSK-3β, -47KDa). Several studies report that both isoforms play similar roles in several signalling pathways and that lithium effectively inhibits both isoforms (24). GSK-3 is one of the few kinases that are constitutively active in cells under resting conditions. GSK-3 activity can be inhibited by a variety of extracellular stimuli including insulin, epidermal growth factor (EGF) and fibroblast growth factor (FGF) and Wnt ligands. GSK-3 is inhibited upon phosphorylation at serine 21 in GSK-3α and serine 9 in GSK-3β and it is regulated by PI3K/Akt, PKC and PKA kinases (23). Several studies suggest that GSK-3 plays a central role in many important signaling pathways and that aberrant GSK-3 signaling is associated with many pathologies including heart failure, bipolar disorder, diabetes mellitus, Alzheimer’s disease, aging, inflammation and cancer. Therefore, GSK-3 is regarded as a prominent therapeutic target and GSK-3 inhibitors, such as lithium could potentially be used for the treatment of these disorders (25, 26).
1.2.2 The effects of lithium on GSK-3

Lithium inhibits GSK-3 through direct and indirect mechanisms. Under normal physiological conditions GSK-3 catalyzes the reaction of multiple protein substrates in the presence of Mg$^{2+}$-ATP. Lithium inhibits the GSK-3 directly through a competitive inhibition of Mg$^{2+}$ leading to disrupted catalytic functioning of GSK-3 (27, 28). However, the indirect mechanism is through the inhibition of GSK-3 phosphorylation. The exact mechanism by which lithium phosphorylates GSK-3 is not exactly known. It could be through the inhibition of phosphatase, which is a protein responsible for GSK-3 reactivity (Fig. 2) (29).

![Fig. 2. Interactions between lithium and GSK-3.](image_url)

GSK-3 catalyzes the phosphorylation of many protein substrates in the presence of Mg$^{2+}$–ATP. Lithium is a competitive inhibitor of the Mg$^{2+}$, which results in inhibition of the activity of GSK-3. Blue arrows indicate the activation of GSK-3 signaling. Red arrows indicate the inhibition of GSK-3 activity.
1.3 Lithium and nephrogenic diabetes insipidus

Lithium salts significantly affects the renal function as it interferes with antidiuretic hormone (ADH), thus resulting in nephrogenic diabetes insipidus (NDI). NDI is a clinical syndrome characterised by reducing the urinary concentrating ability due to the resistance of the kidney against the action of arginine-vasopressin (AVP). NDI occurs in 40% of lithium treated patients (18). NDI has been detected as early as 8 weeks in humans (30) and within 5 - 7 days in rodents following lithium treatment (31). The mechanisms through which lithium induces NDI remain incompletely understood. Recent studies demonstrate that GSK-3 could play an important role in the mechanism of lithium induced NDI. This speculation is based on the observation that the time course of GSK-3 inhibition coincides with the decrease of AQP2 expression and polyuria in lithium treated mice (32).

1.3.1 Lithium transporters protein

Lithium is not like the other anti-depressant drugs; it is not bound to the cellular receptor, rather, it enters the cells predominantly via different sodium or potassium transporters, channels or exchangers (1). Two key entry points for lithium are through ENaC and Na/H exchanger.

ENaC is the major channel for sodium reabsorption in collecting duct. Sodium crosses the apical membrane through ENaC and is actively exported from the cell in basolateral membrane through sodium/potassium adenosine triphosphatase (Na+/K+-ATPase). In the collecting duct, lithium can cross the apical membrane through ENaC transporters, which is more permeable to lithium than sodium. Unlike sodium, lithium is not exported from the cells by Na+/K+-ATPase. ENaC is upregulated by aldosterone and is inhibited by low concentrations of amiloride (33-35).
Na/H exchanger is responsible for sodium reabsorption across the proximal tubule. Na/H exchanger is also an amiloride sensitive channel. Blocking of both ENaC and Na/H exchanger by amiloride results in the reduction of lithium transport within the cell, and ultimately prevents the development of lithium induced NDI (36). Another lithium transporter is the sodium potassium chloride cotransporter 2 (NKCC2), it is found in the apical membrane of the thick ascending limb of the loop of Henle and it is inhibited by furosemide (1).

1.3.2 Pathophysiology of lithium induced NDI

Under normal physiological conditions, specialized membrane channel proteins called aquaporin 2 (AQP2) facilitate water transport across the apical membrane. The AQP2 channel, which is regulated by AVP, is a major physiological regulator of renal water excretion. In response to anti-diuretic stimuli, the pituitary gland secretes AVP, which binds to the V2 vasopressin receptor (V2R) in the basolateral membrane of the principle cell in the collecting duct. This results in the activation of adenylate cyclase through G protein and increases intracellular cAMP, and ultimately, cAMP activates the protein kinase A (PKA), which in turn stimulates the AQP2 transcription and insertion into the apical membrane of the collecting duct allowing water reabsorption and an increase of urine concentration (Fig. 3 a) (30, 37-39).

Several studies have demonstrated that lithium reduces AQP2 protein abundance in the renal collecting duct via the inactivation of adenylyl cyclase and subsequent inhibition of PKA. The down-regulation of AQP2 channel leads to reduced water reabsorption and an increased urinary excretion. It continues to be debated whether lithium induces NDI through a direct effect or as result of GSK-3 inhibition (40-42). GSK-3 inactivation modulating adenylate cyclase activity and cAMP generation (43). Furthermore, Rao et al., 2005 demonstrated that the inhibition of GSK-3 by LiCl up-regulates renal medullary interstitial cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2), which are known to antagonize AVP (Fig. 3 b) (44).
Fig. 3. Sodium and water reabsorption.

A. Under physiological conditions sodium crosses the apical membrane through ENaC and is actively exported from the cell by the Na⁺,K⁺-ATPase.

B. Lithium crosses the apical membrane through ENaC and prevents sodium entry. It is not exported from the cell by the Na⁺,K⁺-ATPase and accumulates intracellularly leading to inhibition of GSK-3.
1.4 Fibroblast growth factors (FGFs)

FGFs are polypeptide, multifunctional growth regulators, affecting a wide variety of physiological events. The FGF family is comprised of 22 members that have a wide variety of functions contributing to organogenesis, tissue remodelling, nervous system control, angiogenesis and regulation of metabolism. FGFs are best known for their regulatory roles in cell growth, differentiation, and morphogenesis in early stages of neural development and have recently been discussed as switch genes, biomarkers, and treatment targets for affective disorders (45, 46). A correlation between antidepressant treatments and FGF expression in the cerebral cortex and hippocampus has been observed (47, 48). Moreover, patients suffering from major depressive disorders have deregulated transcript levels of several FGFs (49). A dysregulation of the FGF system in major depressive disorders involves two specific FGF receptors, FGFR2 and FGFR3 (50, 51).

FGFs exhibit diverse modes of action, mechanisms of secretion and ultimate biological consequences. Therefore, FGF families are grouped into three groups according to their mechanisms of action: intracrine, paracrine and endocrine FGFs (51).

- **Paracrine FGFs** mediate their biological responses in FGFR-dependent manner and carry out an important function in developmental processes. The members of paracrine FGFs subfamilies include FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18 and FGF9/16/20.

- **Intracrine FGFs** are intracellular molecules that regulate the function of voltage gated sodium channels in FGFR-independent manner.

- **Endocrine FGFs** include FGF15/19, FGF21 and FGF23. They emerge as regulators of metabolism and are thought to mediate their biological responses in FGFR-dependent manner (52).
1.4.1 Fibroblast growth factor 23 (FGF23)

FGF23 is a phosphaturic peptide hormone with an N-terminal and C-terminal region, belonging to the FGF19 subfamily of endocrine FGFs. FGF23 has been identified as a gene mutation in patients afflicted with autosomal dominant hypophosphatemic rickets (ADHR) (53). FGF23 has been found to be highly identical in both humans and mice. In humans, the FGF23 gene is located in chromosome 12, while in mice, it is located in chromosome 6 (54, 55). FGF23 is a powerful inhibitor of ageing, lack of FGF23 can lead to premature appearance of a wide variety of age related disorders such as osteopenia, osteoporosis, impaired angiogenesis, enhanced erythrocyte turnover, pulmonary emphysema, skin atrophy, infertility, hearing loss, neuron degeneration, Parkinson’s disease, cognitive impairment, neoplasm and inflammation (56, 57). FGF23 is predominantly expressed in osteocytes and the osteoblasts in the bone. It is also expressed in the parathyroid gland, small intestine, salivary gland, and to a lower extent, in the thymus, skeletal muscle, brain, liver, mammary gland and heart (58, 59).

1.4.1.1 FGF23 function

FGF23 is an important regulator of phosphorus and 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃) metabolism. It is also required for the maintenance of normal bones and mineral homeostasis. Transgenic mice with FGF23 over-expression develop hypophosphatemia, phosphaturia and low serum 1,25(OH)₂D₃ levels (60, 61). Knockout mice with FGF23 ablation and humans with homozygous missing mutations in FGF23 exhibit hyperphosphatemia, an increased 1,25(OH)₂D₃ serum level, a shortened life span and vascular calcifications (62, 63). The main known physiological function of FGF23 is to regulate serum phosphate and 1,25(OH)₂D₃ level. In order to do these functions, FGF23 requires a cofactor known as α-klotho to mediate its effects through FGFRs (57).
1.4.1.2 FGFR - α klotho complex

1.4.1.2.1 Klotho

Klotho was identified as an aging-suppressor gene, which extends life span when over-expressed and accelerates the development of aging-like phenotypes when knockout in mice (64-66). The klotho family includes two isoforms, α-klotho and β-klotho. α-klotho is a single-pass transmembrane protein functioning as an obligate co-receptor for FGF23 and is mainly expressed in the kidney and the epithelium of the choroid plexus in the brain and to a lower extent in the pituitary gland, placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary and colon (67).

The klotho knockout mice exhibit many ageing-like phenotypes after 3 - 4 weeks including growth retardation, extensive soft tissue calcification, a decrease in life span paralleled by osteopenia/osteoporosis, endothelial dysfunction, impaired angiogenesis, sinoatrial node dysfunction with sudden cardiac arrest, enhanced erythrocyte turnover, pulmonary emphysema, skin atrophy, hypogonadotropic hypogonadism, infertility, muscle atrophy, hearing loss, neuron degeneration, Parkinson’s disease, cognition impairment, neoplasm, inflammation and tissue fibrosis (66-78).

Identification of klotho as an obligate co-receptor for FGF23 was based on the observations that both klotho and FGF23 knockout mice exhibit very similar phenotypes. The importance of α-klotho in FGF23 signaling is illustrated by several studies on humans and animals, where the loss of α-klotho results in the end-organ resistance to FGF23. Recent studies have identified α-klotho as being a necessary cofactor for FGF23, since it increases the affinity of FGF23 to their receptors by changing the FGFR into a specific FGF23 receptor. Therefore, FGF23 target organs are defined by the co-expression of α-klotho and FGFRs.
1.4.1.2.2 FGF receptors

Four types of FGFR have been identified in humans and mice. Only FGFR1, FGFR3, and FGFR4 are expressed in the renal proximal tubule (79). FGF23 binds to FGFR, but with low affinity, while it binds to FGFR/α-klotho complex with much higher affinity. Several studies have demonstrated that FGF23 signals via renal FGFR1 and 3 regulate phosphate reabsorption (80), but other studies have suggested that only FGFR1 is the predominant receptor for the FGF23 phosphaturic effect. At the same time, FGF23 has been found to regulate the renal synthesis of vitamin D₃ via renal FGFR3 and 4 (81, 82).

1.4.1.3 FGF23 target organs

1.4.1.3.1 Renal

The kidney is a principal target organ for FGF23. The primary physiological function of FGF23 is to inhibit the renal phosphate reabsorption via NaPT and suppress the renal synthesis of 1,25(OH)₂D₃. FGF23 regulates 1,25(OH)₂D₃ synthesis through inhibition of Cyp27b1 to decrease its production and stimulation of Cyp24A1 in a bid to increase its degradation (83).

1.4.1.3.2 Extra-renal

Recent experiments on FGF23 knockout mice revealed that FGF23 acts in a systemic manner rather than in an autocrine/paracrine manner. Target organs of FGF23 are tissues that express FGFR and klotho like parathyroid glands and brain or tissues that only express FGFR like thymus, spleen, or bones (54). The parathyroid gland expresses FGF receptors and klotho, thus it is regarded as FGF23 target tissue. The effect of FGF23 on parathyroid gland remains controversial. Some studies suggest that FGF23 suppresses the PTH secretion by the creation of PTH - FGF23 feedback loop (increased PTH → increased FGF23 → decreased PTH), but other studies suggest that FGF23 stimulates the production of PTH (84, 85). The effects of FGF23 on other extra-renal organs remain uncertain (Fig. 4) (54).
Fig. 4. Renal and extra-renal functions of FGF23.
A. FGF23 activates FGFR/klotho complexes in the distal tubules (DT) leading to predominant events in the proximal convoluted tubules (PCT).

B. Extra renal targets of FGF23 including heart, brain, thymus, spleen and bone.

1.4.1.4 FGF23 regulation

The regulation of FGF23 is complex and not completely understood. FGF23 regulators include PTH, \(1,25(OH)_2D_3\), secreted klotho, glucocorticoids, calcium and phosphate. \(1,25(OH)_2D_3\) is the main regulator of FGF23. It stimulates FGF23 production thereby creating a negative feedback loop regulating \(1,25(OH)_2D_3\) production (increased \(1,25(OH)_2D_3\) level → stimulate FGF23 → decreased \(1,25(OH)_2D_3\)) (86). In humans, the role of phosphate in FGF23 remains conflicting, with some studies demonstrating that high phosphate diet is associated with an increase in FGF23 serum level, while other studies demonstrate a negative correlation between FGF23 and oral \(P_i\) (87, 88). PTH stimulates the synthesis and production of FGF23 from the bones (89). There are no in vitro data to date to support a direct effect of klotho on FGF23 production, but the results from in vivo experiments suggest that klotho influences the FGF23 production in bones. This finding is based on the fact that both primary (genetic deletion or mutation) and secondary (CKD) klotho deficiencies are associated with an increase in the FGF23 level (90).
Other FGF23 regulators include PHEX, and DMP-1. The exact mechanism(s) through which DMP1 and PHEX regulate FGF23 are yet to be determined. However, recent studies demonstrate the absence of either PHEX or DMP-1 associated with an increase in FGF23 level in the osteocyte and in the circulation, leading to an increased phosphate excretion by the kidney (58, 91, 92).

1.4.1.5 FGF23 and phosphate homeostasis

1.4.1.5.1 Phosphate homeostasis

Phosphorus is a common anion, distributed ubiquitously throughout the body. It plays an important role in a variety of biological processes, such as cell signalling, nucleic acid synthesis, energy homeostasis, maintenance of acid–based balance, formation of lipid bilayers, and bone formation. 80% - 85% of the total body phosphorus is present in the bone; the remaining is present in soft tissue (14%) and extracellular fluid (1%). (Fig. 5) (93, 94).

**Fig. 5. Phosphorus exchange.**

The body phosphate homeostasis is determined by modulation of the intestinal uptake of dietary phosphate, renal phosphate excretion and the exchange of phosphate between extracellular and bone storage pools.
1.4.1.5.2 Phosphate absorption

Intestinal phosphate absorption plays a more significant role in phosphate homeostasis than was previously recognized. Phosphorus is absorbed from the intestine, predominantly in the jejunum, through passive and transcellular pathways. Passive pathway (also known as paracellular pathway) is dependent on the phosphate gradient in the gut, while the transcellular mechanism is mediated by sodium-dependent phosphate co-transporters (NaPi-IIb) and controlled by vitamin D$_3$ (VD$_3$) (95).

At normal physiological state, 90% of serum phosphate is filtered at the level of the glomerulus, 60% - 70% of the filtered phosphate is reabsorbed in the apical membrane of proximal tubule and 20% - 30% is reabsorbed beyond the proximal tubule. Phosphate is reabsorbed in the renal proximal tubule through NaPi-II. Three types of NaPi have been identified (95, 96).

**Type 1 NaPi**: belongs to the SLC17 family and is expressed in the kidney, liver, brain and parathyroid gland. In the kidney, it is expressed in the proximal tubules and to a lesser extent in the distal tubules. NaPi-1 is not thought to play an important role in renal phosphate reabsorption, since its expression in NaPi-IIa knockout mice fails to compensate for hypophosphatemia (97).

**Type 2 NaPi**: belongs to the SLC34 family. Three isoforms of type 2 NaPi transports have been identified:

- **NaPi-IIa** is expressed mainly in the brush border membrane (BBM) of the proximal tubule. The results of the previous studies demonstrate that NaPi-IIa is a key mediator of P$_i$ reabsorption in the renal proximal tubules. It mediates 70% of total renal phosphate reabsorption and it is under the control of PTH, FGF23 and dietary phosphate (98-100).

- **NaPi-IIb** is primarily expressed in the small intestine. It plays a crucial role in intestinal phosphate absorption and it is under the control of 1,25(OH)$_2$D$_3$ and dietary phosphate (101-103).
• **NaPi-IIc** is expressed primarily in the proximal tubule (104) and mediates approximately 30% of renal phosphate reabsorption. It is regulated by dietary phosphate, magnesium, metabolic acidosis, and FGF23 (105, 106).

**Type 3 NaPi:** is a member of SLC20 family. Type III transporters, specifically PiT1 and PiT2; have recently been shown to mediate phosphate transport. Type III NaPi transporters are expressed ubiquitously in several species. It is now clear that PiT2 is present at the renal BBM and PiT1 at the intestinal BBM and that these proteins are controlled by dietary phosphate load and FGF23 (97, 104, 107).

### 1.4.1.5.3 Phosphotoxicity

Elevated serum phosphate level has emerged recently as a risk factor for the vascular calcification and cardiovascular disease in CKD as well as in general population. High phosphate level in the blood can lead to ectopic calcification and arteriosclerosis, COPD, CKD and loss of hearing in a person with normal renal function (108). Moreover, P<sub>i</sub>-lowering therapy has been demonstrated to improve mortality and clinical outcome in patients with CKD. Recent in vivo studies provide evidence that phosphate toxicity accelerates the aging process in mammals (56), an effect which may be in part due to vascular calcification (109). The role of phosphate in vascular calcification has come under intense investigation (110). Therefore, maintaining a normal serum phosphate concentration is very important to prevent calcification and prolong survival (111, 112).

### 1.4.1.5.4 The effects of FGF23 on phosphate homeostasis

Phosphate homeostasis is a complex process that under tight hormonal control. It was previously thought that 1,25(OH)<sub>2</sub>D<sub>3</sub> and PTH are the sole regulators of inorganic phosphate (P<sub>i</sub>) homeostasis, but recent studies have identified several new factors that also play a role in the regulation of phosphate homeostasis among which a mention can be made of FGF-23, FRP-4, FGF7, and MEPE.
Several studies demonstrate that FGF23 remains to be the key regulator of phosphate homeostasis (113-116). Dietary phosphate and age have also been found to influence plasma P\textsubscript{i} concentrations. Studies on animals and humans demonstrate that aging process is associated with a reduction in both the renal and intestinal expression of type II NaPi co-transporter (117). FGF23 inhibits the phosphate reabsorption more directly through inhibition of NaPi-IIa and NaPi-IIc expression in the proximal renal tubule. FGF23 regulates the serum level of phosphate and 1,25(OH)\textsubscript{2}D\textsubscript{3}. PTH and 1,25(OH)\textsubscript{2}D\textsubscript{3} stimulates the release of FGF23 from the bones (Fig. 6) (118-121).

PTH and 1,25(OH)\textsubscript{2}D\textsubscript{3} are two additional hormones which play an important role in phosphate homeostasis. PTH down-regulates the expression of NaPi-IIa and NaPi-IIc in BBM of proximal tubule and causes phosphaturia similar to FGF23, but unlike the FGF23, the PTH increases the production of 1,25(OH)\textsubscript{2}D\textsubscript{3}, which then acts on the intestine to enhance the absorption of calcium and phosphate. Thus, PTH and FGF23 have opposing effects on 1,25(OH)\textsubscript{2}D\textsubscript{3} production. FGF23 inhibits PTH; therefore, it abolishes the activation of CYP27B1 by PTH. At the same time, 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibits the PTH production (115, 122).

FGF23 is recognized as a key regulator of phosphate homeostasis acting independently of the two main endocrine factors, PTH and calcitriol. In addition, PTH and calcitriol synthesis are also regulated by FGF23. Thus, FGF23 regulates the phosphate homeostasis directly through its effects on the renal phosphate transporters or indirectly through the regulation of PTH and calcitriol, (Fig. 6) (122-124).
Fig. 6. Feedbacks regulating serum phosphate level.

Inorganic phosphate is tightly regulated by the three organs depicted here, parathyroid, bone, and kidney, interacting with one another through three feedback loops.

1.4.1.6 The effects of FGF23 on 1,25(OH)$_2$D$_3$

1.4.1.6.1 Vitamin D$_3$ (VD$_3$) metabolism

Vitamin D is a group of fat-soluble vitamins, which plays a key role in controlling the renal excretion of both calcium and phosphorus. The two major forms of vitamin D are vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol). VD$_3$ synthesizes in the skin from 7-dehydrocholesterol through a photochemical process, or it can be obtained from various dietary sources such as fortified dairy products and fish oils. Cholecalciferol is an inactive form and it is converted to its active form via two hydroxylation steps. The first step occurs in the liver, where the VD$_3$ is converted into 25-hydroxycholecalfirol 25(OH)D by 25-hydroxylase. The second step occurs in the kidney, where 25(OH)D is converted to 1,25(OH)$_2$D$_3$ by the enzyme 1α-hydroxylase, which is expressed primarily in the renal proximal tubule (125-128).
FGF23 is regarded as a $1,25(OH)_2D_3$ counter regulatory hormone. It down-regulates the renal 1α-hydroxylase and up-regulates 25 hydroxyvitamin D-24hydroxylase, therefore leading to decreased formation and increased catabolism of $1,25(OH)_2D_3$ (129, 130).

1.4.1.6.2 Role of $1,25(OH)_2D_3$ in phosphate and calcium metabolism

$1,25(OH)_2D_3$ is a powerful stimulator of intestinal and renal phosphate and calcium transport. Moreover, it is an important regulator of PTH. Several studies have demonstrated that $1,25(OH)_2D_3$ influences calcium and phosphate homeostasis by affecting their metabolism and absorption in the intestines, kidneys and bones. Biologic effects of VD$_3$ result largely from binding of VD$_3$ to its vitamin D receptor (VDR), which is found almost in all tissues (131).

Serum calcium level is very tightly regulated by the calcium-sensing receptors (CaSR), which are directly regulated by PTH and calcitriol levels. The exact role of FGF23 in Ca$^{2+}$ homeostasis is not completely understood yet.

High $1,25(OH)_2D_3$ serum level leads to increase the intestinal Ca$^{2+}$ absorption, and reduce the renal Ca$^{2+}$ excretion. The major action of $1,25(OH)_2D_3$ on the small intestine is the stimulation of calcium absorption; a process that occurs via active and passive mechanisms (132, 133). The $1,25(OH)_2D_3$ regulates the serum phosphate level directly through an increase in the intestinal phosphate absorption by enhancing the expression of NaPi-IIb (134) and also indirectly by increasing its renal reabsorption through the suppression of PTH (135-137). $1,25(OH)_2D_3$ has also been found to affect the expression of NaPi-IIa in the renal cortex (137). Several studies have demonstrated that vitamin D$_3$ may exacerbate the vascular calcification by direct and indirect mechanisms, direct by an increase at the calcium and phosphorus blood level or indirect through transforming the VSMCs into osteoblastic-like cells, since it stimulates the calcium flux and modulates the expression of several proteins associated with calcification into VSMCs (138).
2 Aims of the study

As aforementioned, GSK-3 has been implicated in different physiological functions such as metabolism, cell cycle, gene expression and development in various biological systems (139). Aberrant GSK-3 levels are associated with several chronic disorders including inflammatory conditions (139), apoptosis (26), vascular calcification (140) and regulation of many age-related pathways (141). GSK-3 is considered as an important therapeutic target in these pathologies. Lithium is a known GSK-3 inhibitor, which is used widely for the treatment of mood disorders. Several studies have now suggested that lithium exerts its effects through GSK-3 inhibition (142, 143).

Recent observations have revealed that renal klotho expression is markedly down-regulated by dehydration (144, 145). Lithium impairs the urinary concentrating ability of the kidney resulting in NDI, a disorder characterized by unresponsiveness of the kidneys to the action of ADH, leading to polyuria, sodium diuresis, and mild dehydration (18). According to these findings, lithium treatment could affect the renal expression of klotho, which is an important FGF23 co-receptor (146).

FGF23 is a powerful inhibitor of ageing, whose lack can lead to premature appearance of a wide variety of age-related disorders. Recent studies demonstrate a correlation between antidepressant treatments and FGF expression in the cerebral cortex (48). The relationship between lithium and FGF23 remains to be elucidated.

The specific aims of this project are:

- To explore the effect of lithium on the klotho / FGF23 hormonal axis.
- To explore whether lithium treatment influence 1,25(OH)₂D₃ formation and renal phosphate and calcium excretion.
### 3 Materials and methods

#### 3.1 Materials

##### 3.1.1 Chart of materials and instruments

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<tr>
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<tr>
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<td>Centrifuge, Rotana 469 R</td>
<td>Hettich, Tuttlingen, Germany</td>
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3.1.2 Chemicals

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<td>Isoflurane</td>
<td>Curamed Pharma GmbH, Karlsruhe, Germany</td>
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Inhibitor cocktail tablet Complete mini, Roche, Mannheim, Germany
LiCl Calbiochem, Merck GmbH, Germany
Nitrogen liquide Linde, Wiesbaden, Germany
Normal saline 0.9% Fresenius Kabi Bad Homburg, Germany
PBS GIBCO, Carlsbad, Germany
Penicillin-Streptomycin GIBCO, Carlsbad, Germany
peqGOLD TriFast PEQLAB Biotechnologie GMBH, Erlangen, Germany
Phosphatase/Protease Complete mini, Roche, Mannheim, Germany
Phosphate-buffered saline PBS tablets, Invitrogen, Karlsruhe, Germany
2-Propanol (Isopropanol) Sigma, Taufkirchen, Germany
Primers Invitrogen, Darmstadt Germany
Random Hexamers Invitrogen, Darmstadt Germany
RIPA lysis buffer Cell Signaling, Frankfurt, Germany
Sodium chloride Sigma-Aldrich, Hannover, Germany
SuperScript III reverse Invitrogen, Darmstadt Germany
Trypan blue solution 0.4% Sigma, Taufkirchen, Germany
Tween-20 Sigma, Taufkirchen, Germany

3.1.3 Kits

3.1.3.1 Animal study

- Mouse C-terminal FGF23 ELISA kit (Immutopics International, San Clemente, CA, USA).
- Calcitriol kit (IDS, Boldon, UK).
- Plasma ADH ELISA kit (Phoenix Pharmaceuticals, Germany).
- Creatinine kit for plasma – creatinine PAP (Labor technik, Berlin, Germany).
• Creatinine kit for urine – creatinine Jaffe (Labor technik, Berlin, Germany).
• Inorganic phosphate (Roche Diagnostics, Mannheim, Germany).

3.1.3.2 Human study

• Human serum C-terminal FGF23 ELISA kit (Immutopics International, San Clemente, CA, USA).
• Calcitriol kit (IDS, Boldon, UK).
• Inorganic phosphate (Roche Diagnostics, Mannheim, Germany).
• Serum soluble α-klotho level (IBL, Minneapolis, USA).

3.1.4 Animal diet

Standard diet containing 1% Calcium, 0.7% phosphorus and 1,000 IU vitamin D₃/kg (Ssniff, Soest, Germany).

3.1.5 Software

• Computerized data acquisition Springs, USA
• Chart version 4.2 Axon Instruments, California, USA
• Data link version 1.0.0 Herbert & Scheneider Software & CAM, Germany
• GraphPad Instat version 3.05 GraphPad Software Inc., San Diego, USA
• Magellan version 3.11 Tecan GmbH, Crailsheim, Germany
• Sigma plot version 7.0 Systat Software Inc., Erkrath, Germany
• Quantity One software Bio-Rad, Munich, Germany.
• Endnote VII Thomson Reuters, CA, USA
3.1.6 Experimental animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. The experiments were performed on female C57Bl/6 mice at 10 week-old. The mice had free access to water and control food containing 1% calcium, 0.7% phosphorus and 1,000 IU vitamin D₃/kg.

3.1.7 Metabolic cages

For evaluation of renal excretion, the mice were placed individually in metabolic cages from day 11–14 of lithium treatment for 24 hours of urine collection to determine the excretion of urine, and to collect uncontaminated samples of urine.

3.1.8 Patient recruitment

The patients were recruited for this study in the Department of Psychiatry and Psychotherapy, Charité Campus Mitte, Berlin.

A total of 95 acute depressive patients (38 males, 57 females, age 48 ± 14 years) were recruited for this study. Inclusion criteria were, unipolar major depression, age older than 18 years, indication for an antidepressant pharmacotherapy, insufficient response to an adequate antidepressant pretreatment and clinical indication for lithium augmentation, Hamilton Depression Scale (HAMD-17) score of 12 or greater, and written informed consent. The diagnosis was confirmed on the basis of the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders. All patients received individual doses of lithium carbonate adjusted individually for each patient targeting lithium serum-level more than 0.4 mmol/L (147). Blood was drawn from patients first before lithium augmentation and then after 4 weeks following the medication with lithium. Detailed clinical data of the patients have already been published (145).
3.2 Methods

3.2.1 Murine experiments

3.2.1.1 Determination of serum and plasma concentrations

To collect blood specimens, animals were lightly anesthetized with diethylether and about 50 - 200 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Serum C-terminal FGF23, ADH and serum 1,25(OH)\textsubscript{2}D\textsubscript{3} concentrations were measured by ELISA. The serum phosphate and total Ca\textsuperscript{2+} concentrations were measured by a photometric method. The free calcium concentration was analyzed by a blood gas analyzer in heparinized plasma samples.

3.2.1.2 Measurement of urinary calcium and phosphate concentrations

For evaluation of the renal excretion, the mice were placed individually in metabolic cages from day 11–14 of lithium treatment for 24h. The mice had free access to drinking water and food. They were allowed 2-day habituation period when food, water intake, urinary flow rate, urinary excretion, and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24 hours collection of urine was performed to obtain the urinary parameters. To assure the quantitative urine collection, the metabolic cages were siliconized and the urine was collected under water-saturated oil (148). The urinary phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit. The urinary calcium was measured by flame photometry.

3.2.1.3 Western blotting

To analyze the klotho and NaPi-IIa protein abundance and GSK-3 phosphorylation in renal tissue, the kidneys were removed and immediately snap-frozen in liquid nitrogen. After homogenization in lysis buffer (54.6 mM HEPES; 2.69 mM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}; 360 mM NaCl; 10 % (v/v) glycerol; 1 % (v/v) NP40 or RIPA lysis buffer containing phosphatase and protease inhibitor cocktail
tablet, the samples were incubated on ice for 30 min and then centrifuged at 14,000 rpm and 4 °C for 20 min. The supernatant was removed and used for Western blotting. The total protein (80 µg) was separated by SDS PAGE, thereafter transferred to nitrocellulose membranes and blocked in 5 % non-fat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1h. The membranes were probed overnight at 4 °C with polyclonal rat anti-klotho antibody (1:1,000 in 5 % fat-free milk in TBST), with rabbit anti-NaPi-IIa antibody (149); 1:3,000 in TBST with 5 % BSA), or with rabbit anti-phospho-GSK-3β (1:1,000 in TBST supplemented with 5 % BSA). After incubation with horseradish peroxides-conjugated anti-rat or anti-rabbit secondary antibodies (1:2,000) for 1 hour at room temperature, the bands were visualized with enhanced chemiluminescence reagents. The membranes were also probed with GAPDH antibody as loading control. The densitometry analysis was performed using Quantity One software.

3.2.1.4 FGF23 transcripts in UMR106 cells

The UMR106 rat osteosarcoma cells were cultured in DMEM high glucose medium supplemented with 10 % FCS and 1 % penicillin/streptomycin under standard conditions. The cells were pretreated with 100 nM calcitriol to induce the FGF23 expression (150). After 24 hours, the cells were in addition treated either with lithium (2 mM) for another 24 hours or with [Arg8]-vasopressin acetate salt (50 nM) for another 12 hours, or with recombinant human klotho protein (30 ng/ml) for another 12 hours, or were left untreated. Then, the entire RNA was isolated with TriFast RNA extraction reagent based on a chloroform extraction protocol. mRNA was transcribed with SuperScript III reverse transcriptase using an oligodT Q2 primer. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed on a Bio-Rad iCycler iQ™ real-time PCR detection system using the following primers:

*Tbp* (TATA box-binding protein)

Forward (5'-3'): ACTCCTGCCACACCAGCC
Reverse (5'-3'): GGTCAGTTTACAGCCAAGATTCA
The final volume of the PCR reaction mixture was 20 µl and contained 2 µl cDNA, 1 µM each primer, 10 µl GoTaq qPCR master mix, and sterile water up to 20 µl. The qPCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 58 °C for 10 s. The calculated mRNA expression levels were normalized to the expression levels of Tbp of the same cDNA sample. Relative quantification of gene expression was performed using the ∆∆ct method.

3.2.1.5 Statistical analysis

Data are provided as means ± SEM; n represents the number of independent experiments. All data were tested for significance using unpaired Student’s t-test unless otherwise stated. Only results with \( p < 0.05 \) were considered statistically significant.

3.2.2 Human experiments

3.2.2.1 Determination of serum concentration

ELISA kits were employed to determine the serum concentrations of FGF23, klotho and 1,25(OH)_2D_3 according to the manufacturer’s instructions. The serum phosphate and Ca^{2+} concentrations were measured by a photometric method.

3.2.2.2 Statistical analysis

Data are provided as means ± SEM, n; represents the number of independent experiments. All data were tested for significance using unpaired Student’s t-test. Only results with \( p < 0.05 \) were considered statistically significant.
4 Results

Lithium has been demonstrated as a GSK-3 inhibitor. It is widely used for the treatment of mood disorders (28, 29). Several studies demonstrate that lithium inhibits GSK-3 directly by competing for an Mg$^{2+}$ binding site or indirectly by increasing the inhibitory serine-phosphorylation of GSK-3 (26). Lithium treatment interferes with renal effects of ADH, thus causing NDI, which is a clinical syndrome characterised by polyuria, sodium diuresis, and mild dehydration (18). Recent observations revealed that renal klotho expression is markedly downregulated by dehydration (144, 145). Klotho is required as co-receptor for mediating the phosphaturic actions of FGF23 (118, 151).

In this chapter, we investigate the effect of lithium on the FGF23 hormonal axis using a murine model and blood samples from acute depressive patients.

4.1 Murine model

4.1.1 The effect of lithium on renal GSK-3 phosphorylation in the murine model

In order to demonstrate the effect of lithium on renal GSK-3 phosphorylation, 10 week-old female C57BL/6 mice were subcutaneously injected with 200 mg/Kg LiCl and vehicle (NaCl) for 14 days. The kidneys were subsequently removed and renal GSK-3 phosphorylation was determined by Western blotting. As illustrated in Fig. 7, lithium treatment was followed by a significant increase in GSK-3 phosphorylation.
Fig. 7. Renal GSK-3 phosphorylation.

Original Western blot showing the protein abundance of phosphorylated GSK-3 and GAPDH (upper panel) and arithmetic means ± SEM (n =4/group) of the phosphorylated GSK-3 over GAPDH protein abundance ratio (lower panel) in renal tissue from sham- (white bar) and lithium treated (black bar) wild type mice. *p <0.05 indicates a significant difference from untreated mice.
4.1.2 The effect of lithium on ADH serum level in lithium treated mice

Lithium treatment is one of the common causes of acquired NDI, which is a clinical syndrome characterized by reducing the renal responsiveness to ADH leading to an increase in water and sodium diuresis; effects which can result in mild dehydration (18). This finding could suggest that lithium treatment may affect the renal klotho expression.

To clarify whether lithium could result in a state of dehydration, 10 week-old female C57BL/6 mice were subcutaneously injected with 200 mg/Kg LiCl and vehicle (NaCl) for 14 days. Whole blood samples were collected, the serum was separated and the serum ADH was measured using ELISA method. As illustrated in Fig. 8, lithium treatment was followed by an increase in ADH serum level.

![Fig. 8. Serum ADH level.](image)

Arithmetic means ± SEM of serum ADH levels (n =4/group) in sham-(white bar) and lithium-treated (black bar) wild type mice. *p <0.05 indicates a significant difference from untreated mice.
4.1.3 Body weight in lithium treated mice

Body weight in mice treated with LiCl and NaCl (control group) was monitored throughout the treatment duration (14 days). As shown in Fig. 9, lithium treatment did not influence the body weight.

**Fig. 9. Body weight.**
Arithmetic means ±SEM of body weight (n=6) in sham- (blue bar) and lithium-treated (pink bar) wild type mice.
4.1.4 The effect of lithium on renal klotho expression in lithium treated mice

Recent observations revealed that renal klotho expression is markedly down-regulated by dehydration (142, 143). To elucidate the effects of lithium treatment on the renal klotho expression, kidneys of the mice were removed 14 days after treatment with NaCl or with LiCl only. The renal klotho protein abundance was determined by Western blotting. As illustrated in Fig. 10, lithium treatment was followed by a strong and statistically significant decrease in the renal klotho protein abundance.

![Western blot showing renal expression of klotho and GAPDH](image)

**Fig. 10. Renal klotho expression.**

Original Western blot showing renal expression of klotho and GAPDH (upper panel) and arithmetic means ± SEM (n =4/group) of the klotho over GAPDH protein abundance ratio (lower panel) in renal tissue from sham- (white bar) and lithium-treated (black bar) wild type mice. *p <0.05 indicates a significant difference from untreated mice.
4.1.5 The effect of lithium on FGF23

4.1.5.1 FGF23 serum level

Several studies have identified klotho as a FGF23 co-receptor. Its forms complexes with FGFRs and increases their affinity to FGF23 (152). The importance of klotho in FGF23 signaling is illustrated by both human and animal studies, where loss of klotho results in an end-organ resistance to FGF23 (54, 153). In order to explore whether lithium treatment regulates FGF23 secretion, the serum FGF23 level was measured in sham and lithium treated mice. Fig. 11 shows that lithium treatment was followed by a dramatic and statistically significant increase in the serum FGF23 compared to the control group.

Fig. 11. Serum FGF23 level.
Arithmetic means ± SEM (n =10/group) of serum FGF23 levels in sham- (white bar) and lithium- treated (black bar) wild type mice. ***p <0.001 indicates a significant difference from untreated mice.
4.1.5.2 FGF23 transcript level in UMR106 cells treated with lithium

As shown in Fig.7, 8, 10 and 11, lithium treatment is found to decrease both the GSK-3 activity (inhibitory phosphorylation) and renal klotho protein abundance and increase the ADH and FGF23 serum level. An increase in serum FGF23 level following lithium treatment could be due to the influence of ADH or klotho on FGF23 transcription level.

To investigate this conjecture, UMR106 osteoblast-like cells were treated with [Arg8]-vasopressin (50 nM) and FGF23 transcript levels were determined by qRT-PCR. As a result, [Arg8]-vasopressin significantly decreased the FGF23 transcript levels (0.00522±0.00100 arb. units (n=15)) compared to untreated cells (0.01157±0.00215 arb. units (n=15); p <0.05, u test). This result indicates that ADH is unlikely to increase the FGF23 serum level in the lithium treated mice.

Next experiments explored whether klotho protein influences the FGF23 transcription in UMR106 osteoblast-like cells. The FGF23 transcript level was 0.01063±0.00341 arb. units (n=15) in klotho protein treated cells (30 ng/ml) and (0.00708±0.00187 arb. units (n=15) in untreated cells. Thus, klotho protein did not significantly influence the FGF23 expression.

However, exposure of UMR106 osteoblast-like cells to (2 mM) LiCl stimulated FGF23 expression as revealed by quantitative RT-PCR. The FGF23 transcript level approached 0.00769±0.00098 arb. units (n=15) in untreated and 0.01245±0.00208 arb. units (n=15) in lithium-treated UMR106 cells (p <0.001, u test).
4.1.6 1,25(OH)$_2$D$_3$ serum level

Experimental studies on FGF23 and klotho knockout have demonstrated that FGF23 and klotho act in concert to down-regulate the expression of 1α-hydroxylase, which is the key enzyme in the synthesis of 1,25(OH)$_2$D$_3$ (63). In order to determine the effect of lithium treatment on 1,25(OH)$_2$D$_3$, blood-serum samples were collected from either lithium treated or control mice 14 days following lithium or NaCl treatment. ELISA was employed to measure the serum level of 1,25(OH)$_2$D$_3$. Fig. 12 reveals that lithium treatment does indeed decrease serum 1,25(OH)$_2$D$_3$ level.

Fig. 12. Serum 1,25(OH)$_2$D$_3$ Level.

Arithmetic means ± SEM (n =4–5/group) of serum 1,25(OH)$_2$D$_3$ levels in sham- (white bar) and lithium-treated (black bar) wild type mice. ** $p<0.01$ indicates a significant difference from the untreated mice.
4.1.7 The effects of lithium on phosphate metabolism

FGF23 acts as a phosphaturic agent that reduces the serum inorganic phosphate (P\textsubscript{i}) directly through the inhibition of renal NaPi-IIa transporter in the proximal tubule or indirectly through the suppression of calcitriol synthesis (118). The effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} include stimulation of intestinal and renal phosphate transport leading to a rise in the serum phosphate concentrations (136). Accordingly, serum and urinary phosphate concentrations were determined in control and lithium-treated mice.

4.1.7.1 Serum phosphate level

To elucidate whether serum phosphate level might be influenced by lithium treatment, the serum phosphate level was measured 14 days after treatment with or without LiCl by a photometric method. As shown in Fig. 13, lithium treatment significantly decreased the serum phosphate concentration.

![Fig. 13. Serum phosphate level.](image)  
Arithmetic means±SEM of serum phosphate concentration (n =10/group) in sham- (white bars) and lithium-treated (black bars) wild type mice. **p <0.01 indicates a significant difference from the sham-treated mice.
4.1.7.2 Urinary phosphate excretion

Theoretically, the low serum phosphate level in lithium treated mice may be the consequence of an increase in the urinary phosphate excretion. Therefore, the mice were placed individually in metabolic cages from day 11-14 of lithium treatment. Urine was collected every 24h for 3 days and the urinary phosphate excretion was measured. As shown in Fig. 14, the lithium treatment significantly increases the urinary phosphate excretion.

**Fig. 14. urinary phosphate excretion.**

Arithmetic means±SEM of urinary phosphate excretion (n =6/group) in sham- (white bars) and lithium-treated (black bars) wild type mice. **p <0.01 indicates a significant difference from sham-treated mice.**
4.1.7.3 Renal expression of NaPi-IIa

In order to investigate whether the phosphaturia in lithium treated mice was paralleled by altered expression of the major renal phosphate transporters (NaPi-IIa), Western blotting was employed to determine the renal expression of NaPi-IIa. As shown in Fig. 15, lithium treatment did not significantly affect the total renal NaPi-IIa protein abundance. The densitometric analysis yielded a renal NaPi-IIa abundance of 0.55±0.04 arb. units (n =6) in sham-treated and 0.59±0.06 arb. units (n =6) in lithium-treated animals.

**Fig. 15. Renal expression of NaPi-IIa.**
Original Western blotting showing renal expression of NaPi-IIa (upper panel) and GAPDH (lower panel).
4.1.8 The effects of lithium on calcium metabolism

Serum calcium is found in three forms: 40% bound predominantly to albumin; 10% is complexes with serum anions and 50% is ionized (154). The ionized serum calcium is the biologically active form of calcium and it constitutes a better indicator of the functional status of calcium metabolism than the total serum calcium. Since 1,25(OH)₂D₃ also leaves an impact on the renal calcium reabsorption, the serum and urinary calcium concentration were measured.

4.1.8.1 Serum calcium level

To explore the effects of lithium treatment on calcium metabolism, blood-serum samples were collected from either lithium treated or control mice 14 days following treatment and the total serum calcium level was measured by a photometric method. The ionized serum calcium concentration was analysed in whole blood samples by a blood gas analyser. As shown in Fig. 16, lithium treatment tends to decrease the total calcium level. However the plasma-free calcium levels were not different between sham-treated (0.93±0.03 mM, n =6) and lithium- treated mice (0.99±0.04 mM, n =5).

![Fig. 16. Serum calcium level.](image)

Arithmetic means ± SEM of total serum calcium concentration (n =10/group) in the sham- (white bars) and lithium-treated (black bars) wild type mice. ***p <0.001 indicates a significant difference from the sham-treated mice.
4.1.8.2 Urinary calcium excretion

To measure the urinary calcium excretion, the mice were placed individually in metabolic cages for 3 days and urine was collected every 24h. The urinary calcium was measured by flame photometry. Fig. 17 demonstrates that the lithium treated mice suffer from an increase in the urinary calcium excretion compared to the control group. Calciuria in the lithium treated mice was not the simple consequence of hypercalcemia as their serum calcium level was not different from that of the control group.

**Fig. 17. Urinary calcium excretion.**

Arithmetic means±SEM of urinary calcium excretion (n =6/group) in the sham- (white bars) and lithium-treated (black bars) wild type mice. ***p <0.001 indicates a significant difference from the sham-treated mice.
In summary, lithium treatment significantly increased renal GSK3 phosphorylation, enhanced serum ADH and FGF23 concentrations, downregulated renal klotho expression, stimulated renal calcium and phosphate excretion, and decreased serum 1,25(OH)₂D₃ and phosphate concentrations in lithium treated mice.

Importantly, this murine study provides preliminary data indicating a novel powerful effect of lithium on the FGF23. Based on this result we want to investigate the effect of lithium treatment on FGF23 hormonal axis in acute depressive patients.
4.2 Human study

Depression is a multifaceted disorder which has been associated with the risk to develop several medical disorders including cancer, dementia, diabetes, epilepsy and stroke (155). Since its introduction into modern psychiatry in the 1950s and 1960s lithium has been mainly considered an effective maintenance treatment for bipolar disorders (156). Moreover, lithium has been identified as an important anti-suicidal and anti-inflammatory agent. Several studies have demonstrated that a small amount of lithium in drinking water reduces the rate of suicide and promotes the longevity in humans (5, 12, 14, 146-149), but the mechanism through which lithium exerts these effects is largely unknown. FGFs are one of the growth factors that involved in early brain development as well as maintenance and repair through adult life. Although the role of FGFs in psychiatric disorders has been growing relatively recently (157).

Based on the results of murine study which showed a new effect of lithium on FGF23 hormonal axis following lithium treated, in this section we want to investigate the effect of lithium on the FGF23 hormonal axis using serum-blood samples from acute depressive patients.

To this end, a total of 95 acute depressive patients (age 48 ± 14 years old) were recruited. All the patients received individual doses of lithium carbonate adjusted to their individual lithium serum levels.
4.2.1 Serum C-terminal FGF23 level

To determine whether lithium treatment effects serum FGF23 levels in depressive patients, serum blood was taken from medicated patients before lithium augmentation and then after 4 weeks of medication with lithium. ELISA was used to measure serum FGF23 levels. Lithium treatment was followed by a marked increase at the serum FGF23 level of patients Fig. (18).

Fig. 18 Serum C-terminal FGF23.
Arithmetic means ± SEM (n =95) of serum FGF23 levels prior to (white bars) and following (black bars) lithium augmentation. **p<0.01 indicates a significant difference from respective value prior to the treatment.
4.2.2 The serum soluble α-klotho level

In order to explore whether an increase in the FGF23 serum level is associated with altered serum klotho level in lithium treated depressive patients, the serum soluble α-klotho level was measured. As shown in Fig. 19, the serum klotho level tended to increase slightly in acute depressive patients following lithium augmentation.

![Fig. 19. Serum soluble α-klotho Level.](image)

Arithmetic means ± SEM (n = 95) of serum klotho level prior to (white bars) and following (black bars) the lithium augmentation.
4.2.3 The serum 1,25(OH)$_2$D$_3$ level

FGF23 down-regulates 1α-hydroxylase, the key enzyme in the production of 1,25(OH)$_2$D$_3$ (129). The serum blood samples were isolated from the acutely depressive patients and the serum 1,25(OH)$_2$D$_3$ level was measured by ELISA. As shown in Fig. 20, the lithium treatment significantly decreased the serum 1,25(OH)$_2$D$_3$ concentration in acute depressive patients.

![Fig. 20. Serum 1,25(OH)$_2$D$_3$ level.](image)

Arithmetic means ± SEM (n =95) of serum 1,25(OH)$_2$D$_3$ levels in the untreated (white bar) and the lithium treated (black bar) patients. **p<0.01 indicates a significant difference from respective value prior to the treatment.
4.2.4 The serum phosphate and calcium level

Decline in serum 1,25(OH)$_2$D$_3$ levels is expected to decrease both, the calcium and phosphate serum concentrations. Accordingly, the serum calcium and phosphate concentrations were determined before and 4 weeks after lithium augmentation in acute depressive patients.

- **Serum phosphate**

As illustrated in Fig. 21, lithium treatment significantly decreased the serum phosphate concentrations of patients.

![Fig. 21. Serum phosphate level.](image)

Arithmetic means ± SEM (n = 95) of serum phosphate concentration in the untreated (white bars) and lithium treated (black bars) patients. **p<0.01 indicates a significant difference from the respective value prior to the treatment.
- **Serum calcium level**

Fig. 22 demonstrates that serum Ca\(^{2+}\) concentration tends to decrease slightly following the lithium treatment in acute depressive patients, an effect, however not reaching statistical significance.

![Serum Calcium Level Chart](image)

**Fig. 22. Serum calcium level.**

Arithmetic means ± SEM (n =95) of the serum calcium level in untreated (white bars) and lithium treated (black bars) patients.
5 Discussion

As aforementioned in the introduction, for more than 150 years lithium has been used as a therapeutic agent for treatment of manic-depressive disorders (18, 44). However, the use of lithium is frequently complicated with an impaired responsiveness of the kidney to the action of ADH causing NDI, which is a clinical syndrome manifested by increased water and sodium diuresis, resulting in mild dehydration (18). Several lines of evidence have implicated that GSK-3 plays a crucial role in the vasopressin-mediated urine concentration by the kidneys and suggested that decrease GSK-3 activity could be one of the initial targets through which lithium induce-NDI (32).

Recent observations have revealed that renal klotho expression is markedly downregulated by dehydration (144, 145). Klotho is a transmembrane protein that acts as an obligatory coreceptor for FGF23. Several lines of evidence indicated that FGF23 and klotho knockout mice exhibit almost identical phenotypes that include abnormal mineral metabolism which characterized by increased blood $P_i$, $Ca^{2+}$ and vitamin D$_3$ levels (145, 158). Excessive 1,25(OH)$_2$D$_3$ formation contributes to hyperphosphatemia which in turn fosters vascular calcification (118, 159).

In this thesis I demonstrate, for the first time, a novel powerful effect of lithium on FGF23. Short term lithium treatment was found to produce a pronounced increase in FGF23 serum level, significantly decrease serum 1,25(OH)$_2$D$_3$ and phosphate concentration in mice and patients. It is noteworthy to point out that these effects were paralleled by a statistically significant decrease of renal klotho expression, and an increase in the renal phosphate excretion in lithium treated mice.
5.1 Murine study

In order to investigate the effect of lithium on FGF23 hormonal axis, 10-week old female C57Bl/6 mice were treated with LiCl (n=6-10; 200 mg/kg subcutaneously) every 24h for 14-day period. In this thesis I provide preliminary data indicating that a 14-day lithium treatment significantly increased renal GSK-3 phosphorylation, enhanced serum ADH and FGF23 concentrations, downregulated renal klotho expression, stimulated renal calcium and phosphate excretion, and decreased serum 1,25(OH)2D3 and phosphate concentrations.

The effect of lithium on serum FGF23 release may be in part due to GSK-3 inhibition. Several experimental and clinical studies demonstrate that lithium exerts its effects through GSK-3 inhibition (157, 158). In the present murine study a 14-day lithium treatment is followed by a significant increase in GSK-3 phosphorylation (inhibitory phosphorylation), suggesting that the neuroprotective effect of lithium might be at least partially mediated by an increase the FGF23 level. In other words, lithium treatment inhibits the GSK-3 activity, which is a known effect of lithium. In animal and cell culture models, lithium has been shown to increase neuronal viability through a combination of mechanisms that include the inhibition of apoptosis, regulation of autophagy, increased mitochondrial function and synthesis of neurotrophic factors (160).

As mentioned previously, lithium treatment is one of the drugs that can cause dehydration. It interferes with the renal effect of ADH causing NDI (161, 162). However, the underlying mechanism by which lithium causes NDI is not completely understood (161). There is strong evidence suggesting that GSK-3 inactivation may account for the vasopressin-resistant polyuria of lithium-induced NDI (18, 32). My observations suggest that the effect of lithium on FGF23 serum concentration may be in part due to polyuria and dehydration, reflected by increased ADH.
Dehydration has previously been shown to downregulate the renal klotho expression (162, 163). In the present study, a short-term administration of LiCl in C57Bl/6 mice was found to downregulate the renal membrane α-klotho expression, an effect which could be due to dehydration. FGF23 also increases the expression of renal klotho abundance (164). Membrane α-klotho is mandatory for FGF23 to activate its downstream signaling pathways. However, there is growing evidence suggesting that FGF23 may stimulate its downstream signaling pathways in cells that lack or have little expression of membrane α-klotho (54, 165, 166).

Serum FGF23 levels are enhanced in gene-targeted mice lacking either SPAK (162) or OSR1 (163). Both SPAK and OSR1 stimulate renal tubular NaCl transporters, and thus lack of those kinases is expected to result in dehydration. However, according to the qRT-PCR results, ADH is unlikely to account for the enhanced FGF23 serum levels following lithium treatment, since the vasopressin analogue [Arg8]-vasopressin downregulates the FGF23 transcript levels in UMR106 osteoblast-like cells.

Decreased klotho expression in lithium treated mice was expected to blunt the effects of FGF23 on 1,25(OH)₂D₃ formation. However, evidence from the murine experiments demonstrate that lithium treatment leads to decrease the 1,25(OH)₂D₃ sera level in spite of the decrease in the expression of the renal klotho abundance. This result is consistent with a scenario which occurs in the early stage of CKD, in which an increase in FGF23 can attenuate phosphate retention and decrease 1,25(OH)₂D₃ formation despite a fall in renal α-klotho levels (167). Together, these lines of data could support the new hypothesis, that FGF23 can activate intracellular signaling pathways in cells that lack or have little expression of membrane α-klotho.

1,25(OH)₂D₃ stimulates the release of FGF23 and the excessive 1,25(OH)₂D₃ formation in klotho deficiency stimulates the FGF23 serum level (168). In the present murine study, a 14-day lithium treatment was found to increase the FGF23 serum level despite the observed decrease in 1,25(OH)₂D₃ serum concentrations, which were expected to result in a decrease of FGF23 release.
This finding implicated that 1,25(OH)\(_2\)D\(_3\) does not account for the increase of FGF23 serum level following lithium treatment. FGF23 downregulates the renal 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) and upregulates 25 hydroxyvitamin D-24 hydroxylase, which are the enzymes responsible for the synthesis and degradation of 1,25(OH)\(_2\)D\(_3\), respectively (118). The increase of FGF23 serum level following lithium treatment presumably accounts for the decrease of serum 1,25(OH)\(_2\)D\(_3\) concentrations.

1,25(OH)\(_2\)D\(_3\) is a potent stimulator of both renal and intestinal phosphate transport (126). Increased FGF23 serum level following the lithium treatment in 10 week-old female C57BL/6 mice was paralleled by hypophosphatemia and phosphaturia. Therefore, the hypophosphatemia could explain the phosphaturia in the lithium treated mice. This observation could be explained by the effect of FGF23 on 1,25(OH)\(_2\)D\(_3\) metabolism and renal tubular phosphate transport. FGF23 has been found to increase the urinary phosphate excretion directly by reducing the expression of sodium/phosphate cotransporter type II (NaPi-II) and indirectly through reducing phosphate absorption in the gut by suppressing 1α-hydroxylase activity (55). Therefore, an increased FGF23 release following lithium treatment is expected to generate phosphaturia and, thus, decreasing the serum phosphate concentration.

According to the Western blot result in the murine experiments, lithium treatment did not reveal a significant decrease in the whole kidney NaPi-IIa protein abundance. The finding does, however, not rule out that the protein abundance in the apical cell membrane is decreased by lithium treatment. Alternatively, the lithium treatment affects the activity of the carrier.

Furthermore, the present murine experiments demonstrate that the phosphaturia of the lithium treated mice is paralleled by increase in the urinary calcium excretion, which is not the simple consequence of hypercalcemia in the lithium treated mice, as their serum calcium level is not significantly different from that of the control group.
Lithium induce-hypercalcemia is a known side effect observed in a fraction of lithium-treated patients (169, 170). Lithium may increase the serum Ca\(^{2+}\) concentration by stimulating the PTH release, and it may decrease the serum Ca\(^{2+}\) concentration by lowering the 1,25(OH)\(_2\)D\(_3\) serum concentrations (Fig. 6 and 14). Thus, the eventual outcome may depend on the magnitude of the alterations in the PTH and 1,25(OH)\(_2\)D\(_3\) release.

Several lines of evidence from \textit{in vitro}, clinical and epidemiological studies suggest that increased serum phosphate concentration predisposes to vascular calcification and eventually leads to an early appearance of age-related disorders and a decrease of life span (110, 112, 159). It would be interesting to speculate whether lithium treatment may attenuate the tissue calcification (171). The present observations may suggest that lithium is capable of counteracting vascular calcification, aging, and early death.

Both klotho and FGF23 are essential regulators of aging. Lack of either klotho or FGF23 accelerates the development of several age-related disorders and eventually leads to early death (172). FGF23 deficiency elevates the serum phosphate, calcium, and 1,25(OH)\(_2\)D\(_3\) levels, effects eventually leading to several disorders, such as vascular calcification, decrease in bone density, growth retardation, infertility, and a dramatic reduction of life span. Together, these lines of data indicate that lithium may counteract at least some of the multiple disorders observed in FGF23 deficiency.

Circulating levels of FGF23 similarly increase in patients with renal insufficiency. Hyperphosphatemia is a risk factor for vascular calcification, cardiovascular disease and mortality in patients with renal insufficiency and FGF23 level is thought to increase as a compensatory mechanism to maintain normal phosphate balance in those patients (173, 174). Unlike renal insufficiency, lithium treatment lowers the serum phosphate concentration.
Taken together, these lines of evidence suggest that lithium treatment significantly increased renal GSK-3 phosphorylation, enhanced serum ADH and FGF23 concentrations, downregulated renal klotho expression, stimulated renal calcium and phosphate excretion, and decreased serum $1,25(OH)_2D_3$ and phosphate concentrations. This study provides preliminary data indicating that the effect of lithium on FGF23 may protect against vascular calcification and ultimately the appearance of age-related disorders.

5.2 Human study

Lithium salt has been widely used as a mood stabilizing drug, particularly in the treatment of bipolar disorders. A recent study has further suggested that lithium may have a potential therapeutic effect against Alzheimer’s disease and other neurodegenerative disorders. However, the exact mechanism of lithium’s neuroprotective effect is largely unknown. Several studies in humans explain these neuroprotective effects by GSK-3 inhibition, increase of BDNF synthesis, increase expression of anti-apoptotic genes, inhibition of cellular oxidative stress, cortical thickening, increased grey matter density, and hippocampal enlargement (160).

FGFs have been demonstrated as an essential factor for cell growth, differentiation and morphogenesis in the early stages of neural development. Moreover, a correlation between antidepressant treatments and FGF expression in the cerebral cortex and hippocampus has been observed (45). FGF23 is one of the most recently discovered FGFs, which maintain the phosphate and $1,25(OH)_2D_3$ metabolism by regulating the renal expression of NaPi-IIa and vitamin D$_3$ synthesis.

Following the murine study which discloses an important effect of lithium on FGF23, I further investigated whether lithium treatment influences the FGF23 serum level in lithium treated patients. To answer this question a total of 95 acute depressive patients were recruited. Those patients were treated with lithium carbonate targeting a lithium serum level of more than 0.4 mmol/L.
Surprisingly, 4-weeks Lithium treatment was found to increase FGF23 serum levels without significantly modifying serum klotho concentrations and significantly decreased serum 1,25(OH)$_2$D$_3$ and phosphate concentrations without significantly modifying serum Ca$^{2+}$ concentrations in those patients.

At least in theory, the effect of lithium on FGF23 serum levels may in part be due to polyuria and dehydration. Serum FGF23 levels are enhanced in gene-targeted mice lacking kinases involved in stimulation of renal tubular NaCl transport, and thus required for adequate renal salt and fluid reabsorption as well as hydration (175). FGF23 serum levels are further increased in renal insufficiency (167, 173).

Contrary to the murine experiments, I observed that lithium treatment tends to increase the serum soluble α-klotho level in acute depressive patients, an effect, however not reaching the statistical significance. The functions of soluble α-klotho in FGF23 signaling have not been identified. Soluble α-klotho displays an enzymatic activity that may be important in regulating ion channels such as the sodium-phosphate co-transporter (NaPi-IIa), renal outer medullary potassium (ROMK) channel and transient receptor potential vanilloid (TRPV5) ion channel(176).

Increased FGF23 serum levels following lithium treatment in acute depressive patients were paralleled with a significant decrease in 1,25(OH)$_2$D$_3$ serum levels. This effect could be explained by an increase FGF23 serum level. FGF23 acts as a counter-regulatory hormone for 1,25(OH)$_2$D$_3$. In other words, FGF23 could suppress 1α-hydroxylase activity in the proximal renal tubule, leading to reduced circulating levels of 1,25(OH)$_2$D$_3$ (129).

1,25(OH)$_2$D$_3$ stimulates the renal and intestinal phosphate transport. FGF23 has been regarded as a main phosphaturic factor. It regulates the phosphate homeostasis through its direct inhibitory effects on the expression of NaPi-IIa and NaPi-IIc in the renal tubule as well as through the inhibition of the renal synthesis of 1,25(OH)$_2$D$_3$. Therefore, the FGF23 reduces the intestinal phosphate absorption indirectly through its effects on 1,25(OH)$_2$D$_3$ (116).
Based on my findings, an increase in FGF23 serum level following lithium treatment in acute depressive patients could account for the observed decrease of serum phosphate concentration. Lithium treatment tends to decrease the serum calcium level in acute depressive patients, an effect, however not reaching the statistical significance. Urinary calcium and phosphate excretion in the clinical samples was not investigated and therefore it is difficult to interpret the relevance of phosphate homeostasis in response to lithium treatment in human (patho)physiology.

As mentioned above, FGF23 ablation results in elevated serum phosphate, calcium, and 1,25-dihydroxyvitamin D₃ levels, vascular calcifications, and early death. Vascular calcification was considered to be a passive, degenerative, and end-stage process of vascular disease. However, several observations have challenged this hypothesis and linked high serum phosphate level (i.e., higher than the normal adult range of 1.0 to 1.5 mmol/L) with the development of vascular calcification (110-112).

This study might partly explain these findings as lithium might decrease the phosphate concentration, vascular calcification and thereby increase the life span. Furthermore, these findings are in line with evidence which implicated that even low levels of lithium in drinking water may protect against suicide and promote longevity in humans (177-180). Moreover, recent studies demonstrate that long-term low-dose exposure to lithium may exert anti-aging capabilities and decrease mortality in distinct species (12).

The anti-depressant mechanism that may underline the observed effect of lithium on FGF23 in those patients might be the proper formation of synaptic connection, maturation and survival of catecholamine neurons and neurogenesis.
5.3 Limitations of the study

During this study I acquired a number of laboratory skills ranging from cell culture to RTQ-PCR, Western blot analysis and ELISA. Nevertheless, I encountered a number of limitations, the majority of which were dealt with through trial and error. For example, the validity of measuring the serum klotho level in the clinical samples using ELISA method. Since soluble α-klotho is being produced by extrarenal tissue. Moreover, the cross reactivity with other analytes cannot be excluded (181, 182).

One drawback of this study was the small sample size for each patient group; however collection and analysis of more samples would strengthen the validity of some of our current data. Furthermore, no placebo-treated group was observed, and the effects on FGF23 and \(1,25(OH)_2D_3\) were rather small in magnitude. However, small effects might cause changes when medications are used chronically.

Furthermore, the main limitation of lithium treatment is its narrow therapeutic index and requirement of close medical supervision. The debate about the risk of kidney damage is another concern which remained controversial. However, a recent 2-year randomized, placebo-controlled trial study demonstrated that chronic use of lithium at low doses did not affect renal function and was clinically safe (177).
5.4 Conclusion

In conclusion, I present conclusive evidence implicating the effect of lithium on FGF23 hormonal axis. As mentioned above, a short-duration lithium treatment was parallel with a significant increase in FGF23 serum level, decrease of serum $1,25(OH)_2D_3$ and phosphate concentration. In the murine experiments, these effects were paralleled by a statistically significant decrease of renal klotho expression, an increase in the renal GSK-3 phosphorylation and the renal phosphate excretion. The lithium treatment tends to decrease the serum calcium level in both experiments, an effect, however not reaching the statistical significance. These effects could be medically useful against vascular calcification and the appearance of age-related disorders.
6 Summary

Lithium is one of the oldest drugs used in Neuropsychopharmacology (183), and still remains a first-line therapy in bipolar mood disorders. Lithium is at least partially effective by inhibiting the glycogen synthase kinase 3 (GSK-3), which in turn regulates aquaporin 2 water channels via adenylate cyclase or prostaglandin-E2 (32, 184). Further, lithium treatment has been demonstrated as one of the most common causes of drug-induced nephrogenic diabetes insipidus (NDI), leading to renal water loss. Dehydration has in turn been shown to downregulate klotho, which is required as a co-receptor for fibroblast growth factor 23 (FGF23) (144, 145).

FGF23 is a potent inhibitor of aging. FGF23 deficiency is followed by increased levels of serum phosphate, calcium, and 1,25(OH)$_2$D$_3$ concentrations with subsequent vascular calcification, decrease of bone density and reduction of life span (118, 158, 185, 186). More recently, there is growing evidence suggesting that low-dose lithium treatment may exert anti-aging capabilities and promote longevity in humans (12, 178, 180).

The present study explored whether lithium treatment influences the renal expression and serum level of klotho, FGF23 serum concentration, 1,25(OH)$_2$D$_3$ formation, urinary excretion and serum concentration of phosphate and calcium.

To this end, 10 week-old female C57BL/6 mice were analysed after a 14-day period of treatment with NaCl or LiCl (200 mg/kg/day subcutaneously). Additionally, a total of 95 acute depressive patients were recruited who received lithium carbonate treatment as an augmenting agent for 4 weeks. All patients reached a lithium serum level of more than 0.4 mmol/l. Blood samples from those patients were drawn before and 4 weeks following lithium augmentation.

In the murine experiments, the serum ADH, FGF23, and 1,25(OH)$_2$D$_3$ concentrations were determined by ELISA. Renal klotho protein abundance and GSK-3 phosphorylation were analysed by Western blot and the urinary and serum phosphate and calcium concentration by photometry.
In the human experiments, the FGF23, soluble α-klotho, 1,25(OH)₂D₃, phosphate and calcium concentrations were measured in serum samples of acutely depressive patients before and 4 weeks after lithium augmentation.

Both lines of evidence from murine and human experiments revealed that lithium treatment resulted in a pronounced increase in the FGF23 serum level, and a significant decrease of serum 1,25(OH)₂D₃ and phosphate concentration. In the murine experiments, these effects were paralleled by a statistically significant decrease in the renal klotho expression, and increase in renal phosphate excretion. The lithium treatment tended to decrease the serum calcium level in mice.

In conclusion, this study presents conclusive evidence that lithium upregulates FGF23 formation, an effect paralleled by a substantial decrease of serum 1,25(OH)₂D₃, and phosphate concentrations. These effects could possibly counteract vascular calcification and the onset of age-related disorders. Our observation might partly explain the mechanism through which lithium improves survival and promotes longevity in humans.
Zusammenfassung


FGF23 unterdrückt effektiv den Alterungsprozess. Ein Mangel an FGF23 führt zu einer Erhöhung der Serumphosphat-, Kalzium- und 1,25(OH)2D3-Konzentrationen, gefolgt von vaskulärer Kalzifikation, Abnahme der Knochendichte und Erniedrigung der Lebenserwartung (118, 158, 185, 186). Seit kurzem gibt es vermehrt Hinweise darauf, dass die Behandlung mit niedrig dosiertem Lithium einen Anti-Aging Effekt ausübt und eine positive Auswirkung auf die Lebenserwartung beim Menschen zeigt (12, 178, 180).

Diese Studie beschäftigt sich mit der Frage, ob eine Lithium-Behandlung die renale Expression und den Serumspiegel von Klotho, die FGF23-Serumkonzentration, die 1,25(OH)2D3-Bildung, sowie die renale Ausscheidung und die Serumkonzentration von Phosphat- und Kalzium beeinflusst. Zu diesem Zweck wurden 10 Wochen alte C57BL/6 Mäuse (Weibchen) nach einer 14-tägigen Behandlungsperiode mit NaCl oder Lithium (LiCl) (200 mg/kg/d subkutan) untersucht. Darüber hinaus wurden 95 akut depressive Patienten rekrutiert, die eine Lithiumkarbonat-Behandlung als zusätzliche Therapie über einen Zeitraum von vier Wochen erhielten. Alle Patienten erreichten ein Serumlevel von mehr als 0.4mmol/l. Von diesen Patienten wurden Blutproben vor und nach der vierwöchigen Behandlung entnommen.
Im Mausexperiment wurden zudem die Serumkonzentrationen von ADH, FGF23 sowie 1,25(OH)₂D₃ mittels ELISA bestimmt. Mit Hilfe der Western Blot-Technik wurde die renale Klotho-Proteinmenge und die GSK-3 Phosphorylierung analysiert. Die Urin- und Serumkonzentrationen von Phosphat und Kalzium wurden photometrisch bestimmt.

Im Humanexperiment wurden die Serumkonzentrationen von FGF23, dem zirkulierenden α-Klotho, von 1,25(OH)₂D₃ sowie die Phosphat- und Kalziumkonzentrationen der Patienten vor der Lithiumbehandlung und vier Wochen nach der Lithiumbehandlung gemessen.


7 Reference list


8 Publications


Posters

• Annual meeting of the Germany Physiological Society “Regulation of mineral metabolism by lithium.” Dresden, Germany, 2012.

• Annual meeting of the Germany Physiological Society “Checkpoint kinase Chk2 controls renal Cyp27b1 expression, calcitriol formation, and calcium-phosphate metabolism.” Magdeburg, Germany, 2015.
9 Declaration

I hereby declare that this thesis is my own original work and it has been written by me in its entirety. I have faithfully and properly cited all sources used in the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institute.

The experimental work which incorporated in this thesis has been carried out in the Institute of Physiology I, University of Tübingen, Germany. The results presented in Figures 7, 10 and 15 have been generated by Mr. Abul Fajol, and Figures 8, 11 and 12 have been generated in collaboration with Mr. Pathare Ganesh. FGF23 transcript level in UMR106 osteoblast-like cells was measured by Ms. Zhang Bingbing.

Parts of this thesis have been published:


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