Beyond lipids and smoking

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Beyond lipids and smoking –
the nephrologist's perspective on
cardiovascular disease associated
with CKD

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**Beyond lipids and smoking –
the nephrologist's perspective on cardiovascular disease
associated with CKD**

Dissertation

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector magnificus Prof. Dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on Friday, September 6th at 10.00 hours

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1.SECTION:

General introduction

Overview on cardiovascular risk in chronic kidney disease

Chronic kidney disease (CKD) is a significant risk factor for the development of cardiovascular disease with a major impact on disease severity and can as such be considered equivalent to diabetes mellitus (1). This association is dose-dependent, meaning that already small declines in renal function, as assessed by subnormal levels of glomerular filtration rate (GFR) or the occurrence of albuminuria, increases the risk for cardiovascular events and mortality (1). Indeed, a patient with early CKD has a risk of developing cardiovascular disease (CVD) approximately twenty times higher than the risk to ultimately require renal replacement therapy (2). In regard to the most severe form of CKD, i.e. end-stage renal disease (ESRD), it has been convincingly shown that dialysis patients suffer from an exorbitantly high risk which brings likelihood of death in an 20-year-old ESRD patient within the same range as in “healthy” octogenarians (1;3). The reasons for the excessive cardiovascular morbidity and mortality in patients with CKD are manifold, and various risk factors in many divergent areas and disease conditions contribute to this risk (4) (Figure 1). Among the previously established risk factors, FGF23 excess/klotho deficiency as well as disturbances in vitamin K metabolism have gained a substantial amount of scientific and clinical interest.

Figure 1: Schematic overview of traditional and non-traditional cardiovascular risk factors; marked in red are the topics of the present thesis

These risk factors and predisposing conditions can be classified into several categories, e.g. disturbed mineral metabolism, deficiency of factors inhibiting ectopic (vascular) mineral deposition and chronic inflammatory states. Many of the uremia-associated cardiovascular risk factors belong to a syndrome known as chronic kidney disease – mineral and bone disorder (5). This syndrome nicely summarizes how different organs, such as the endocrine glands or the skeleton, contribute to the cardiovascular risk in CKD. Cardiovascular disease (CVD) in CKD patients differs in various ways from CVD in non-renal patient populations. These differences encompass the age of occurrence and speed of development as well as the clinical consequences. The vascular phenotype in CKD patients exhibits some specific features undetectable in the typical coronary artery disease patient with healthy kidneys. While a major proportion of the cardiovascular risk in patients without significant CKD can be attributed to the so-called traditional Framingham risk factors such as arterial hypertension and smoking, non-traditional risk factors are considered to be the major driving force behind cardiovascular risk in CKD patients (Figure 1). The complexity of CVD disease in CKD is in many ways reflected by the unsuccessful attempt to transfer well-established CVD-modifying drugs such as statins into the ESRD population, whereas statins have failed to reduce mortality in these patients (6).

Vascular calcification as a hallmark of CKD-associated cardiovascular disease

Accelerated and premature vascular calcification is one of the non-traditional Framingham cardiovascular risk factors among CKD patients (7). Vascular calcification in CKD is the final common pathway of various disturbances ranging from abnormalities in local cellular functions (8) to skeletal demineralization processes (9). These demineralization processes stimulate the deposition of calcium and phosphate in the vascular wall due to the inhibited mineral buffering capacities of bone (9). Vascular calcification is not only physico-chemical precipitation but represents a regulated and cell-mediated process as well (8). All anatomical cardiovascular structures are susceptible to the development of premature and accelerated calcifications in CKD and ESRD patients. Calcifications in arteries and heart valves, which ultimately directly impair cardiovascular function such as elasticity and valvular hemodynamics, are of specific clinical importance. A specific subtype of cardiovascular calcification is a rare disease coined calciphylaxis.

Calciphylaxis as an extreme variant of vascular calcification in CKD

Calciphylaxis (calcific uremic arteriolopathy, CUA) is a life-threatening disease which is increasingly acknowledged as a challenging condition at the interface of nephrology, dermatology and cardiology (10). Calciphylaxis predominantly occurs in ESRD patients. The primary CUA diagnosis is most often made in nephrology care units because the vast majority of affected cases are detected in patients undergoing chronic hemodialysis therapy. The typical clinical cascade starts with severe pain often in initially inconspicuous skin areas and may ultimately progress to deep tissue ulcerations (10). Figure 2 depicts prototypic

macroscopic skin findings at the lower thigh in a calciphylaxis patient. Clinically, the lesions were painful and discomfort started before lesions were visible.

Ulcer development is a severe complication with particularly high morbidity and mortality. Unfortunately, the nephrology community must lament a certain stagnancy in regard to the slow progress in our understanding of how and why CUA develops. Moreover, there are several important open issues regarding therapy that have yet to be successfully addressed (10). The pathohistological hallmarks of the disease are circumferential calcifications in small skin arterioles which contribute to endothelial shedding and luminal thrombosis (10).

Figure 2: Macroscopic appearance of calciphylaxis at the lower thigh.

A major risk factor for calciphylaxis is previous vitamin K antagonist treatment such as warfarin or phenprocoumon (11). Application of vitamin K antagonists apparently predisposes the vascular wall to the development of calcification (12;13). Vitamin K deficiency is increasingly acknowledged as a risk factor that can trigger the development of vascular calcification.

Collecting and analyzing data from calciphylaxis patients is difficult due to the rare and unpredictable occurrence in these patients. In countries where centralized data storage for all incident and prevalent dialysis patients is not compulsory (as in most European countries) alternative routes of data collection are required. A nation-wide and prospectively international internet-based registry approach is such an alternative (www.calciphylaxis.net) (14). One of the reasons EuCalNet was founded was to benefit the analysis of potential risk factors, among them VKA use which has become recongnized as a modifiable risk factor of major importance (10;15).

A major goal of the present thesis is to provide epidemiological, cross-sectional data derived from a national survey on calciphylaxis in Germany based on an internet-based registry (16). These data were in part integrated into a summarizing review which gives a clinically focused update on calciphylaxis. This update is intended to represent a state-of-the-art summary regarding etiology, pathogenesis, and clinical presentation. Moreover, therapeutic strategies (e.g. the potential benefits of VKA avoidance and vitamin K application, see below) were specifically reviewed (10).

Vitamin K and its implications for cardiovascular health

Vitamin K is best known as a purely hemostasiological co-factor required to activate clotting factors. However, it is also a key factor in the regulation of bone and soft tissue calcification based on the anti-calcific property of vitamin K due to its ability to activate matrix-Gla protein (MGP) (17). Comparable to other clotting factors, MGP needs to undergo posttranslational gamma-glutamylcarboxylation of specific protein-bound glutamate residues, for which vitamin K is an essential co-factor. Hence, these proteins are called vitamin K-dependent proteins (Gla-proteins) (18). Data from epidemiological studies have pointed to an important role of Vitamin K as a potential protective factor for cardiovascular health (19). Research investigating the role of vitamin K in vascular biology started some 20 years ago with a first description of the MGP knock-out animal model. Luo and coworkers were the first to show that MGP deficiency in mice resulted in vascular calcification at the elastic lamellae of the aortic wall starting as early as two weeks after birth (20). This deficiency had a deleterious consequence: MGP-deficient animals died prematurely due to rupture of the heavily calcified aorta and internal hemorrhage (20). The next step was initiated by Price and colleagues showing that the chemical inhibition of MGP function by the vitamin K-antagonist warfarin resulted in a similar calcification phenotype (21). Hence, application of warfarin mimicks to some extent the MGP knock-out phenotype. It is important to understand that vitamin K exists in two subtypes, vitamin K1 and K2. Both exhibit strong overlap in biological function but do have some specificities in their mode of action with K1 predominantly stabilizing hepatic synthesis of active clotting factors and K2 being responsible for peripheral MGP activation in the arterial wall. Rodent experiments with warfarin as inductor of vascular calcification require some vitamin K1 application in order to avoid fatal bleeding in the animals prior to the development of calcification (13). Mutagenesis of the Glu residues into aspartate residues indeed resembled the warfarin-induced calcification phenotype (22). These findings established MGP as an important inhibitor of vascular calcification, and more precisely, as a local calcification inhibitor (22). Noteworthy, the severe calcification phenotype induced by MGP deficiency or MGP inactivity could not be rescued by systemic, rather only by local arterial wall knock-in of MGP. From these data, a paradigm shift for vitamin K was induced. The sole function of vitamin K as coagulation cofactor was expanded by its novel function in protecting the vascular wall against calcification, hence contributing to vascular health (18).

Vitamin K antagonists as potential inducers of vascular calcification

Vitamin K deficiency can occur via two routes: insufficient oral supply and / or treatment with vitamin K antagonists. The first evidence for a link between nutritional vitamin K status and vascular health was provided by the Rotterdam study (19). In this study, dietary intake of phylloquinone (vitamin K-1) and menaquinone (vitamin K-2) was related to aortic calcification and coronary heart disease (CHD) in 4,807 elderly subjects recruited from the general population. Risk of incident CHD, all-cause mortality, and vascular pathology (aortic calcification) was studied in relation to vitamin K intake. Intake of vitamin K-2 was inversely related to both cardiovascular mortality and presence of aortic calcification. Interestingly, no such benefits could be detected with respect to different levels of vitamin K-1 intake. Since the Rotterdam study (19), several additional associative cohort studies have supported the hypothesis that insufficient dietary intake of vitamin K is associated with increased risk of cardiovascular calcification, whereas high dietary intake of vitamin K exerts cardiovascular protection. However, these studies cannot prove causality since there might be residual confounding – e.g. vitamin K intake might just be a reflector of overall healthy nutrition and life style including a high intake of leafy green vegetables. Spinach and broccoli are amongst the most important vitamin K suppliers in the Western diet.

However, as already discussed, these epidemiological data are supported by fundamental *in vitro* and *in vivo* work demonstrating that the use of vitamin K antagonists (VKA) for oral anticoagulation therapy (OAT) accelerates cardiovascular calcification (12;13). This has significant implications for human therapeutic strategies (23) since the application of VKA is still a common finding in general practice despite the availability of alternatives, i.e. the so-called non-vitamin K antagonists (NOAKs). In animals, it has been shown that vitamin K application can reverse VKA-induced calcification. In rats with warfarin-induced medial elastocalcinosis, high intake of vitamin K-1 and K-2 for 6 weeks reversed calcification, indicating that the process of vascular calcification is amendable through intervention (24). In line with these increasingly available experimental findings (25), several human non-interventional studies point towards an association between OAT with VKA and amount of vascular calcification, while NOAKs promise to be a safer alternative (23;25-27). Remarkably, VKA-induced ectopic calcification in humans was first described in tracheal cartilage (28). MGP also protects cartilage from ectopic, non-physiological calcification. Importantly, clinical associations between VKA use and calcification were described for both the arterial vessel wall and heart valves (29-31). Additionally, as mentioned above, VKA use is considered a major risk factor for the development of calciphylaxis (32) (Figure 2). Major implications for health care emerge, since some degree of cardiovascular calcifications in the elderly morbid population might actually arise from usage of VKA despite alternatives for OAT.

Several aspects regarding the association of vitamin K and protection against vascular calcification are under debate: First, a significant association between VKA use and cardiovascular calcification was not confirmed by all previous studies (33;34). Secondly, most studies do not allow true causality between VKA use and calcification to be determined since we cannot definitively exclude bias by indication. Thirdly, vascular calcification might also cause a disease condition requiring VKA use (reverse causality). In summary, while speculations about long-term VKA use (i.e. >12 months) as a causative factor for vascular calcification in patients are intriguing, true proof of concept in humans is still lacking. Indeed, the potential importance of VKA for anticoagulant treatment could certainly exceed arteriosclerosis development (i.e. vascular calcification in the medial layer of the vascular wall). It may also negatively affect plaque composition and therefore intimal atherosclerosis development (reviewed extensively in (35)). Warfarin use in ApoE-deficient mice provided experimental evidence that VKA-induced vascular deficiency resulted in a vulnerable plaque phenotype (36). Human randomized controlled studies are an adequate tool to close the gaps of knowledge:

Another major goal of the present work is to present data about the usefulness of vitamin K supplementation in the setting of calcific aortic stenosis in which a slower progression rate of valvular calcification was the primary endpoint (37).

The issue of accelerated cardiovascular calcification by VKA compared to NOAK application is covered by an ongoing trial that will present data from an interim analysis in 2019 (23).

Left ventricular hypertrophy in CKD

Besides vascular calcification, another cardiovascular pathology, actually myocardial pathology, is a hallmark of CKD patients, i.e. left-ventricular hypertrophy (LVH) (38). The pathophysiology of CKD-associated LVH is complex and multifactorial, and LVH occurs even in the absence of severe and long-standing uncontrolled arterial hypertension or aortic valvular disease in CKD patients. Instead, increased vascular stiffness (which relates to a significant extent to vascular calcification), anemia, hypervolemia, activation of the renin-angiotensin and sympathetic nervous system as well as direct toxic effects of uremia-associated circulating factors (uremic toxins) also contribute to the development of LVH in the setting of CKD (38). Among the latter, fibroblast growth factor 23 (FGF23) is a candidate that is currently undergoing intense debate.

Fibroblast growth factor 23 (FGF23)

An important milestone in our understanding of cardiovascular disease in CKD and in determining how skeletal and mineral disturbances relate to this increased risk was the discovery of the profound metabolic, specifically mineral effects of FGF23 (39). FGF23 is a 32kDa bone-derived potent regulator of vitamin D and phosphorus metabolism. Circulating FGF23 is mainly produced by osteocytes and osteoblasts. FGF23 primarily targets the renal tubular and parathyroid cells (40). These canonical effects of FGF23 (i.e. regulating phosphate, vitamin D metabolism and PTH) rely upon its interaction with FGF23 receptors and its obligate renal co-receptor alpha-klotho. In renal tubular cells, upon binding to the FGF-receptor and alpha-klotho, FGF23 (1) stimulates the excretion of phosphate, (2) reduces the activation of calcidiol to calcitriol and (3) increases the degradation of the latter (40;41).

FGF23 was originally identified less than 20 years ago as phosphaturic hormone in renal phosphate wasting syndromes such as oncogenic osteomalacia (42). Shortly thereafter, it was found that plasma concentration of FGF23 rises dramatically with increasing severity of CKD (43), and moreover, it is independently associated with poorer outcome among non-dialysis CKD patients as well as dialysis patients (44). Soon, FGF23 left the nephrology niche and proved to be of particular interest also for the cardiology community, since the dismal association between high FGF23 levels and poor prognosis is also detectable in patients selected primarily via cardiac disorders (45). Abundant cohort studies followed, all pointing towards the same direction – a morbid association between elevated FGF23 levels and fatal outcome (mortality and morbidity) (46). The next evolutionary step in our FGF23 understanding was the transfer from association towards causality. In addition to the above-mentioned epidemiological and associative studies, recent experimental data have established a causal pathway and linked extra-cardiac FGF-23 directly to the development of cardiovascular pathologies, specifically cardiomyopathy (47). However, even this point of view is again moving forward as evidence grows that the cardiovascular system itself may be able to modulate and even influence FGF23 levels with potential, yet to be determined, local and systemic effects (48). Much of the previous FGF23-related work in cohort studies is based on the assumption that the predictive power of FGF23 is comparable between the two forms, intact (iFGF23) and c-terminal FGF23 (c-term FGF23) (44).

We sought to investigate whether c-term FGF23 can independently predict outcome (cardiovascular events and mortality) in a cohort of patients included by virtue of the need to undergo coronary angiography due to a recent onset of acute coronary syndrome (LURIC cohort) (49).

Much less data is available as to whether a comparable association exists between the circulating part of the obligate FGF23 coreceptor klotho (soluble klotho) and outcome. Hence, we analyzed the same cohort also with regard to s-klotho (50).

FGF23 with specific focus upon iron metabolism and anemia

Another interesting aspect of FGF23 is the close association of anemia, inflammation and iron deficiency with substantially increasing levels of FGF23, especially the c-term FGF23 form (51;52). An increasing level of evidence points towards the fact that FGF23 production and release are not only dependent on phosphate intake and serum phosphate levels (53;54), but also upon systemic inflammation, anemia and hypoxia.

Of particular interest, and also of practical importance, is the association between anemia and FGF23. Iron deficiency and anemia associate with high levels of FGF23 in both directions meaning that anemia causes FGF23 increases and high levels of FGF23 aggravate anemia (54-56). While anemia correction and iron replenishment lower FGF23 levels in the long run, very recently a novel disease entity came to the attention of physicians: i.v. iron-associated osteomalacia (57). Apparently, some i.v. formulations cause a transient sharp rise in intact FGF23 levels which cause renal phosphate wasting, hypophosphatemia and finally, in some at risk patients, induce osteomalacia with an increased risk for Looser's fractures (58). The latter complication was described after repetitive usage over several months to years of i.v. iron. This potential side effect of i.v. iron upon phosphate metabolism has direct clinical implications since iron replenishment is increasingly recommended for various disease conditions with heart failure as a prominent example (59). However, uncertainties exist about the true dimension of the problem and the exact etiology since detailed investigations of FGF23 metabolism shortly after i.v. iron administration have not been performed. Moreover, i.v. iron-associated osteomalacia has not yet been investigated in heart failure patients (rather predominantly in patients with underlying chronic bowel disease (57)) despite the fact that heart failure patients are at risk for osteoporosis which might further increase with widespread usage of i.v. iron (60).

Hence, we identified an unsolved question concerning the issue if and why i.v. iron in heart failure patients could induce hypophosphatemia, and we sought to investigate the exact role of FGF23 metabolism in this setting (61).

Aim of this thesis

As shown above, FGF23 excess and vitamin K deficiency are prototypic examples of factors representing the so-called bone-heart-kidney axis. This term describes a condition in which various organs interact and multiply the deleterious effects induced by reduced kidney function. The major aim of the research summarized in the present thesis is an in-depth description of how FGF23 excess and vitamin K deficiency contribute to cardiovascular risk in specific patient cohorts. Importantly, the underlying work addresses not only descriptive data but also focuses upon potential therapeutic influence, i.e. can FGF23 levels be modified and how does vitamin K replenishment influence vascular calcification processes.

The specific focus of this thesis was addressed in the following workpackages:

- Section A, Chapter 1 presents a cross-sectional description of data on calciphylaxis (incidence, clinical presentation, potential risk factors) in Germany with a focus on the association between calciphylaxis and VKA usage.
- Section A, Chapter 2 is a state-of-the-art review about calciphylaxis.
- Section A, Chapter 3 summarizes data from a human pilot interventional study regarding the efficacy of vitamin K application to slow the progression of valvular calcification.
- Section B, Chapter 1 and 2 show results from an investigation of the association between circulating FGF23 levels as well as soluble klotho levels and the outcome in an observational prospective cohort study (LURIC) including patients with acute coronary syndrome.
- Section B, Chapter 3 presents data on the impact of iron replenishment upon FGF23 metabolism in heart failure patients in order to gain information about the threat of hypophosphatemia development.

2.SECTION:
Fibroblast growth factor 23 (FGF23) and
cardiovascular risk

a)

Fibroblast growth factor 23 (FGF23) and mortality: the Ludwigshafen Risk and Cardiovascular Health Study (IF 4)

Fibroblast growth factor 23 (FGF23) and mortality: the Ludwigshafen Risk and Cardiovascular Health Study.

Brandenburg VM, Kleber ME, Vervloet MG, Tomaschitz A, Pilz S, Stojakovic T, Delgado G, Grammer TB, Marx N, März W, Scharnagl H. Atherosclerosis. 2014 Nov; 237 (1): 53-9.

Abstract

Background: Fibroblast growth factor 23 (FGF23) is an important regulatory hormone in phosphate and vitamin D metabolism. Here, we investigated the associations of FGF23 with traditional cardiovascular risk factors and with bone metabolism parameters as well as the impact of FGF23 upon long-term mortality in a large cohort of patients referred for coronary angiography.

Methods: We examined whether c-term FGF23 concentrations at baseline were associated with cardiovascular and total mortality in 2974 patients from the Ludwigshafen Risk and Cardiovascular Health Study (LURIC). We investigated if these associations were independent from established cardiovascular risk factors as well as from other mineral regulating factors and bone biomarkers such as calcium, parathyroid hormone (PTH), alkaline phosphatase (AP), vitamin D, and serum phosphate.

Results: Mean age of participants was 63 ± 10 years; median c-term FGF23 serum levels were 54 (40 - 78) RU/ml. During a median follow-up of 9.9 years, 884 deaths (30%) occurred, 545 (18%) of which were cardiovascular. FGF23 significantly and inversely correlated with eGFR. AP, phosphate, and PTH increased in parallel with quartiles of FGF23. Age- and sex-adjusted hazard ratios (HRs) in the fourth quartile compared to the first quartile of FGF23 were 2.54 (95%CI, 2.09 - 3.09; $p < 0.001$) for all cause and 2.56 (95% CI, 1.99 - 3.28; $p < 0.001$) for cardiovascular mortality. These associations remained significant after additional adjustments for cardiovascular risk factors and bone biomarkers (calcium, PTH, AP, vitamin D, and phosphate): Adjusted HRs were 1.38 (95%CI, 1.26 - 1.52; $p < 0.001$) for all-cause and 1.35 (95%CI, 1.20 - 1.52; $p < 0.001$) for cardiovascular mortality for each increase by one standard deviation of c-term FGF23.

Conclusions: In patients undergoing coronary angiography baseline c-term FGF23 levels predict the risk for all-cause and cardiovascular mortality over 9.9 years of follow-up. These associations were independent of established cardiovascular risk factors and serum phosphate.

1. Introduction

Fibroblast growth factor 23 (FGF23) was initially described as a hormone synthesized by osteocytes with stimulating effects upon renal phosphate excretion and down-regulation of 1.25 dihydroxyvitamin D synthesis.¹ However, FGF23 activities reach beyond mineral metabolism. Both increased FGF23 concentrations, elevated serum phosphate, and low vitamin D negatively influence cardiovascular health in various patient populations.^{2,3} While the negative effects of high serum phosphate and high FGF23 were originally detected in patients with severely impaired renal function,^{4,5} more recent data also point to dismal effects of elevated serum phosphate and FGF23 in cohorts without overt renal insufficiency.^{6,7} In the Heart and Soul study patients in the highest FGF23 tertile revealed a hazard ratio of 2.07 (95% CI, 1.36e3.13) for overall mortality during prospective follow-up as compared to patients in the lowest FGF23 tertile.⁷ The vast majority of previously published data confirm an independent risk of high FGF23 levels for increased cardiovascular morbidity, events and mortality effects in the investigated cohorts.⁷⁻¹³ This is of particular interest given the fact that FGF23 helps controlling serum phosphate levels which per se contribute to cardiovascular risk.² Since FGF23 is in the center of complex bone and mineral regulatory systems such as phosphate, alkaline phosphatase, vitamin D or PTH careful adjustments need to be made for possible confounding. Moreover, it is incompletely understood how the association between FGF23 concentrations and outcome is influenced by the background cardiovascular risk and might be different between population-based cohorts compared to those selected for elevated cardiovascular risk or previous events. In the present study we analyzed data from the LURIC (the Ludwigshafen Risk and Cardiovascular Health Study) study including long-term outcome in patients who had been referred for coronary angiography and underwent long-term follow-up. Thorough adjustments for confounders were performed in order to study the independent role of FGF23 in cardiovascular events in this population at intermediate to high risk.

2. Methods

2.1. Participants, study design, and definition of comorbidities

The baseline examination of the LURIC study was performed between 1997 and 2000 at a coronary care tertiary referral center (Herzzentrum Ludwigshafen). Overall 3316 patients with coronary angiogram at baseline were included. All patients were Caucasians and were required to be in clinical stable conditions except for acute coronary syndromes. Indications for coronary angiogram were cardiac chest pain, acute coronary syndrome or cardiac ischemia as suspected with non-invasive testing. The patients gave written and informed consent. A diagnosis of coronary artery disease (CAD) was established if at least one stenosis of at least one out of 15 coronary arterial segments according to visual estimation of the maximal luminal narrowing $\geq 20\%$ was present. The severity of CAD was quantified with the Friesinger score.¹⁴ Acute myocardial infarction (MI) was defined as a MI that had occurred within the four weeks prior to enrollment. A definite STsegment elevation MI (STEMI) was diagnosed if typical ECG changes were present along with prolonged chest pain, refractory to sublingual nitrates and/or enzyme or troponin T

elevations (>0.1 mg/L). Non-ST elevation MI (NSTEMI) was diagnosed, if symptoms and/or troponin T criteria weremet without STEMI ECG criteria. Diabetes mellitus was diagnosed by the previous prescription of antidiabetic medication, or fasting blood glucose levels greater than 7.0 mmol/l, or if HbA1c was $> 6.5\%$. Arterial hypertension was present if the mean blood pressure in one out of five measurements exceeded either 140 mm Hg systolic or 90 mmHg diastolic.

Alternatively, current prescription of antihypertensive drugs confirmed the diagnosis of arterial hypertension. Details about participants, recruitment, comorbidities and baseline examinations have been previously described.¹⁵

2.2. Outcome assessment during follow-up

FGF23, lipid, and vitamin D measurements were available in 2974 individuals with coronary angiograms (90% of the entire cohort). During a median follow-up time of 9.9 years 884 (30%) of the patients died. No patient was lost to follow-up. Death certificates were missing in 18 decedents who were included in the analysis for total mortality, but excluded from analysis of cardiovascular mortality. Two physicians without knowledge of FGF23 levels and baseline characteristics of the study participants classified causes of death by reviewing hospital records and death certificates. Cardiovascular deaths included sudden cardiac death (SCD), fatal myocardial infarction, deaths due to heart failure, death after intervention to treat CAD, stroke, and other deaths due to heart disease. SCD was defined as a sudden unexpected death either within 1 h of symptom onset or within 24 h of having been observed alive and symptom free.

2.3. Laboratory analyses

Standard laboratory measurements were performed as previously described.¹⁵ In brief, venous blood was drawn in the morning before coronary angiography and standard laboratory parameters were immediately determined. Serum and plasma was harvested according to standard procedures and was snap frozen for further determinations and stored at -80°C until further analysis. FGF23 (c-term) was measured by enzyme-linked immunosorbent assay (ELISA, Immundiagnostik AG, Bensheim, Germany), intra- and inter-assay coefficient of variation were 2.4 and 3.1%, respectively. Intact PTH, and N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) were determined by Electro-ChemiLuminescence (ECL) on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). Serum concentrations of 25-hydroxyvitamin D were measured by a radioimmunoassay (Dia-Sorin Antony, France; Stillwater, USA) with an intra- and inter-assay coefficient of variation of 8.6 and 9.2%, respectively. 1.25-dihydroxyvitamin D was measured by radioimmunoassay (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) using a multicrystal counter (Berthold LB2014, Dia- Sorin, SA, USA). Estimated glomerular filtration rate (eGFR) was calculated according to the CKD-EPI study equation.^{16,17}

2.4. Statistical analysis

FGF23 concentration quartiles were generated according to the values of the entire study cohort. Baseline characteristics were analyzed by FGF23 quartiles. Depending on their distribution, continuous parameters are either presented as means $\pm$ standard deviation (normally distributed variables) or as medians with interquartile range (skewed variables).

Categorical data are shown as proportions. All skewed continuous parameters were logarithmically transformed before use in parametric procedures. Comparisons across FGF quartiles were carried out by analysis of variance (ANOVA) with p for trend for continuous parameters and by X²-test with p for linear-by-linear test for categorical variables. For all-cause and cardiovascular mortality, Kaplan-Meier curves followed by a log-rank test were calculated to show the differences in event-free survival between FGF23 quartiles. Hazard ratios (HRs) with 95% confidence intervals (95% CI) for all-cause and cardiovascular mortality were calculated with Cox proportional hazard models both according to quartiles of FGF23 and per increase of 1 SD. To test for interaction of FGF23 with BMI and eGFR we used Cox proportional hazard models allowing for interaction. Finally, the predictive value of multivariate models with and without FGF23 for total and cardiovascular mortality was calculated using receiver operating characteristic (ROC) curves, net reclassification improvement (NRI) and integrated discrimination improvement (IDI). A p-value below 0.05 was considered statistically significant and all statistical tests were two-sided. Data were analyzed using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient characteristics

A total of 2974 patients with available FGF23, vitamin D, and lipid levels at baseline were included in the present cohort study (68.7% male, mean age 63 ± 10 years). 648 patients (21.8%) were free of coronary artery disease. Reduced renal function (defined as an eGFR < 60 ml/min) was present in 405 patients (13.6%). Details regarding patients' characteristics, baseline demographic data and laboratory data of the entire cohort are listed in Table 1. During a median follow-up of 9.9 years 884 (30%) patients had died. Among these 545 (18%) deaths were due to cardiovascular reasons. Cardiovascular risk factors, demographic data, and laboratory data were compared between patients with GFR <60 ml/min and those with GFR > 60 ml/min (Table 1).

3.2. Associations between FGF23 levels and cardiovascular risk factors and cardiovascular status and markers of mineral and bone metabolism

Between group comparisons revealed the following statistically significant differences in FGF23 levels between patient subgroups (Table 2): Higher FGF23 levels were detected in female patients compared to male, younger patients compared to elderly patients, those without lipid-lowering drugs compared to those with such medication, those with diabetes compared to non-diabetics, and smokers compared to non-smokers. FGF23 rose constantly in parallel to decreasing renal function (Table 2). There was an almost linear decrease of FGF23 levels detectable with increasing quartiles of HDL-cholesterol, LDL-cholesterol, and triglycerides. We examined markers of mineral and bone metabolism according to quartiles of FGF23 concentrations (first quartile: n = 742, <40.2 RU/ml; second quartile: n = 743, 40.2 - 54.1 RU/ml; third quartile: n = 745, 54.2 - 78.0 RU/ml; fourth quartile: n = 743, >78 RU/ml) (Table 3). Levels of AP, phosphate, and PTH showed linear and clinically meaningful increases with increasing quartiles of FGF23. 1,25-dihydroxyvitamin D levels constantly decreased from the lowest to the highest FGF23 quartile (Table 3).

	All individuals (n = 2974)	eGFR ≥ 60 ml/min/1.73 m ² (n = 2569)	eGFR < 60 ml/min/1.73 m ² (n = 405)	P ^a
Age, years, means $\pm$ SD	63 $\pm$ 10	62 $\pm$ 10	71 $\pm$ 8	<0.001
Male sex, %	69	70	58	0.004 ^b
Lipid-lowering drugs, %	49	49	49	0.864
Body mass index, kg/m ² , means $\pm$ SD	27 $\pm$ 4	27 $\pm$ 4	28 $\pm$ 4	0.193
Diabetes mellitus, %	40	37	56	<0.001
Hypertension, %	73	71	84	0.067
Smoking, %				
Never	37	36	43	
Past	44	44	46	
Current	19	21	11	0.096
Previous myocardial infarction, %	42	40	51	0.235
Peripheral vascular disease, %	9	9	13	<0.001
Cerebrovascular disease, %	9	8	18	0.025
Systolic blood pressure, mm Hg, means $\pm$ SD	141 $\pm$ 24	141 $\pm$ 23	145 $\pm$ 26	<0.001
Diastolic blood pressure, mm Hg, means $\pm$ SD	81 $\pm$ 11	81 $\pm$ 11	79 $\pm$ 11	0.103 ^c
Fasting blood glucose (mg/L), means $\pm$ SD	113 $\pm$ 35	112 $\pm$ 34	119 $\pm$ 43	<0.001 ^c
NT-pro-BNP, ng/L	296	247	1157	0.056
Median (25th and 75th percentile)	(108–882)	(96–676)	(459–2612)	<0.001 ^d
LDL cholesterol, mg/dl, means $\pm$ SD	116 $\pm$ 34	117 $\pm$ 34	111 $\pm$ 34	
HDL cholesterol, mg/dl, means $\pm$ SD	39 $\pm$ 11	39 $\pm$ 11	37 $\pm$ 11	<0.001 ^e
Triglycerides, mg/dl	144	143	160	<0.001 ^{d,e}
Median (25th and 75th percentile)	(107–197)	(107–197)	(120–210)	
FGF-23, RU/ml	54	51	96	<0.001 ^d
Median (25th and 75th percentile)	(40–78)	(39–70)	(64–159)	
Alkaline phosphatase, U/L, means $\pm$ SD	122 $\pm$ 46	119 $\pm$ 42	139 $\pm$ 63	<0.001
Calcium, mmol/L, means $\pm$ SD	2.33 $\pm$ 0.11	2.33 $\pm$ 0.10	2.33 $\pm$ 0.13	0.539
Phosphate, mg/dl, means $\pm$ SD	3.53 $\pm$ 0.57	3.51 $\pm$ 0.53	3.69 $\pm$ 0.77	<0.001
Parathyroid hormone, pg/mL, means $\pm$ SD	34 $\pm$ 29	31 $\pm$ 14	54 $\pm$ 67	<0.001
25-Hydroxyvitamin D, μ g/L, means $\pm$ SD	17.4 $\pm$ 9.7	17.9 $\pm$ 9.7	14.7 $\pm$ 9.3	<0.001
1,25-Dihydroxyvitamin D, ng/L, means $\pm$ SD	35.2 $\pm$ 14.0	36.8 $\pm$ 13.7	25.0 $\pm$ 11.2	<0.001

^a Comparison between individuals with high and low eGFR; analysis of variance or logistic regression, respectively, adjusted for age and gender.

^b Logistic regression, adjusted for age only.

^c Adjusted for use of beta blockers, ACE inhibitors, AT1 receptor antagonists, calcium channel blockers, diuretics and lipid-lowering agents.

^d ANOVA of logarithmically transformed values.

^e Adjusted for use of lipid-lowering agents.

Table 1: Clinical and biochemical characteristics of study participants according to eGFR

	<i>n</i>	FGF-23 (ng/mL) ^a	Difference (%) ^b	<i>P</i> ^b
Gender				
Men	2042	59.3 (57.6–61.1)		
Women	932	63.1 (60.3–66.)	+6.4	0.028
Age (years)				
<60	1079	66.0 (63.2–68.9)		
60–70	1085	57.6 (55.4–59.9)	–12.6	<0.001
>70	810	57.5 (54.7–60.3)	–12.9	<0.001
Lipid-lowering drugs				
No	1508	62.1 (60.0–64.3)		
Yes	1466	58.9 (56.8–60.9)	–5.3	0.033
Coronary artery disease				
None	648	60.2 (57.0–63.6)		
Stable CAD	1380	61.3 (59.2–63.4)	+1.8	0.596
Unstable CAD (Troponin T-)	573	60.0 (56.9–63.2)	–0.4	0.923
Unstable CAD (Troponin T+)	373	58.7 (54.9–62.9)	–2.5	0.595
Friesinger Score				
1st quartile (0–1)	614	57.8 (54.2–61.7)		
2nd quartile (2–4)	675	62.5 (59.6–65.6)	+8.1	0.054
3rd quartile (5–8)	976	60.0 (57.5–62.6)	+3.8	0.390
4th quartile (9–15)	709	61.6 (58.6–64.8)	+6.6	0.171
Body mass index (kg/m ²)				
<26 or 27 ^c	1400	61.8 (59.7–63.9)		
>26 or 27 ^c	1574	59.3 (57.5–61.3)	–4.0	0.092
Type 2 Diabetes mellitus				
None	1799	57.7 (55.9–59.4)		
Diabetes mellitus	1175	65.0 (62.6–67.6)	+12.7	<0.001
Hypertension				
No	807	61.9 (59.1–64.9)		
Yes	2167	59.9 (58.3–61.6)	–3.2	0.252
Smoking				
Never	1089	56.9 (54.7–59.3)		
Former	1311	61.6 (59.4–63.9)	+8.2	0.006
Current	574	64.8 (61.3–68.6)	+13.9	<0.001
eGFR (ml/min/1.73 m ²) ^d				
>60	2569	55.1 (53.7–56.5)		
30–59	359	94.3 (88.0–101.2)	71.3	<0.001
<30	46	337.6 (279.5–407.9)	512.9	<0.001
LDL cholesterol (mg/dL)				
1st quartile (<94)		64.3 (61.3–67.6)		
2nd quartile (94–113)		59.1 (56.4–61.9)	–8.1	0.013
3rd quartile (114–137)		60.0 (57.3–62.9)	–6.7	0.045
4th quartile (>138)		58.7 (56.0–61.5)	–8.8	0.009

	<i>n</i>	FGF-23 (ng/mL) ^a	Difference (%) ^b	<i>P</i> ^b
HDL cholesterol (mg/dL)				
1st quartile (<32)		66.0 (62.9–69.3)		
2nd quartile (32–37)		61.4 (58.7–64.3)	–6.9	0.033
3rd quartile (38–44)		58.7 (56.0–61.6)	–11.0	0.001
4th quartile (>44)		56.2 (53.5–59.0)	–14.9	<0.001
Triglycerides (mg/dL)				
1st quartile (<109)		64.0 (61.0–67.2)		
2nd quartile (109–145)		59.7 (57.1–62.6)	–6.7	0.039
3rd quartile (146–199)		58.7 (56.0–61.5)	–8.2	0.035
4th quartile (>199)		59.6 (56.8–62.6)	–6.9	0.036

^a Estimated marginal means and 95% confidence intervals obtained in a general linear model (ANOVA), adjusted for each of the other factors, whereby age, body mass index, eGFR, LDL cholesterol, HDL cholesterol and triglycerides (log transformed), were included as continuous rather than categorical covariables.

^b Compared to the first category of each variable.

^c Thresholds of 26 and 27 kg/m² apply to males and females, respectively.

^d eGFR was calculated on the basis of the equation of the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration).

Table 2: Association of FGF-23 with cardiovascular risk factors and coronary artery disease

	FGF-23 (RU/mL)			P ^a
	<40.2 n = 743	40.2-54.1 n = 743	54.2-78.0 n = 745	>78.0 n = 743
Alkaline phosphatase (U/L)	113 ± 35	115 ± 38	120 ± 39	138 ± 62
Calcium (mmol/L)	2.32 ± 0.10	2.33 ± 0.10	2.34 ± 0.11	2.34 ± 0.12
Phosphate (mg/dL)	3.39 ± 0.52	3.46 ± 0.52	3.59 ± 0.53	3.68 ± 0.67
Parathyroid hormone (pg/mL)	29 ± 13	31 ± 14	32 ± 16	43 ± 51
25-Hydroxyvitamin D (µg/L)	18.4 ± 8.9	17.8 ± 9.1	17.5 ± 9.1	16.1 ± 11.4
1,25-Dihydroxyvitamin D (ng/L)	39.9 ± 13.8	37.3 ± 13.4	33.9 ± 13.6	29.5 ± 13.1
Values are given as mean ± SD.				
^a ANOVA, adjusted for age and sex.				

Table 3: Association of FGF-23 with characteristics of bone and kidney metabolism

3.3. Associations between FGF23 levels and cardiovascular or allcause mortality

The hazard ratios for all cause mortality increased with increasing quartiles of FGF23 concentration (Table 4A). Compared to the lowest quartile, the unadjusted hazard ratios (HRs) for death from all causes in the second, third, and fourth quartile were 1.20 (95% CI: 0.96 - 1.50), 1.53 (95% CI: 1.23 - 1.89), and 3.22 (95% CI: 2.66 - 3.91), respectively (Table 4A, model 1). In the multiple adjusted model (including cardiovascular risk factors, phosphate, AP, calcium, PTH, and 25-hydroxyvitamin D) the HR in the highest compared to the lowest FGF23 quartile was 1.72 (95% CI: 1.38 - 2.14). An increase of one SD in FGF23 concentration was associated with a HR for all-cause and cardiovascular mortality of 1.38 (95% CI: 1.26 - 1.52) and 1.35 (1.20 - 1.52), respectively, in the multiple adjusted model. We obtained similar results for the association of FGF23 with cardiovascular mortality (Table 4B). HRs per increase of one SD were 1.84 (95% CI: 1.69 - 1.99) in the unadjusted model and 1.35 (95% CI: 1.20 - 1.52) in the multiple adjusted model, respectively (Table 4B, model 1 and model 5). Additional inclusion of NTproBNP as covariate only slightly decreased the HRs for both, total and cardiovascular mortality (Table 4A and B, model 6). Figure 1 shows the KaplanMeier curve analyses for total (left panel) and cardiovascular mortality (right panel) according to quartiles of FGF-23. Increasing FGF23 concentration was significantly associated with increased death from all causes (log rank $p < 0.001$) and cardiovascular causes (log rank $p < 0.001$) during a median follow-up period of 9.9 years. We analyzed the effect of the interaction of BMI and FGF23 on clinical outcomes (total and cardiovascular mortality). Cox proportional hazard model allowing for interaction of these parameters revealed that the interaction was not significant. The significant association between increasing FGF23 concentration expressed as quartiles or as increasing SD was not substantially modified by renal function, i.e. eGFR. Table 5 (Suppl. Material) indicates that HRs for all cause and cardiovascular mortality were similar after stratifying patients at an eGFR threshold of 60 ml/min. Further, statistical models allowing for interaction of FGF23 and eGFR did not reveal any significant interaction.

FGF23 (RU/ml)	Deaths n (%)	Model 1 HR (95% CI)	P	Model 2 HR (95% CI)	P	Model 3 HR (95% CI)	P	Model 4 HR (95% CI)	P	Model 5 HR (95% CI)	P	Model 6 HR (95% CI)	P
A.													
All individuals (n = 2974)													
1st quartile (<40)	143 (19)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	167 (23)	1.20 (0.96–1.50)	0.107	1.11 (0.88–1.38)	0.382	1.04 (0.83–1.31)	0.705	1.04 (0.83–1.31)	0.712	1.04 (0.83–1.32)	0.712	1.10 (0.86–1.38)	0.420
3rd quartile (55–78)	208 (28)	1.53 (1.23–1.89)	<0.001	1.33 (1.08–1.65)	0.008	1.14 (0.92–1.41)	0.245	1.11 (0.89–1.38)	0.337	1.15 (0.92–1.43)	0.236	1.16 (0.93–1.45)	0.179
4th quartile (>78)	366 (49)	3.22 (2.66–3.91)	<0.001	2.54 (2.09–3.09)	<0.001	1.82 (1.48–2.25)	<0.001	1.79 (1.45–2.21)	<0.001	1.72 (1.38–2.14)	<0.001	1.68 (1.36–2.08)	<0.001
1 SD		1.81 (1.69–1.94)	<0.001	1.71 (1.59–1.84)	<0.001	1.48 (1.36–1.62)	<0.001	1.46 (1.34–1.60)	<0.001	1.38 (1.26–1.52)	<0.001	1.34 (1.23–1.46)	<0.001
B.													
All individuals (n = 2956)													
1st quartile (<40)	88 (12)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	99 (13)	1.16 (0.87–1.54)	0.323	1.06 (0.80–1.42)	0.683	0.99 (0.74–1.32)	0.991	0.98 (0.74–1.31)	0.983	0.96 (0.72–1.30)	0.805	1.06 (0.80–1.43)	0.676
3rd quartile (55–78)	130 (18)	1.54 (1.18–2.02)	0.002	1.35 (1.03–1.77)	0.031	1.10 (0.84–1.45)	0.495	1.07 (0.81–1.41)	0.651	1.09 (0.82–1.44)	0.557	1.14 (0.87–1.51)	0.345
4th quartile (>78)	228 (31)	3.24 (2.53–4.15)	<0.001	2.56 (1.99–3.28)	<0.001	1.71 (1.31–2.24)	<0.001	1.66 (1.27–2.17)	<0.001	1.57 (1.19–2.07)	0.002	1.56 (1.19–2.05)	0.001
1 SD		1.84 (1.69–1.99)	<0.001	1.74 (1.59–1.90)	<0.001	1.47 (1.32–1.63)	<0.001	1.44 (1.29–1.61)	<0.001	1.35 (1.20–1.52)	<0.001	1.28 (1.15–1.43)	<0.001
Model 1: Unadjusted.													
Model 2: Adjusted for age and gender.													
Model 3: In addition adjusted for coronary artery disease (none, stable CAD, unstable CAD, NSTEMI, STEMI), body mass index, Type 2 diabetes, hypertension, smoking status, glomerular filtration rate (CKD-EPI), use of lipid-lowering drugs, LDL-C, HDL-C, and triglycerides.													
Model 4: model 3 additionally adjusted for phosphate.													
Model 5: model 4 additionally adjusted for alkaline phosphatase, calcium, parathyroid hormone, 25-hydroxyvitamin D.													
Model 6: model 5 additionally adjusted for NTPROBNP.													

Table 4A and 4B: A. Hazard ratios (HR) for death from all causes according to FGF23; B. Hazard ratios (HR) for death from cardiovascular causes according to FGF-23

Total mortality	all individuals (n = 2974)		eGFR $\geq$ 60 ml/min/1.73 m ² (n = 2569)		eGFR < 60 ml/min/1.73 m ² (n = 405)	
Deaths, n (%)	884 (30)		640 (25)		244 (60)	
	HR per 1SD	P	HR per 1SD	P	HR per 1SD	P
Model 1	1.81 (1.69 - 1.94)	<0.001	1.67 (1.51 - 1.85)	<0.001	1.47 (1.31 - 1.65)	<0.001
Model 2	1.71 (1.59 - 1.84)	<0.001	1.59 (1.43 - 1.77)	<0.001	1.55 (1.38 - 1.74)	<0.001
Model 3	1.48 (1.36 - 1.62)	<0.001	1.48 (1.32 - 1.66)	<0.001	1.37 (1.19 - 1.59)	<0.001
Model 4	1.46 (1.34 - 1.60)	<0.001	1.47 (1.30 - 1.64)	<0.001	1.42 (1.22 - 1.65)	<0.001
Model 5	1.38 (1.26 - 1.52)	<0.001	1.40 (1.25 - 1.58)	<0.001	1.37 (1.17 - 1.61)	<0.001
Cardiovascular mortality	all individuals (n = 2956)		eGFR $\geq$ 60 ml/min/1.73 m ² (n = 2556)		eGFR < 60 ml/min/1.73 m ²	
Deaths, n (%)	545 (18)		383 (15)		162 (40)	
	HR per 1SD	P	HR per 1SD	P	HR per 1SD	P
Model 1	1.84 (1.69 - 1.99)	<0.001	1.69 (1.48 - 1.92)	<0.001	1.47 (1.27 - 1.69)	<0.001
Model 2	1.74 (1.59 - 1.90)	<0.001	1.62 (1.41 - 1.86)	<0.001	1.53 (1.33 - 1.76)	<0.001
Model 3	1.47 (1.32 - 1.63)	<0.001	1.49 (1.29 - 1.73)	<0.001	1.35 (1.13 - 1.62)	0.001
Model 4	1.44 (1.29 - 1.61)	<0.001	1.47 (1.26 - 1.70)	<0.001	1.40 (1.17 - 1.69)	<0.001
Model 5	1.35 (1.20 - 1.52)	<0.001	1.39 (1.19 - 1.63)	<0.001	1.33 (1.09 - 1.61)	0.004
MODEL 1: UNADJUSTED						
MODEL 2: ADJUSTED FOR AGE AND GENDER						
MODEL 3: IN ADDITION ADJUSTED FOR CORONARY ARTERY DISEASE (NONE, STABLE CAD, UNSTABLE CAD, NSTEMI, STEMI), BODY MASS INDEX, TYPE 2 DIABETES, HYPERTENSION, SMOKING STATUS, GLOMERULAR FILTRATION RATE (CKD-EPI), USE OF LIPID-LOWERIMG DRUGS, LDL-C, HDL-C, AND TRIGLYCERIDES						
MODEL 4: MODEL 3 ADDITIONALLY ADJUSTED FOR PHOSPHATE						
MODEL 5: MODEL 4 ADDITIONALLY ADJUSTED FOR ALKALINE PHOSPHATASE, CALCIUM, PARATHYROID HORMONE, 25-HYDROXYVITAMIN D						

Table 5: Hazard ratios (HR) for total and cardiovascular mortality according to FGF23 in subgroups with eGFR above and below 60 ml/min/1.73 m²

Figure 1: Kaplan-Meier curve for total (left panel, log rank $p < 0.001$) and cardiovascular mortality (right panel, log rank $p < 0.001$) according to quartiles of FGF-23. For hazard ratios and confidence intervals, see Table 4A and B (model 1)

3.4. Reclassification analysis

The predictive value of FGF23 for total and cardiovascular mortality was assessed using ROC and reclassification analysis (Table 6; Suppl Material). The addition of FGF23 to a model consisting of cardiovascular risk factors including eGFR did not significantly increase the area under the ROC curve for total mortality (0.772 vs. 0.785) and cardiovascular mortality (0.748 vs. 0.756), respectively. However, reclassification analysis revealed significant improvement of the predictive model on addition of FGF23. The net reclassification improvement (NRI) for total mortality was 30.1% (95% CI: 22.2 - 37.9, $p < 0.001$), the integrated discrimination improvement (IDI) was 0.020 (95% CI: 0.014 - 0.027, $p < 0.001$). Similar results were obtained for cardiovascular death: NRI was 21.9% (95% CI: 12.7 - 31.2, $p < 0.001$), IDI was 0.010 (95% CI: 0.004 - 0.014, $p = 0.002$).

	Total mortality			Cardiovascular mortality		
	Model 1	Model 2	P	Model 1	Model 2	P
AUC	0.772 (0.754- 0.790)	0.785 (0.768- 0.803)	ns	0.748 (0.726- 0.770)	0.756 (0.735- 0.777)	ns
NRI, % (95% CI)	30.1 (22.2 - 37.9)		<0.001	21.9 (12.7 - 31.2)		<0.001
IDI (95% CI)	0.020 (0.014 - 0.027)		<0.001	0.010 (0.004 - 0.014)		0.002
Model 1: cardiovascular risk factors including age, sex, BMI, hypertension, HDL- and LDL-cholesterol, triglycerides, smoking, coronary artery disease, type 2 diabetes mellitus, eGFR (CKD-EPI)						
Model 2: model 1 plus FGF-23						
AUC indicates area under the receiver-operating characteristic (ROC) curves; NRI, net reclassification improvement; IDI, integrated discrimination improvement; NS, not significant						

Table 6: ROC and reclassification analysis for total and cardiovascular mortality

4. Discussion

The major finding of the present study is the independent association of FGF23 at baseline with future cardiovascular and allcause mortality in patients from the LURIC cohort, which includes Caucasian patients who underwent coronary angiography with comprehensive long-term prospective follow-up. These significant associations proved to be independent from kidney function at baseline and remained stable also after adjustments for levels of potentially confounding markers of bone and mineral metabolism (calcium, vitamin D, alkaline phosphatase, and PTH). Remarkably, also adjustments for serum phosphate did not substantially mitigate the association between FGF23 and outcome, suggesting that GF23 is not just a proxy for phosphate, or other markers of mineral metabolism, in predicting risk for mortality. Important characteristics of LURIC patients are their clinical homogeneity as high risk patients for future cardiovascular events based on the previous clinical indication for coronary angiography and the high proportion of patients with documented CAD. Thus, the current work is a valuable extension to previous ones indicating that FGF23 qualifies as a marker for increased mortality risk in future.^{5,7,8,11,12,18} However, despite the consistency of clinical observations, there is less certainty as to the exact mechanism underlying the impact of higher FGF23 concentrations. Several studies demonstrated that higher FGF23 levels are associated with an increased risk for prevalent and incident left-ventricular hypertrophy and dysfunction. This association was found in patients with and without severely impaired renal function.^{11,19,20} In a set of elegant in vivo and in vitro experiments Faul and coworkers showed that FGF23 directly induces cardiomyocyte hypertrophy.¹⁹ We also would like to emphasize that when we added NTproBNP as a marker of left-ventricular dysfunction to our prognostic models the predictive power of FGF23 was only slightly attenuated suggesting an independent contribution of FGF23 to allcause and cardiovascular mortality. In this context it may be important to mention that classical atherosclerotic disease conditions such as peripheral arterial disease are only moderately associated with higher FGF23 levels²¹ while left-ventricular hypertrophy revealed a strong association.²² It has been speculated that the presence of CKD modifies the association between FGF23 and outcome.²³ Indeed, median FGF23 were about twice as high in patients with GFR below 60 ml/min compared to those

with GFR above 60 ml/min. However, the presence of CKD as defined with an eGFR below 60 ml/min did not substantially modify FGF23 effects upon mortality, nor was there any indication for a significant interaction between FGF23 and eGFR. The LURIC cohort allows for the first time analyzing the effects of FGF23 elevations upon cardiovascular outcomes in a very high risk CAD population, since the LURIC cohort is not a population-based screening cohort.^{7,9,11} The currently most similar cohort study for LURIC is the Californian Heart and Soul study.⁷ However, the LURIC and the Heart and Soul study reveal important differences in various aspects including the threefold larger sample size of the LURIC study. Moreover, in contrast to LURIC, the patient selection in Heart and Soul was based on the historic diagnosis of confirmed and presumed CAD and in contrast to LURIC the baseline visit was not timely related to the qualifying event. After multivariate adjustment in Heart and Soul the third tertile of FGF23 was associated with a HR of 2.07 (95% CI 1.36 - 3.13) for all-cause mortality. Thus our data extend these previous findings⁷ by several essential aspects. Among these novel aspects special attention is warranted on the ability to analyze separately all-cause and cardiovascular mortality, and most importantly, the ability to adjust for important potentially confounding bone and mineral metabolism factors. All of these bone biomarkers may independently influence cardiovascular events: High levels of PTH, alkaline phosphatases, and phosphate^{6,24} and low levels of vitamin D²⁵ have been previously identified as risk factors for increased morbidity and mortality. Moreover, these bone biomarkers and FGF23 are closely interrelated and part of a complex feed-back loop underlining the need for adjustments in order to avoid confounding.^{1,3} So the particular strength of the LURIC database is the homogenous character of the cohort and the comprehensive adjustments that put substantial and unique strength to the core result: FGF23 baseline levels predict independently future cardiovascular and especially all-cause mortality in patients referred to coronary angiography. However, the exact impact of FGF23 in predicting future cv events and also its contribution to risk stratification remains to be clarified. Our study faces some limitations. The results are cohort-dependent and generalizability to cohorts with lower cv risk is limited. We could not assess the impact of time-averaged FGF23 concentrations since only single spot measurements were obtained. Data upon urinary phosphate excretion e.g. are not available. Moreover, it is unclear if coronary angiography and the acute clinical setting itself could influence individual FGF23 levels. A recent study in patients with acute heart failure and cardiogenic shock showed significantly elevated FGF23 levels upon admission in the intensive care unit²⁶ and therefore reverse causality²⁷ cannot be excluded.

5. Conclusion

In summary, the present large-scale prospective cohort data clearly indicate that the risk for all-cause and cardiovascular mortality increases significantly and independently with increasing baseline levels of FGF23 in persons at the time of, or at risk for an acute coronary ischemic event, even after adjustments for a large array of confounders of bone metabolism.

6. Author contributions

W.M. designed the study, contributed to the interpretation of the results and reviewed the manuscript. V.B. contributed to data analysis and interpretation and wrote the manuscript. M.K. was responsible for sample handling and database management and supported writing of the manuscript. T.S. and H.S. organized and supervised the FGF23 measurements. H.S. conducted the statistical analyses. All co-authors contributed to the interpretation of the results and reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript as submitted.

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8. Disclosure/conflict of interest

The authors have no conflict of interest to declare.

9. Acknowledgments

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10. Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2014.08.0>

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b)

Soluble klotho and mortality: the Ludwigshafen Risk and Cardiovascular Health Study (IF 4)

Soluble klotho and mortality: the Ludwigshafen Risk and Cardiovascular Health Study.

Brandenburg VM, Kleber ME, Vervloet MG, Larsson TE, Tomaschitz A, Pilz S, Stojakovic T, Delgado G, Grammer TB, Marx N, März W, Scharnagl H. Atherosclerosis. 2015 Oct; 242(2): 483-9.

Abstract

Background: Experimental evidence suggests that soluble klotho (s-klotho), a co-receptor for fibroblast growth factor 23 (FGF23), may modulate cardiovascular risk through multiple mechanisms. However, the predictive value of s-klotho in patients remains unclear. Therefore, the present study examined in a large cohort of patients referred for coronary angiography whether s-klotho is associated with cardiovascular and total mortality.

Methods: The longitudinal associations between baseline s-klotho and FGF23 concentrations and mortality were evaluated in 2948 participants of the Ludwigshafen Risk and Cardiovascular Health Study (LURIC), referred for coronary angiography.

Results: Mean age of participants was: 63 ± 10 years. Patients with diabetes mellitus (n=1136) had elevated s-klotho: [440 (430 - 449) versus 414 (406 - 421) pg/ml, $p < 0.001$]. S-klotho decreased in parallel to glomerular filtration rate (GFR) and increased in parallel to FGF23. During a median follow-up of 9.9 years, 874 deaths (30%) occurred, 539 (18%) of which were cardiovascular. After adjustment for cardiovascular risk factors, the hazard ratios in the fourth quartile compared to the first quartile of s-klotho were 1.14 (95%CI, 0.94 - 1.38; $p = 0.187$) for all-cause mortality and 1.03 (95%CI, 0.80 - 1.31; $p = 0.845$) for cardiovascular mortality. Excess mortality prediction by high levels of baseline FGF23 was not modified by adjustment for baseline s-klotho levels.

Conclusions: Klotho does not add predictive power to cardiovascular and mortality risk assessment in patients with normal renal function.

1. Introduction

Klotho was first identified as a protein involved in aging-like processes and reduced life-span detectable in klotho-deficiency models, whereas an extended life span was seen when klotho was over-expressed.^{1,2} Close interactions between fibroblast growth factor 23 (FGF23) and klotho exist, since classical FGF23 signal transduction requires membrane-klotho to increase binding affinity to FGF receptors.³ FGF23 increases renal phosphate excretion and decreases 1 α ,25 dihydroxyvitamin D synthesis.⁴ Additionally, klotho-FGF23 is strongly involved in cardiovascular (patho-) physiology. Studies in various patient populations have consistently shown that high levels of FGF23 are associated with dismal outcome and elevated cardiovascular event rates.⁵⁻¹² In vivo FGF23 application directly leads to myocardial hypertrophy in murine models.¹³ The klotho gene encodes a single-pass transmembrane protein with a large extracellular domain, which is synthesized predominantly in the kidney.¹ The extracellular domain of klotho can be cleaved and released into extracellular fluids including plasma,¹⁴ where soluble klotho (s-klotho) is detectable via ELISA techniques.¹⁵ Circulating klotho is considered to be involved in preservation of endothelial function and arterial wall integrity and hence may be protective against endothelial dysfunction and arteriosclerosis,¹⁶ reduced vascular elasticity or calcification.^{17,18} Recent in vivo findings point towards direct cardioprotective effects of s-klotho.^{19,20} A small human study showed that decreased klotho was an independent determinant of arterial stiffness as measured by pulsewave velocity.²¹ It is noteworthy that klotho also interacts with calcium and phosphate homeostasis which per se influences cardiovascular health.¹⁶ Moreover, it is intriguing to speculate about the presence of reciprocal effects between klotho and FGF23. This hypothesis of concerted klotho-FGF23 action is fueled by recent experimental in vivo work showing that high FGF23 concentration positively correlated with cardiac remodeling only in a klothodeficient state but not in a klotho-replete state.²² So klotho deficiency in humans might mediate at least in part FGF23-associated mortality excess. However, recent longitudinal cohort studies in pre-dialysis and dialysis patients did not detect any significant relationship between s-klotho and cardiovascular outcome.^{23,24} Notably, since renal failure is a condition of severely impaired klotho-FGF23-mineral-metabolism with remarkable FGF23 excess these previous negative human study results^{23,24} are presumably not generalizable to patients with preserved renal function. Therefore, we explored the predictive value of s-klotho in large cohort comprising patients with (close to) normal kidney function by analyzing the Ludwigshafen Risk and Cardiovascular Health Study (LURIC), i.e. patients identified via referral for coronary angiography.

2. Methods

2.1. Participants, study design, and definition of comorbidities

The LURIC study is a prospective cohort study. The baseline examination was performed between 1997 and 2000 in south-west Germany at a coronary care tertiary referral center (Herzzentrum Ludwigshafen). Details about participants, recruitment, comorbidities and baseline examinations have been described previously.²⁵ Overall 3316 patients with coronary angiogram at baseline were included. All patients were Caucasians and were

required to be in clinical stable conditions except for acute coronary syndromes. Indications for coronary angiography were cardiac chest pain, acute coronary syndrome or cardiac ischemia as suspected with non-invasive testing. Chest pain with non-cardiac origin was an exclusion criterion as well as the predominance of any noncardiac disease and malignancy within the previous 5 years. Kidney function was not relevant for inclusion. The patients gave written and informed consent. The study protocol was designed according to the Declaration of Helsinki. The Ärztekammer Rheinland-Pfalz was the responsible ethics committee, which approved the study. We diagnosed coronary artery disease (CAD) based on at least one stenosis of at least one out of 15 coronary arterial segments according to visual estimation of the maximal luminal narrowing > 20%. Myocardial infarction (MI) was classified as acute if the event was within the four weeks prior to enrolment. A definite ST-segment elevation MI (STEMI) was diagnosed if typical ECG changes were present along with prolonged chest pain, refractory to sublingual nitrates and/or enzyme or troponin T elevations (>0.1 mg/L). Non-ST elevation MI (NSTEMI) was diagnosed, if symptoms and/or troponin T criteria were met in the absence of ECG criteria defining STEMI. The diagnosis diabetes mellitus was based on at least one of the following findings: the (previous) prescription of antidiabetic medication, fasting blood glucose levels greater than 7.0 mmol/l, HbA1c was >6.5%, or if the 2 h blood glucose level exceeded 11.1 mmol/L in an oral glucose tolerance test. Arterial hypertension was diagnosed if the mean systolic and diastolic blood pressure exceeded either 140 mmHg systolic or 90 mmHg diastolic in three consecutive measurements at the upper arm. Alternatively, current prescription of antihypertensive drugs confirmed the diagnosis. Cerebrovascular disease (CVD) was defined clinically by documented history of a previous cerebrovascular event or by documented carotid plaques (>50% luminal obstruction).

2.2. Outcome assessment during follow-up

S-klotho measurements were available in 2948 patients representing 89% of the entire LURIC cohort. Median follow-up time was 9.9 years. Death certificates were missing in 18 decedents who were included in the analysis for total mortality, but excluded from analysis of cardiovascular mortality. Information on survival status was obtained from local person registries. Two physicians blinded to baseline characteristics of the study participants classified causes of death by reviewing hospital records and death certificates. Cardiovascular deaths included sudden cardiac death (SCD), fatal myocardial infarction, deaths due to heart failure, death after intervention to treat CAD, stroke, and other deaths due to heart disease. SCD was defined as a sudden unexpected death either within 1 h of symptom onset or within 24 h of having been observed alive and symptom free. Persons whose sudden death was most likely attributable to a non-cardiac disease and patients who suffered from any predominant non-cardiac and terminal disease (e.g. cancer) so that their death was not unexpected were not classified as having died due to SCD. In the case of disagreement about classification, the final decision was made by one of the principal LURIC investigators after appropriate review of the data.

2.3. Laboratory analyses

Standard laboratory measurements were performed as previously described.²⁵ In brief, venous blood was drawn in the morning before coronary angiography and standard

laboratory parameters were immediately determined. Serum and plasma was harvested according to standard procedures and was snap frozen for further determinations and stored at -80°C until further analysis. Soluble klotho levels were assessed by human soluble alpha-klotho assay, Immuno-Biological Laboratories Ltd. (Japan). Within the LURIC analysis the assay revealed an intra- and interassay coefficient of variation of 3.4 and 4.9%, respectively. FGF23 (c-terminal) was measured by enzyme-linked immunosorbent assay (ELISA, Immundiagnostik AG, Bensheim, Germany), intra- and inter-assay coefficient of variation were 2.4 and 3.1%, respectively. Intact PTH, and N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) were determined by ElectroChemiluminescence (ECL) on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). 25-hydroxyvitamin D was measured with a radioimmunoassay (DiaSorin Antony, France; Stillwater, USA) with an intra- and interassay coefficient of variation of 8.6 and 9.2%, respectively. 1,25-dihydroxyvitamin D was measured by radioimmunoassay (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) using a multicrystal counter (Berthold LB2014, Dia Sorin, SA, USA). Estimated glomerular filtration rate (GFR) was calculated according to the CKD-EPI study equation.^{26,27}

2.4. Statistical analysis

Soluble klotho concentration quartiles were generated according to the values of the entire study cohort. Depending on their distribution, continuous parameters are either presented as means $\pm$ standard deviation (normally distributed variables) or as medians with interquartile range (skewed variables). Categorical data are shown as proportions. All skewed continuous parameters were logarithmically transformed before use in parametric procedures. Characteristics of bone and kidney metabolism were analyzed by s-klotho quartiles. Comparisons across s-klotho quartiles were carried out by analysis of variance (ANOVA) with p for trend for continuous parameters and by χ^2 -test with p for linear-by-linear test for categorical variables. For all-cause and cardiovascular mortality, Hazard ratios (HRs) with 95% confidence intervals (95% CI) for all-cause and cardiovascular mortality were calculated with Cox proportional hazard models after negative testing for violation of the proportional hazard assumption using Cox models with time-dependent covariates. Cox proportional hazards were calculated both according to quartiles of s-klotho and per increase of 1 SD (calculated from the logarithmically transformed values). We present results of different adjustment models: Model 1: unadjusted; Model 2: adjusted for age and gender; Model 3: in addition adjusted for coronary artery disease (none, stable CAD, unstable CAD, NSTEMI, STEMI), body mass index, type 2 diabetes, hypertension, smoking status, use of lipid-lowering drugs, LDL-C, HDL-C, triglycerides, and glomerular filtration rate (CKDEPI); Model 4: model 3 additionally adjusted for FGF23; Model 5: model 3 additionally adjusted for interaction of FGF-23 and s-klotho. Additionally we performed outcome assessment with parallel analysis of s-klotho and FGF23 by stratifying patients according to the median levels of s-klotho and FGF23 into four groups: low s-klotho and low FGF23, low s-klotho and high FGF23, high s-klotho and low FGF23, and high s-klotho and high FGF23, respectively. A p-value below 0.05 was considered statistically significant and all statistical tests were two-sided. Data were analyzed using the SPSS 22.0 statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

Mean age of the LURIC participants was 63 ± 10 years, 69% were male and 39% were diabetics. During the median follow-up time of 9.9 years 874 (30%) of these 2948 patients died. 539 (18%) deaths were due to cardiovascular causes. The median s-klotho level was 419 (334 - 525) pg/ml and the median FGF23 was 54 (40 - 78) RU/ml.

3.1. Association of s-klotho with clinical data, cardiovascular risk factors, status of coronary artery disease and FGF23 (Table 1)

S-klotho was not related to age, hypertension, body mass index, and the lipid status of the patients. Women, presence of diabetes, and smokers revealed significantly higher s-klotho concentrations. There was a linear and significant trend for increasing s-klotho levels with increasing quartiles of FGF23. About 14% of all LURIC participants exhibited CKD defined as GFR <60 ml/min/1.732. Sklotho levels declined significantly in parallel with CKD-stages: Patients with a GFR <30 ml/min/1.722 exhibited about 15% lower s-klotho levels compared to those with a GFR >90 ml/min/1.732 (Table 1).

3.2. Association of s-klotho levels with biomarkers of chronic kidney disease - mineral and bone disorder (CKD-MBD) (Table 2)

Persons in the highest quartile of s-klotho showed significantly higher concentrations of total serum calcium, lower phosphorus, and higher 1.25-dihydroxyvitamin D (Table 2). Alkaline phosphatase, PTH, and calcidiol were not related to s-klotho.

3.3. Associations of s-klotho with all-cause and cardiovascular mortality (Tables 3a and 3b)

S-klotho levels at baseline were not associated with total and cardiovascular mortality in all models (models 2, 3, and 4). An increase of 1 SD was associated with fully adjusted HRs for death from any cause of 1.05 (0.98 - 1.13, $p = 0.142$) and for cardiovascular death of 1.03 (0.95 - 1.13, $p = 0.480$).

3.4. Combined analysis for FGF23 plus s-klotho for mortality (Tables 4a and 4b).

We analyzed the impact of high/low-s-klotho levels on mortality of FGF23 levels. Patients within the highest quartile of FGF23 revealed the highest hazard ratio for death independent of the s-klotho being above or below the median. These findings were similar for all-cause and for cardiovascular mortality (Tables 4a and 4b).

	n	Klotho (pg/mL) ^a	Difference (%) ^b	P ^a
Gender				
Men	2021	419 (412–426)		
Women	927	435 (424–446)	+3.8	0.025
Age (years)				
<60	1071	424 (413–435)		
60–70	1074	425 (415–435)	+0.3	0.881
>70	803	422 (410–435)	–0.4	0.844
Lipid-lowering drugs				
No	1492	431 (422–439)		
Yes	1456	417 (409–426)	–3.1	0.038
Coronary artery disease				
None	641	417 (403–431)		
Stable CAD	1367	440 (431–449)	+5.5	0.006
Unstable CAD (Troponin T-)	571	406 (394–420)	–2.5	0.303
Unstable CAD (Troponin T+)	369	404 (388–421)	–3.1	0.260
Diuretics				
No	2103	424 (417–431)		
Yes	845	423 (412–435)	–0.2	0.919
Body mass index (kg/m ²)				
<26 or 27 ^c	1387	422 (413–430)		
>26 or 27 ^c	1561	426 (418–434)	1.0	0.466
Type 2 Diabetes mellitus				
None	1785	414 (406–421)		
Diabetes mellitus	1163	440 (430–449)	+6.3	<0.001
Hypertension				
No	801	424 (412–435)		
Yes	2147	424 (417–431)	0.0	0.965
Smoking				
Never	1081	436 (426–447)		
Former	1300	420 (412–429)	–3.6	0.030
Current	567	409 (395–422)	–6.3	0.003
eGFR (ml/min/1.73m ²) ^d				
>90	1077	444 (433–456)		
60–89	1468	420 (412–428)	–5.4	0.001
30–59	359	388 (372–404)	–7.7	<0.001
<30	44	378 (338–424)	–14.8	0.007
LDL cholesterol (mg/dL)				
1st quartile (<95)	750	422 (411–434)		
2nd quartile (95–113)	713	432 (420–445)	+2.3	0.260
3rd quartile (114–137)	742	418 (407–429)	–1.1	0.566
4th quartile (>137)	743	423 (412–435)	+0.2	0.918
HDL cholesterol (mg/dL)				
1st quartile (<32)	734	420 (408–433)		
2nd quartile (32–37)	751	427 (415–439)	+1.6	0.447
3rd quartile (38–44)	690	423 (412–436)	+0.7	0.744
4th quartile (>44)	773	424 (412–437)	+0.9	0.692

	n	Klotho (pg/mL) ^a	Difference (%) ^b	P ^c
HDL cholesterol (mg/dL)				
1st quartile (<32)	734	420 (408–433)		
2nd quartile (32–37)	751	427 (415–439)	+1.6	0.447
3rd quartile (38–44)	690	423 (412–436)	+0.7	0.744
4th quartile (>44)	773	424 (412–437)	+0.9	0.692
Triglycerides (mg/dL)				
1st quartile (<109)	739	434 (422–446)		
2nd quartile (109–145)	739	423 (411–434)	–2.5	0.202
3rd quartile (146–199)	738	420 (408–431)	–3.1	0.110
4th quartile (>199)	732	419 (408–431)	–3.3	0.112
FGF-23 (RU/ml)				
1st quartile (<40)	736	416 (404–428)		
2nd quartile (40–54)	738	418 (407–430)	+0.6	0.020
3rd quartile (55–78)	737	424 (412–435)	+1.3	0.020
4th quartile (>78)	737	438 (425–451)	+5.4	0.022

^a Estimated marginal means and 95% confidence intervals obtained in a general linear model (ANOVA), adjusted for each of the other factors, whereby age, body mass index, eGFR, LDL cholesterol, HDL cholesterol and triglycerides (log transformed), were included as continuous rather than categorical covariables.

^b Compared to the first category of each variable.

^c Thresholds of 26 and 27 kg/m² apply to males and females, respectively.

^d eGFR was calculated on the basis of the equation of the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration).

Table 1: Association of Klotho with cardiovascular risk factors, coronary artery disease, usage of diuretics, and FGF-23

	Klotho (pg/mL)			p ^a
	<334 n = 737	334-418 n = 737	419-525 n = 737	
Alkaline phosphatase (U/L)	121 ± 49	121 ± 47	124 ± 49	0.404
Calcium (mmol/L)	2.32 ± 0.11	2.33 ± 0.10	2.33 ± 0.10	0.004
Phosphate (mg/dL)	3.56 ± 0.60	3.52 ± 0.56	3.53 ± 0.55	0.007
Parathyroid hormone (pg/mL)	34 ± 22	35 ± 40	35 ± 32	0.433
25-Hydroxyvitamin D (µg/L)	17.4 ± 9.2	17.7 ± 9.5	17.5 ± 9.3	0.897
1,25-Dihydroxyvitamin D (ng/L)	33.1 ± 13.2	35.2 ± 14.1	35.5 ± 14.9	<0.001

Values are given as mean ± SD.

^a ANOVA, adjusted for age and sex.

Table 2: Association of Klotho with characteristics of bone and kidney metabolism

s-klotho (pg/ml)	Deaths n (%)	Model 1 HR (95% CI)	P	Model 2 HR (95% CI)	P	Model 3 HR (95% CI)	P	Model 4 HR (95% CI)	P	Model 5 HR (95% CI)	P
All individuals (n = 2948)											
1st quartile (<334)	239 (32)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (334–418)	200 (27)	0.81 (0.67–0.98)	0.025	0.91 (0.75–1.10)	0.320	0.95 (0.78–1.14)	0.567	0.94 (0.78–1.14)	0.514	0.93 (0.77–1.12)	0.438
3rd quartile (419–525)	221 (30)	0.90 (0.75–1.08)	0.246	1.02 (0.85–1.22)	0.841	1.11 (0.92–1.34)	0.266	1.11 (0.92–1.34)	0.268	1.09 (0.91–1.31)	0.362
4th quartile (>525)	214 (29)	0.85 (0.71–1.02)	0.081	1.03 (0.85–1.23)	0.797	1.14 (0.94–1.37)	0.179	1.14 (0.94–1.38)	0.187	1.10 (0.90–1.33)	0.355
Per 1 SD		0.94 (0.88–1.01)	0.082	1.01 (0.94–1.08)	0.890	1.05 (0.98–1.13)	0.141	1.05 (0.98–1.13)	0.142	1.04 (0.97–1.12)	0.232

Table 3A: Hazard ratios (HR) for death from all causes according to quartiles of s-klotho

s-klotho (pg/ml)	Deaths n (%)	Model 1 HR (95% CI)	P	Model 2 HR (95% CI)	P	Model 3 HR (95% CI)	P	Model 4 HR (95% CI)	P	Model 5 HR (95% CI)	P
All individuals (n = 2930)											
1st quartile (<334)	153 (21)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (334–418)	118 (16)	0.74 (0.59–0.95)	0.016	0.84 (0.66–1.06)	0.143	0.87 (0.68–1.11)	0.246	0.86 (0.68–1.10)	0.222	0.85 (0.67–1.09)	0.197
3rd quartile (419–525)	144 (20)	0.91 (0.73–1.15)	0.430	1.03 (0.82–1.30)	0.783	1.13 (0.89–1.42)	0.319	1.12 (0.89–1.42)	0.322	1.11 (0.88–1.40)	0.363
4th quartile (>525)	124 (17)	0.77 (0.61–0.98)	0.031	0.93 (0.73–1.18)	0.532	1.03 (0.81–1.31)	0.826	1.03 (0.80–1.31)	0.845	1.01 (0.80–1.28)	0.940
per 1 SD		0.92 (0.84–1.01)	0.079	0.99 (0.91–1.08)	0.769	1.03 (0.95–1.13)	0.478	1.03 (0.95–1.13)	0.480	1.02 (0.94–1.11)	0.621
Model 1: unadjusted.											
Model 2: adjusted for age and gender.											
Model 3: in addition adjusted for coronary artery disease (none, stable CAD, unstable CAD, NSTEMI, STEMI), body mass index, type 2 diabetes, hypertension, smoking status, use of lipid-lowering drugs, LDL-C, HDL-C, triglycerides, and glomerular filtration rate (CKD-EPI).											
Model 4: model 3 additionally adjusted for FGF-23.											
Model 5: model 3 additionally adjusted for interaction of FGF-23 and s-klotho.											

Table 3B: Hazard ratios (HR) for death from cardiovascular causes according to quartiles of s-klotho

FGF-23 (RU/ml)	Deaths n (%)	Model 1 HR (95% CI)	P	Model 2 HR (95% CI)	P	Model 3 HR (95% CI)	P	Model 4 HR (95% CI)	P
Individuals: s-klotho $\leq$ 419 pg/ml									
1st quartile (<40)	67 (19)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	82 (22)	1.20 (0.87–1.65)	0.277	1.11 (0.80–1.53)	0.534	1.03 (0.75–1.43)	0.849	1.04 (0.75–1.43)	0.828
3rd quartile (55–78)	106 (28)	1.57 (1.16–2.14)	0.004	1.40 (1.03–1.90)	0.032	1.16 (0.85–1.58)	0.351	1.17 (0.92–1.60)	0.329
4th quartile (>78)	185 (50)	3.33 (2.52–4.40)	<0.001	2.67 (2.01–3.54)	<0.001	1.86 (1.37–2.53)	<0.001	1.85 (1.36–2.51)	<0.001
Individuals: s-klotho > 419 pg/ml									
1st quartile (<40)	76 (20)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	83 (23)	1.17 (0.86–1.60)	0.324	1.06 (0.78–1.10)	0.710	0.99 (0.72–1.34)	0.985	0.98 (0.72–1.14)	0.895
3rd quartile (55–78)	100 (28)	1.46 (1.08–1.96)	0.014	1.23 (0.92–1.22)	0.155	1.07 (0.79–1.45)	0.682	1.06 (0.78–1.34)	0.725
4th quartile (>78)	175 (48)	2.90 (2.28–3.91)	<0.001	2.30 (1.75–1.23)	<0.001	1.67 (1.24–2.24)	0.001	1.56 (1.16–2.10)	0.004

Model 1: unadjusted.
Model 2: adjusted for age and gender.
Model 3: in addition adjusted for coronary artery disease (none, stable CAD, unstable CAD, NSTEMI, STEMI), body mass index, type 2 diabetes, hypertension, smoking status, use of lipid-lowering drugs, LDL-C, HDL-C, triglycerides, and glomerular filtration rate (CKD-EPI).
Model 4: model 3 additionally adjusted for the interaction of FGF-23 and s-klotho.
[†]The study cohort was stratified according to the median of s-klotho (419 pg/mL).

Table 4A: Hazard ratios (HR) for death from all causes according to FGF-23 and s-klotho

Klotho (pg/ml)	Deaths n (%)	Model 1 HR (95% CI)	P	Model 2 HR (95% CI)	P	Model 3 HR (95% CI)	P	Model 4 HR (95% CI)	P
Individuals: s-klotho ≤419 pg/ml									
1st quartile (<40)	41 (11)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	46 (12)	1.09 (0.72–1.67)	0.679	1.01 (0.66–1.54)	0.955	0.94 (0.62–1.44)	0.782	0.95 (0.62–1.22)	0.791
3rd quartile (55–78)	69 (18)	1.67 (1.13–2.45)	0.010	1.48 (1.00–2.18)	0.048	1.24 (0.84–1.84)	0.289	1.25 (0.84–1.85)	0.270
4th quartile (>78)	116 (31)	3.38 (2.37–4.82)	<0.001	2.71 (1.89–3.87)	<0.001	1.92 (1.30–2.83)	0.001	1.91 (1.30–2.82)	0.001
Individuals: s-klotho >419 pg/ml									
1st quartile (<40)	47 (12)	1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}		1.0 ^{reference}	
2nd quartile (40–54)	51 (14)	1.16 (0.79–1.73)	0.454	1.05 (0.71–1.57)	0.800	0.96 (0.64–1.43)	0.839	0.95 (0.64–1.42)	0.816
3rd quartile (55–78)	60 (17)	1.41 (0.96–2.07)	0.077	1.21 (0.82–1.77)	0.340	0.95 (0.64–1.41)	0.810	0.95 (0.64–1.40)	0.781
4th quartile (>78)	109 (30)	3.00 (2.13–4.23)	<0.001	2.31 (1.63–3.23)	<0.001	1.43 (0.98–2.09)	0.061	1.34 (0.91–1.96)	0.135
Model 1: unadjusted.									
Model 2: adjusted for age and gender.									
Model 3: in addition adjusted for coronary artery disease (none, stable CAD, unstable CAD, NSTEMI, STEMI), body mass index, type 2 diabetes, hypertension, smoking status, use of lipid-lowering drugs, LDL-C, HDL-C, triglycerides, and glomerular filtration rate (CKD-EPI).									
Model 4: model 3 additionally adjusted for the interaction of FGF-23 and s-klotho.									
¹ The study cohort was stratified according to the median of s-klotho (419 pg/mL).									

Table 4B: Hazard ratios (HR) for death from cardiovascular causes according to FGF-23 and Klotho

4. Discussion

Analyzing the impact of s-klotho upon outcome within the prospective cohort study LURIC provides two novel and important findings: First, s-klotho levels at baseline did not predict cardiovascular and all-cause mortality in a cohort with normal and mildly impaired renal function but at high risk for future cardiovascular events. Second, s-klotho did not modify the corroborated strong relationship between FGF23 and mortality. The present findings about the absence of an association between s-klotho and outcome of particular interest considering our recent publication about an independent and strong association between FGF23 serum levels and future mortality coming from the same cohort, the LURIC study (n = 2974 patients).¹² So LURIC has revealed already convincing data about a strong impact of high FGF23 with poor outcome which is perfectly in line with other prospective cohorts.²⁸ Our previous data showed a hazard ratio of 1.34 (95%CI, 1.23 - 1.46) for all-cause mortality [1.28 (95%CI, 1.15 - 1.43) for cardiovascular mortality] for each standard deviation increase in baseline FGF23 concentration. Based on the strong biological interactions between FGF23 and klotho it is therefore reasonable to investigate the association between s-klotho and outcomes. Klotho is the obligatory co-receptor mediating FGF23 receptor activity especially in the kidneys e a key organ for FGF23 mediated systemic effects.^{3,29} Although klotho is absent in the heart in vivo experiments by Hu and coworkers pointed towards reciprocal klotho-FGF23 effects: Preserved klotho activity ameliorated deleterious cardiac effects associated with phosphate load, CKD, or FGF23 excess.²² The authors came to the conclusion that klotho deficiency may be a promoter and modifier of cardiomyopathy and that FGF23 excess is especially noxious to the heart only in states of klotho deficiency.²² These experimental findings are strongly supported by various human and in vivo studies indicating that s-klotho associates in a protective way with different cardiovascular surrogates such as kidney function development, aging, fibrosis, or oxidative stress.^{2,28,30} According to in vivo findings by Xie and coworkers¹⁹ klothodeficiency aggravated CKD-induced cardiomyopathy and restoration of soluble klotho via transgenic expression ameliorated signs of cardiomyopathy independent from phosphate and FGF23 levels.¹⁹ The lack of association between s-klotho and mortality in the LURIC cohort, in particular cardiovascular mortality, and also the non-existence of any modulatory effect of s-klotho upon FGF23 risk prediction therefore deserves careful interpretation.^{16,28} There may be several causes for this observation. First, it is imaginable that previously reported klotho effects, especially in isolated in vitro systems, are not clinically relevant in the context of competing risk factors. Second, most of the reported effects of klotho in animal studies were generated in various models of pronounced klotho deficiency that may not translate into similar effects in humans with (close to) normal klotho levels. In this regard, all relevant animal models of klotho deficiency (e.g. models of CKD and genetically induced klotho deficiency) are accompanied by predominant secondary alterations including high FGF23 levels that are known to elicit many of the “aging-like” phenotypes attributed to klotho deficiency.³¹ Finally, it remains uncertain to what extent s-klotho reflects its tissue levels and it remains elusive whether currently available s-klotho assays appropriately assess circulating s-klotho.²⁸ Several lines of evidence suggest that such assays may suffer from limitations concerning specificity, most importantly because circulating klotho levels in

animal CKD studies invariably are strongly decreased when measured with alternate techniques, whereas human studies provide inconsistent data on the relationship between klotho and kidney function.^{16,28,32} In agreement, we found only a weak correlation between eGFR and sklotho levels. The present klotho data from the LURIC cohort in terms of absence of association to mortality support two previous reports^{23,24} with remarkable differences in terms of patient characteristics. These two studies were performed in patients either with moderate to severe CKD²⁴ or end-stage renal disease²³ in whom causes for morbidity and mortality are presumably different from LURIC patients. Accordingly median FGF23 levels in LURIC are much lower [median c-term FGF23 serum levels were 54 (40 - 78) RU/ml] compared to the Care for Home cohort [102 (64 - 164) RU/ ml]²⁴ or especially to the ICD-2 cohort [7247 ± 16815 RU/ml].²³ However, all these studies were all performed in patients with a significant degree of comorbidities such as chronic kidney disease, prevalent coronary artery disease or diabetes. Thus, our cohort does not include basically healthy individuals and LURIC is not a prototypic screening cohort. Hence, we cannot assess the potential link between s-klotho and survival in patients at very low cardiovascular risk. Analyses of the LURIC cohort comprising high-risk patients provide evidence that smoking habits (higher levels in nonsmokers), gender (higher levels in females) and diabetes status (higher levels in diabetics) associate with s-klotho levels. These associations have not yet been addressed in previous prospective cohort studies.^{23,24} The nature of our cohort does not allow conclusions about causality. While female gender and non-smoking are prototypic cardioprotective factors, the associated higher sklotho levels do not translate into reduced mortality in the entire cohort and thus, these findings remain of undetermined biological significance. A previous study reported comparable associations between s-klotho levels and diabetes status but this study was too small to provide conclusive evidence (n = 62 patients).³³ Further studies are clearly needed to evaluate the potential link between smoking and s-klotho. Overall, the present data derived from LURIC extend our understanding that s-klotho concentrations are not associated with future mortality to a patient cohort which is at intermediate to high risk of cardiovascular events, but without overt chronic kidney disease. Some potential limitations of our study need to be acknowledged. Only few participants of the LURIC study are in the low GFR range (CKD stage IV was present in only 44 patients). Renal function impairment was only assessed via GFR determination, while data upon proteinuria are missing. Additionally, our data solely rely upon measurements of s-klotho, of which specificity is not firmly established. Moreover, these levels may not sufficiently reflect tissue levels of klotho which cannot be obtained and analyzed in the absence of a clinical indication for renal biopsy. Based on the inclusion criteria of the present study we can only assess the impact of s-klotho upon mortality in a diseased population (secondary prevention) while our data do not allow conclusions about cardioprotective effects of elevated s-klotho in healthy people or those at very low cardiovascular risk (primary prevention). Strengths of the current study is its magnitude, homogeneous inclusion criteria, prospective data collection, stringent long-term follow-up and meticulous assessment of clinical endpoints. Moreover a wide range of standard cardiovascular risk factors were captured.

5. Conclusion

In conclusion, higher S-klotho levels are not associated with the long-term risk of death in persons undergoing coronary angiography and it does not modify the strong and established association of FGF23 with mortality. Thus, it is unlikely that s-klotho will qualify as a novel biomarker for cardiovascular risk.

6. Disclosure/conflict of interest

The authors have no conflict of interest to declare.

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**High-Dose Ferric Carboxymaltose in Patients
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High-Dose Ferric Carboxymaltose in Patients with HFrEF Induces Significant Hypophosphatemia.

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Iron deficiency is a frequent comorbidity in up to 50% of patients who have heart failure with reduced ejection fraction (HFrEF).¹ Intravenous iron replenishment (specifically by ferric carboxymaltose [FCM]) increases exercise tolerance and quality of life in HFrEF patients with iron deficiency.¹ FCM is currently graded as a Class IIa recommendation by heart failure guidelines in iron-deficient HFrEF patients.^{2,3} However, preliminary data have linked FCM administration to the development of hypophosphatemia.⁴ Since circulating fibroblast growth factor (FGF)-23 is a key regulator of phosphate and vitamin D metabolism, we sought to determine if FCM modifies FGF-23 levels and how this associates with hypophosphatemia induction. Our single-center prospective interventional pilot trial included 23 stable iron-deficient HFrEF patients (NCT03079518). Patients were divided into groups based on presence or absence of chronic kidney disease (CKD) (cut-off estimated glomerular filtration rate 60 ml/min/1.73 m²): group HFrEF(-)CKD (n = 12), and group HFrEF(+)CKD (n = 11). Iron deficiency was defined as ferritin <100 mg/dl or ferritin 100 to 300 mg/dl and transferrin saturation (TSAT) <20%. Patients received a single dose of 1,000 mg FCM intravenously. We assessed FGF-23 levels by both intact FGF-23 (iFGF-23) as well as the degraded C-terminal FGF-23 (c-termFGF-23) (both by enzymelinked immunosorbent assay) over a period of 28 days. Furthermore, we measured serum phosphate, 1,25-OH vitamin D, parathyroid hormone, tartrateresistant acid phosphatase 5b, and bone-specific alkaline phosphatase as well as fractional excretion of phosphate (FEPi). FCM infusion resulted in a concordant increase in ferritin and TSAT. Baseline serum phosphate, iFGF-23, c-termFGF-23, and FEPi were higher in HFrEF(+)CKD compared to HFrEF(-)CKD patients. iFGF-23 levels rose rapidly in both groups reaching their peak at day 1 (in HFrEF(+)CKD patients by a factor of 1.8; p = NS; in HFrEF(-)CKD patients by a factor of 11; p <0.001). In HFrEF(-)CKD patients, iFGF23 remained significantly elevated until day 28. c-termFGF23 levels decreased constantly toward day 28 in both groups. Serum phosphate significantly decreased only in HFrEF(-)CKD patients (nadir at day 14, mean decrease 0.33 - 0.2 mmol/l compared to baseline) and returned to baseline at day 28. FEPi was induced in both groups, although more in the HFrEF(-)CKD group. Transient hypophosphatemia (<0.8 mmol/l) developed in 14 of 23 patients (9 HFrEF(-)CKD patients and 5 HFrEF(+)CKD). Levels of 1,25-OH vitamin D significantly decreased in both groups by more than 50% with a similar time course as serum phosphate (Table 1). Bone markers parathyroid hormone, tartrate-resistant acid phosphatase 5b, and bone-specific alkaline phosphatase remained stable over the follow-up period (data not shown). We thus show that the currently recommended intravenous iron supplementation in HFrEF patients has a relevant hypophosphatemic effect through increased iFGF-23 with relevant drops in serum phosphate and 1,25-OH vitamin D levels lasting over several weeks in HFrEF(-)CKD patients. HFrEF(+)CKD patients appear partially protected from these effects, presumably caused by lower relative iFGF23 increases and assumed renal Klotho deficiency, which renders the tubular phosphate reabsorption system less responsive to FGF-23. The present findings are in line with previous reports about the hypophosphatemic actions of FCM mediated by iFGF-23 increases in females with a history of heavy uterine bleeding.⁴ Our data obtained in a selected HFrEF cohort are of particular interest because increased FGF-23 is associated with increased cardiovascular mortality;⁵ moreover, FGF-23 in itself may induce or aggravate left ventricular hypertrophy and heart failure. Long-lasting or repetitive

hypophosphatemia and active vitamin D deficiency may further associate with the risk of osteomalacia. Our data emphasize the need to assess long-term safety of repetitive FCM administration in HFrEF patients. It must be determined if the observed derangements in FGF-23-phosphate metabolism may partly outweigh beneficial effects of FCM, particularly with repetitive dosing. Potential negative skeletal effects should also be assessed. Based on our findings, we recommend monitoring of serum phosphate for 2 to 3 weeks after high-dosage FCM infusion. Further studies should investigate if these changes are formulation or dosing effects of intravenous iron. In summary, despite convincing evidence regarding a net symptomatic benefit of FCM therapy in iron-deficient HFrEF patients, we recommend a careful assessment of the (transient) phosphate-FGF-23 derangements after the administration of FCM.

HFref (+)CKD						
	Baseline	Post-Infusion	Day 1	Day 7	Day 14	Day 28
Ferritin, µg/l	45.1 ± 25.9	45.9 ± 24.9	204.9 ± 127.7*	804.4 ± 309.9*	554.1 ± 320.9*	281.9 ± 153.1*
TSAT, %	13.0 ± 6.0	155.1 ± 56.1*	109.3 ± 13.1*	31.6 ± 10.1*	23.7 ± 6.2†	22.4 ± 3.8†
Phosphate, mmol/l	1.22 ± 0.25	1.25 ± 0.26	1.12 ± 0.33	0.97 ± 0.40	1.08 ± 0.47	1.06 ± 0.30
iFGF-23, pg/ml	214.2 ± 188.6	228.4 ± 210.1	384.6 ± 316.2	289.7 ± 335.3	222.7 ± 168.8	169.0 ± 176.3
c-termFGF-23, RU/ml	805.6 ± 535.8	751.1 ± 493.2	756.8 ± 493.2	693.7 ± 499.1	605.4 ± 486.1	452.9 ± 403.1
FEPI, mEq/l	30.9 ± 11.8	31.6 ± 16.4	39.4 ± 17.6	37.7 ± 9.9	42.2 ± 8.8	33.3 ± 9.9
1,25-OH vitamin D, ng/l	30.9 ± 13.5	29.6 ± 14.6	26.8 ± 15.9†	16.2 ± 10.8*	16.7 ± 10.4*	27.9 ± 5.34
25-OH vitamin D, ng/l	16.3 ± 9.6	16.2 ± 9.2	16.2 ± 8.9	16.3 ± 8.8	17.0 ± 8.8	16.4 ± 7.3
GFR, ml/min/m²	28.2 ± 9.1	28.1 ± 7.7	29.9 ± 8.4	29.7 ± 9.9	28.2 ± 8.7	32.3 ± 9.9
Values are mean ± SD. p values are compared to baseline. *p < 0.001. †p < 0.05. ‡p < 0.01.						
CKD = chronic kidney disease; c-termFGF23 = C-terminal FGF23; FEPI = fractional excretion of phosphate; FGF = fibroblast growth factor; GFR = glomerular filtration rate; HFref = heart failure with reduced ejection fraction; iFGF = intact FGF; TSAT = transferrin saturation.						

HFref (-)CKD						
	Baseline	Post-Infusion	Day 1	Day 7	Day 14	Day 28
Ferritin, µg/l	82.9 ± 53.2	74.5 ± 46.8	259.6 ± 55.2*	1,032.0 ± 231.6*	719.1 ± 271.5*	438.9 ± 176.0*
TSAT, %	18.8 ± 10.4	186.1 ± 41.7*	115.8 ± 16.1*	31.6 ± 11.6*	32.1 ± 8.9*	31.2 ± 10.6‡
Phosphate, mmol/l	1.03 ± 0.19	1.09 ± 0.21	1.06 ± 0.21	0.76 ± 0.20†	0.69 ± 0.21‡	0.88 ± 0.20
iFGF-23, pg/ml	17.3 ± 11.0	10.6 ± 11.8	178.9 ± 131.6*	126.5 ± 80.5*	65.9 ± 56.0‡	24.5 ± 23.4
c-termFGF-23, RU/ml	302.5 ± 464.8	329.9 ± 501.5	342.1 ± 424.2	178.2 ± 154.4	83.5 ± 36.1	82.3 ± 99.7‡
FEPI, mEq/l	12.20 ± 3.05	14.30 ± 6.14	18.00 ± 9.79	24.0 ± 12.3	24.9 ± 12.4	19.4 ± 10.3
1,25-OH vitamin D, ng/l	40.5 ± 11.3	40.0 ± 10.6	28.2 ± 8.36*	13.30 ± 6.99*	19.6 ± 12.1*	34.2 ± 14.6
25-OH vitamin D, ng/l	15.4 ± 8.7	14.7 ± 7.9	14.9 ± 8.2	15.2 ± 7.1	14.0 ± 7.2	13.3 ± 6.7
GFR, ml/min/m²	74.7 ± 15.9	76.2 ± 17.4	74.4 ± 17.2	77.3 ± 23.4	81.5 ± 20	80.6 ± 22.6

Table 1: Serum parameters over time

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3.SECTION:
Vitamin K and cardiovascular disease

a)

**Calcific uraemic arteriopathy (calciphylaxis):
data from a large nationwide registry (IF 4)**

Calcific uraemic arteriopathy (calciphylaxis): data from a large nationwide registry.

Brandenburg VM, Kramann R, Rothe H, Kaesler N, Korbiel J, Specht P, Schmitz S, Krüger T, Floege J, Ketteler M. Nephrol Dial Transplant. 2017 Jan 1; 32(1): 126-132.

Abstract

Background: Calcific uraemic arteriopathy (CUA, calciophylaxis) is a rare disease predominantly in dialysis patients and associated with high mortality. Painful skin ulcerations and calcification of cutaneous arterioles characterize calciophylaxis.

Methods: We established an observational, Internet-based registry allowing online notification for all German CUA cases. The registry recorded data about patient characteristics, biochemistry and therapies. Blood samples were stored in a central biobank.

Results: Between 2006 and 2015, 253 CUA patients were recorded: median age 70 [interquartile range (IQR) 61-76] years, 60% females and 86% (n = 207) dialysis patients, translating into an estimated annual incidence rate of 0.04% in German dialysis patients. Fifty-two per cent received vitamin K antagonists (VKAs) prior to CUA. Skin lesions were localized in 71% on the legs or gluteal region. In dialysis CUA patients median total serum calcium was 2.20 (IQR 2.06-2.37) mmol/L, phosphorus 1.67 (IQR 1.35-2.03) mmol/L, intact parathyroid hormone 147 (IQR 72-276) pg/ml and fetuin-A 0.21 (IQR 0.16-0.26) g/L (normal range 0.35-0.95). Median sclerostin, osteoprotegerin, TRAP5b, bone-specific alkaline phosphatase and c-terminal FGF23 levels were all elevated. The most frequently recorded therapeutic procedures in dialysis CUA patients were as follows: wound debridement (29% of cases), stopping VKA (25%), lowering calcium supply (24%), sodium thiosulphate (22%), application of vitamin K (18%), increase of dialysis duration/frequency (17%) and stopping active vitamin D (16%).

Conclusions: Approximately 50% of CUA patients used VKA. Our data suggest that uncontrolled hyperparathyroidism is not the key determinant of calciophylaxis. Therapeutic strategies were heterogeneous. The experience of the German registry will help substantially to initiate a large-scale multinational CUA registry. Keywords: anticoagulation, calcification, calciophylaxis, cardiovascular, ESRD

1. Introduction

Calcific uraemic arteriopathy (CUA), also known as calciophylaxis, is a rare disease (ORPHA292147) predominantly affecting patients with end-stage renal disease (ESRD) on dialysis.^{1,2} The exact incidence and prevalence are unknown. Although rare, two clinical and aetiological aspects qualify CUA as an exceptional research target in chronic kidney disease-mineral and bone disorder (CKD-MBD).³ First, the unmet clinical need of how to prevent and how to treat calciophylaxis is urgent since the condition is clinically devastating: debilitating pain, potentially large areas of skin ulcerations and markedly reduced quality of life dominate the clinical picture. Moreover, CUA is associated with a massive reduction in longterm survival.^{1,2,4} Second, CUA might serve as a potential 'high-speed' template for general cardiovascular calcification processes in CKD involving larger arteries or heart valves.

Therefore, CUA research might add valuable insights into the causative pathways of accelerated uraemic calcifying arteriosclerosis, since circumferential calcification in the medial layer of (in this case small cutaneous) arteries is also the histological hallmark of CUA.⁵ Unfortunately, CUA treatment options are not evidencebased since prospective randomized controlled trials are barely possible in such a devastating, rare disease.^{1,2} Overcoming these limitations is not easy since centralizing clinical as well as research expertise in the field of CUA is difficult: the severe clinical condition and the high degree of comorbidities in the often elderly CUA patients complicate establishing tertiary referral centres (centres of expertise), requiring frequent long-distance travel. As an alternative, we established an Internet-based registry in 2006 (<http://www.calciophylaxie.de>). Within this online registry, we invited treating physicians to register all cases of established or suspected CUA. The registry consists of a comprehensive database of various parameters, including patient characteristics, laboratory data, clinical background and presentation as well as therapeutic strategies. The aim of the registry was to collect data on potential risk factors and good clinical practice as well as the creation of a biobank allowing research biomarker analyses in the core facility [University Hospital Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen]. The aim of the present analyses is to provide a detailed and comprehensive summary of the first 9 years of data collection within the German calciophylaxis registry.

2. Methods

The German calciophylaxis registry is accessible at www.calciophylaxie.de. It was established in December 2006 (first patient in). The nature of the registry is non-interventional and purely observational. For study participation, written informed consent of the patient is required. The storage of patient data is anonymous. The registry activity was approved by the University Hospital RWTH Aachen ethical committee (vote no. 10-024). Most of the query items allow simple selection via dropdown menus. In contrast, the field with the question about applied treatment strategies allows a free text answer, since drop-down list might incompletely reflect the real-world situation. Moreover, such a pre-selection may have created the impression of plausibility or a preference for a certain intervention.

Incoming data underwent centralized plausibility checking and quality control. Numerical outliers were double checked with the peripheral centre, and in case of doubts, ambiguous data, implausible data or incomplete data, such issues were discussed with the peripheral centre and the treating physician locally in charge. In case such a request could not clarify implausible data, such data or the entire data set was deleted from the database. Whenever possible, photo documentation and transfer of the images to the registry centre in Aachen was requested to allow second-opinion evaluation. The registry team at the Aachen University Hospital requested full blood, plasma and serum sampling of the patients according to standard procedures and asked for immediate freezing at the peripheral study site. Long-term storage was done at -80°C immediately after arrival at the University Hospital Aachen. The registry activities were supported by an unrestricted grant from Amgen (2006 until present) and Sanofi (2012 until present). The registry is under the patronage of the German National Society of Nephrology (DGfN) and the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) CKD-MBD Scientific Working Group. Marketing activities were undertaken by the study team to increase awareness of the registry and to increase motivation for participation. At the start of the registry, Amgen field staff distributed flyers about the aims and scope of the registry to virtually all German nephrologists. Since then, the study team has regularly presented data summaries at annual national as well as international nephrology congresses.

2.1. Laboratory measurements

The registry recorded online levels of serum albumin, total calcium, alkaline phosphatase, haemoglobin, serum creatinine, inorganic phosphate, intact parathyroid hormone (iPTH), 25-hydroxyvitamin D [25(OH)D, calcidiol] and total protein. For those parameters, standard procedures were applied according to the laboratory routine in the peripheral centres. Total serum calcium was corrected for albumin content. In order to increase comparability between biochemistry results, centralized laboratory analysis was performed using Aachen biobank samples for the following CKD-MBD parameters: iPTH, C-reactive protein (CRP) and calcidiol [25(OH)]. Additionally, non-routine (research) CKD-MBD parameters were measured from the same biobank samples: fetuin-A, osteoprotegerin (OPG), sclerostin, tartrate-resistant alkaline phosphatase 5b (TRAP5b), bone-specific alkaline phosphatase (BSAP) and c-terminal fibroblast growth factor 23 (c-FGF23). Commercially available enzyme-linked immunosorbent assays (ELISAs) were used to determine levels of fetuin-A, OPG and BAP (TECOmedical AG, Sissach, Switzerland). PTH was measured as iPTH by an assay provided by Biomerica (Irvine, CA, USA). Serum sclerostin was assessed by the TECO Sclerostin EIA Kit, which is a 96-well immunocapture ELISA product. TRAP5b was assessed via ELISA (Quidel, San Diego, CA, USA). c-FGF23 was measured in plasma by an assay from Immutopics (San Clemente, CA, USA). CRP was measured by ELISA (Biomerica).

2.2. Statistical analysis

Descriptive statistics were performed for the entire cohort as well as for subgroups of patients: separate analyses were done for dialysis patients, for peritoneal dialysis (PD) patients and for haemodialysis (HD)/haemodiafiltration (HDF) patients stratified for gender. Results are expressed as number (%) for categorical variables, as mean $\pm$ SD for normally distributed continuous variables and as median [interquartile range (IQR)] for continuous

variables with skewed distribution. For differences between groups, Student's t-test and the Mann-Whitney U test were performed for normally and non-normally distributed variables, respectively. The statistical analysis was performed with SPSS 21 (IBM, Armonk, NY, USA).

3. Results

3.1. The entire registry cohort: clinical data

From December 2006 to March 2015 (100 months), 265 patients were recorded. Twelve patients were deleted from the database after negative plausibility control and feedback discussion with the referring physician revealing low probability of CUA or after an alternative diagnosis was established. Overall, 253 patients were finally classified as calciphylaxis. The median age of the CUA patients was 70 years (IQR 61-76; minimum 21 and maximum 88). The male/female ratio was 40-60%. Most (n = 252) patients were Caucasian, and one patient was Asian. The median annual notification rate in the complete years 2007-2014 was 30 cases per year [ranging from 24 (2012) to 35 (2009) per year]. In 210 cases (83%), a date for the occurrence of first symptoms was recorded, hence allowing calculation of days between onset of the disease and online notification to the registry. The median time interval was 28 days (IQR 13-60). Overall, 19 centres recorded more than one patient into the registry. The maximum of registered patients per centre was five patients (period from 2008 to 2015). A skin biopsy was performed in 45% of patients, whereas in 55% the diagnosis was established without histological examination of the specimen. In 88% (n = 222) of cases, the treating physician stated the predominant anatomic site of cutaneous CUA manifestation. The dominant lesions were located on the legs in 179 (71%) cases. In the vast majority of these cases, the thighs were affected either alone or in combination with the lower legs. The trunk was the predominant location in 45 (18%) cases, with the abdominal wall and the hips being the most frequent locations. In 12% of cases, a dominant anatomic location was not recorded. The character of the skin lesions was described as ulcerative in 63% versus non-ulcerative in 37% [rate of return n = 199 (79%)]. In 66 (26%) cases, the referring physician speculated about the presence of an acute triggering event prior to calciphylaxis development. Physical trauma (e.g. fall, subcutaneous injections, injury, surgical wound, compression trauma and haematoma) was the single most recorded triggering event (n = 27). The referring physician was asked to classify the presence/absence and degree of kidney disease prior to CUA development into categories (Table 1).

Status of kidney disease prior to CUA development (n = 253)	n (%)
Normal or mildly impaired renal function	7 (3)
CKD, non-dialysis	18 (7)
Functioning kidney graft	10 (4)
PD	25 (10)
Dialysis (HD + HDF)	193 (76)
ESRD (dialysis + transplantation)	228 (90)

Table 1: Stage of kidney disease at the time of CUA development

The majority of patients were ESRD patients. Cardiovascular comorbidities at baseline are listed in Supplementary data, Table S1, among which arterial hypertension, diabetes mellitus and coronary heart disease were most frequently recorded. In 250 patients, data on previous or ongoing vitamin K antagonist (VKA) use at the time of CUA development were available. The overall proportion of patients on VKA was 130 (52%) versus 120 (48%) without previous VKA use (Supplementary data, Table S2). The patients were stratified according to the VKA prescription 'yes' versus 'no'. Those two groups did not reveal statistically significant differences in terms of anthropomorphic data, clinical data or biochemistry (data not shown).

Comorbidity (n=253)	yes, n (%)	no, n (%)
Arterial hypertension	224 (89%)	26 (10%)
Diabetes mellitus (type 1/2)	137 (55%)	115 (45%)
Coronary heart disease	123 (49%)	78 (31%)
Peripheral vascular disease	99 (39%)	111 (44%)
Cerebrovascular disease	50 (20%)	182 (72%)
History of myocardial infarction	48 (19%)	192 (76%)
Any bone fractures	26 (10%)	221 (87%)

Table S1: Overview about the prevalence of comorbidities (% related to n = 253 pts; in the remaining patients the question was not answered or answered with "unknown")

VKA usage in subgroups of CUA patients (n=250)	VKA: yes	VKA: no
	n (%)	n (%)
Total	130 (52%)	120 (48%)
Chronic kidney disease, non-dialysis	8 (44%)	10 (56%)
Normal or mildly impaired renal function	3 (43%)	4 (57%)
Functioning kidney graft	6 (60%)	4 (40%)
Peritoneal dialysis (PD)	15 (62.5%)	9 (37.5%)
Dialysis (HD + HDF), male (n= 71)	33 (46%)	38 (54%)
Dialysis (HD + HDF), female (n=120)	65 (54%)	55 (46%)

Table 2S: Proportion of VKA users in the entire CUA population (*data missing for 3 patients)

3.2. The subgroup of CUA dialysis patients

For further analysis, especially regarding laboratory values and background medication usage, we focussed on dialysis patients. The group of dialysis patients was defined as HD/HDF patients (n = 193) and PD patients (n = 25) [n = 218 (86% of the entire cohort)]. The HD/HDF patient group was split into males and females for further analysis. Table 2 provides an overview of the clinical data for the group of dialysis patients. We also recorded prescription of prototypic nephrology medication. Table 3 gives an overview of medication prescriptions in the dialysis subgroup of the entire cohort. The laboratory values of the dialysis CUA patients are depicted according to the place of measurement, either in a peripheral treatment centre (Table 4) or results were obtained from the central biobank after measurement in the core lab (Table 5). In 180 dialysis patients, both albumin and total calcium levels were available, allowing for calculation of albumin-corrected calcium. Biochemistry data regarding calcium, phosphate and iPTH were stratified according to target ranges defined in the Kidney Disease Outcomes Quality Initiative (KDOQI) or Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (Supplementary data, Table S3 and Table 6). We analysed the recorded data from the free text field regarding therapeutic strategies in the questionnaire. Data were recorded in 165 cases (76%; in the other 24% of patients no data were recorded) (Table 7).

Parameter	All HD/HDF patients, <i>n</i> = 193	PD, <i>n</i> = 25	Male HD/HDF, <i>n</i> = 72	Female HD/HDF, <i>n</i> = 121
Age (years)	70 (62–76)	64 (55–74)	69 (61–76)	71 (64–77)
Caucasians	All	All		
Females	121 (63%)	12 (48%)		
Time between onset to diagnosis (days)	24 (11–50)	24 (12–42)	30 (14–60)	21 (10–46)
Time interval since start of dialysis to CUA diagnosis (months)	30 (7–56)	46 (29–69)	25 (4–50)	34 (7–64)
Kt/V ^a	1.4 (1.3–1.7)	n.a.	1.3 (1.2–1.5)	1.5 (1.3–1.7)
Previous fracture	23 (12%)	2 (8%)	5 (7%)	17 (14%)
Previous parathyroidectomy	19 (10%)	4 (16%)	6 (8%)	13 (11%)

Values are given as median (IQR) or *n* (%).

^aKt/V: multiple of the volume of plasma cleared of urea divided by the distribution volume of urea.

Table 2 Clinical parameters in the dialysis patients with calciophylaxis (*n* = 218)

Medication ^a	All HD/HDF, patients, n = 193		PD, n = 25		Male HD/HDF, n = 72		Female HD/HDF, n = 121	
	Yes	No	Yes	No	Yes	No	Yes	No
Active vitamin D (calcitriol, paricalcitol, others)	102 (53%)	87 (45%)	15 (60%)	9 (36%)	41 (57%)	30 (42%)	61 (50%)	57 (47%)
Cinacalcet	50 (26%)	137 (71%)	12 (48%)	12 (48%)	20 (28%)	49 (68%)	30 (25%)	88 (73%)
Any phosphate binder (PB) ^b	149 (77%)	41 (21%)	20 (80%)	4 (16%)	56 (78%)	15 (21%)	93 (77%)	26 (21%)
Calcium-containing PB ^b			11 (44%)		19 (26%)		35 (29%)	
Sevelamer ^b			7 (28%)		21 (29%)		31 (26%)	
Lanthanum carbonate ^b			1 (4%)		13 (18%)		18 (15%)	
Other PB ^b			2 (8%)		7 (10%)		20 (17%)	
VKAs	98 (51%)	93 (48%)	15 (60%)	9 (36%)	33 (46%)	38 (53%)	65 (54%)	55 (45%)
Erythropoietin and other	160 (83%)	29 (15%)	21 (84%)	2 (8%)	57 (79%)	13 (18%)	103 (85%)	16 (13%)
Erythropoiesis-stimulating agents, ESAs								

^aDue to missing values, patient figures may be smaller than the entire subgroup and cumulative percentage figures may not reach 100%.

^bSingle use as well as part of combination therapy.

Table 3: Medication use in the group of dialysis patients at the time of CUA development

Parameter (unit)	Normal range	All HD/HDF patients, <i>n</i> = 143–184	PD, <i>n</i> = 19–25	Male HD/HDF, <i>n</i> = 51–70	Female HD/HDF, <i>n</i> = 92–114
Albumin (g/L)	35–55	34 (29–38)	29 (24–34)	34 (29–40)	35 (29–38)
Alkaline phosphatase (U/L)	65–220	112 (80–168)	100 (91–133)	108 (73–158)	112 (81–168)
Total calcium (mmol/L)	2.30–2.60	2.20 (2.06–2.37)	2.20 (2.05–2.28)	2.20 (2.10–2.37)	2.22 (2.06–2.40)
Haemoglobin (g/L)	male: 14–18 female: 12–16	103 (94–115)	96 (87–104)	105 (96–122)	102 (94–111)
Serum phosphorus (mmol/L)	0.77–1.55	1.65 (1.31–2.04)	1.80 (1.38–2.00)	1.65 (1.42–2.13)	1.67 (1.30–2.02)
Protein (g/L)	66–83	66 (60–71)	65 (60–70)	68 (62–71)	64 (58–70)

Values are given as median (IQR); *n* indicates the number of patients in whom the biochemical parameters were recorded (*n* = *a* – *b* indicates the range between the minimum and maximum numbers of patients with available data for each parameter).

Table 4: Locally obtained biochemical parameters at the time of blood drawing after online notification in dialysis CUA patients (*n* = 218)

Parameter	All HD/HDF patients, n = 123	PD, n = 14	Male HD/HDF, n = 45	Female HD/HDF, n = 78
Study centre research parameters ^a				
Fetuin-A (g/L)	0.21 (0.16–0.27)	0.17 (0.16–0.22)	0.21 (0.17–0.28)	0.22 (0.16–0.28)
iPTH (pg/mL)	143 (66–275)	201 (115–329)	167 (87–275)	130 (62–272)
Calcidiol, 25(OH)D (ng/mL)	22.3 (11.7–34.3)	15.5 (12.1–20.3)	24.8 (12.2–32.3)	20.8 (11.2–33.4)
OPG (pmol/L)	19.2 (15.1–23.8)	20.4 (14.8–27.2)	19.2 (16.0–23.9)	18.9 (15.1–23.3)
Sclerostin (ng/mL)	0.94 (0.64–1.53)	1.51 (0.84–2.3)	1.01 (0.68–1.54)	0.94 (0.61–1.54)
TRAP5b (U/L)	3.8 (2.6–5.3)	5.0 (4.1–9.4)	3.8 (2.6–4.8)	3.7 (2.6–5.7)
Bone-specific AP (U/L)	36.5 (25.8–57.2)	33.8 (27.5–54.9)	39.0 (20.3–65.3)	36.0 (27.9–55.5)
c-FGF23 (RU/mL)	1760 (819–9092)	6709 (4546–18 977)	2796 (583–11 481)	1592 (833–7643)
CRP (mg/L)	43.9 (12.3–99.3)	84.4 (22.3–173.8)	53.1 (13.6–105.5)	38.5 (11.8–92.8)

^aNormal ranges or mean values according to the manufacturer's instructions: fetuin-A: 0.35–0.95 g/L; iPTH: 10.4–66.5 pg/mL; calcidiol: >30 ng/mL (target range); OPG: 5.7 ± 0.42 pmol/L; sclerostin: pre-menopausal 0.59 ± 0.23 ng/mL, post-menopausal 0.66 ± 0.22 ng/mL, male 0.83 ± 0.22 ng/mL; TRAP5b: pre-menopausal 0.54–3.23 U/L, post-menopausal 1.15–4.14 U/L, male 0.61–3.45 U/L; bone-specific AP: pre-menopausal 11.6–29.6 U/L, post-menopausal 14.2–42.7 U/L, male 15.0–41.3 U/L; c-FGF23: pre-menopausal 20.9–91.1 RU/L, post-menopausal 44.0–139.9 RU/L, male 33.7–96.5 RU/L; CRP: 0.07–8.2 mg/L.

Table 5: Centrally obtained (Aachen biobank) biochemical research parameters at the time of blood drawing after online notification in dialysis CUA patients

Parameter	n	Target range KDIGO ^a	Below	Within target range	Above target range
Serum calcium	207	2.30–2.60 mmol/L	133 (64%)	65 (31%)	9 (4%)
Albumin-corrected calcium	180		74 (42%)	79 (44%)	27 (15%)
Serum phosphorus	201	0.77–1.55 mmol/L	12 (6%)	68 (34%)	121 (60%)
iPTH, study core lab	138	133–600 pg/mL ^b	65 (47%)	65 (47%)	8 (6%)
iPTH, peripheral centre	197	n.a. ^c	n.a.	n.a.	n.a.

^aKDIGO suggests normal range for calcium and phosphate as the target range.
^bTarget range is defined as PTH levels between 2- and 9-fold of the upper limit of normal range (=66.5 pg/mL).
^cCalculation of the target range is not applicable since assays and consecutively normal PTH levels vary between centres.

Table 6: Proportion of dialysis patients (PD, HD and HDF) within and outside biochemistry target ranges according to KDIGO guidelines 2009 (serum calcium and phosphorus were measured in transferring centres, iPTH was measured in a core lab) (laboratory data were obtained at the time of registry inclusion)

Intensifying dialysis therapy	
Increase of dialysis duration and/or frequency	16.9%
Switch from haemodialysis to haemodiafiltration	0.8%
Switch from PD to HD	1.5%
Reduction of calcium supply/intake	
Lowering/stopping of calcium-containing PB	23.8%
Lowering dialysis bath calcium concentration	1.5%
Reduction/stop active vitamin D treatment including VDRA	16.2%
Vitamin K metabolism	
Stop VKA treatment	25.4%
Give vitamin K	17.7%
Secondary hyperparathyroidism	
Initiate cinacalcet	10.8%
Stop cinacalcet	2.3%
Parathyroidectomy	2.3%
Initiate native vitamin D	2.3%
Calcification inhibition	
Apply STS	21.5%
Give bisphosphonates	2.3%
Fresh frozen plasma	1.7%
Tissue oxygen supply	
Revascularization (surgical and angioplasty)	3.8%
Antibiotics	16.1%
Surgical wound management	
Including necrosectomy, debridement and skin transplantation	29.2%
Others	
Application of glucocorticoids	3.1%
Prostaglandin infusion	0.7%

Table 7: Recorded therapeutic strategies in 165 CUA dialysis patients (76% of the entire dialysis CUA cohort) [multiple answers possible; single answers in n = 16 (10%) cases]

4. Discussion

We report data from a long-term nationwide online CUA registry that represents the largest CUA cohort documented so far. Overall, the German CUA registry allows presentation of far-reaching data regarding the clinical picture, additional background data, comprehensive data on biomarkers (CKD-MBD standard as well as research biomarkers) as well as applied therapeutic strategies. We recorded a stable notification rate of about 30 dialysis patients per year, which equals an annual incidence of about 0.04%, assuming that the number of German dialysis patients has been about 70 000 during the registry period. Thus, our data do not support estimated incidence figures as high as 4-5% among dialysis

patients.⁶ Also, we need to acknowledge that we have to accept a potentially high estimated number of unreported cases. Our cohort of CUA patients is characterized by a striking predominance of dialysis patients. It is generally accepted that non-renal CUA is even rarer than dialysis-related CUA.⁷ We acknowledge that our registry and our scientific activities primarily address the German nephrology community, and therefore we might have missed cases seen by other medical specialists. Additionally, lack of awareness of the disease might have contributed to a difficult to quantify number of hidden cases. On the other hand, our registry approach might carry the risk of including a few false-positive cases, which cannot be excluded completely due to the absence of systematic diagnostic standards for calciophylaxis and decentralized patient care within the registry. All available data, including the present registry, indicate that ESRD predisposes patients to develop CUA. The registry allows comprehensive analyses of CKD-MBD parameters; at the time of CUA diagnosis, serum calcium levels in affected dialysis individuals were clinically unremarkable. Total serum calcium levels were indeed quite low, looking at the distribution of calcium levels according to the historical KDOQI target ranges or according to the current KDIGO target ranges. Hypercalcaemia above the upper limit of normal (2.60 mmol/l) was present in only 15% of CUA dialysis patients in terms of albumin-corrected calcium. In contrast, serum phosphate levels were more often above target ranges (42% for the KDOQI range and 60% for the KDIGO range), reflecting the well-documented prevalence of hyperphosphatemia in dialysis patients. Of note, these laboratory data were obtained about 1 month after CUA development. Hence, the current disease burden and nutritional status may have influenced the recorded calcium and phosphate levels. More strikingly, the PTH levels in our dialysis CUA cohort were unexpectedly low, which deserves thorough comments and further evaluation. Relatively low PTH levels are in line with previous case-control series, which could not document uncontrolled hyperparathyroidism being a risk factor for CUA development.¹ The fact that our CUA cohort exhibited low PTH levels is also reflected by the low rate of co-medication with cinacalcet in our cohort: 71% of our patients did not receive cinacalcet at the time of CUA diagnosis. Adynamic bone disease might predispose to CUA development since the buffering capacity for an excess of circulating minerals is thought to be impaired [8]. One potential reason for low PTH levels in dialysis patients is oversuppression of hyperparathyroidism prior to CUA development. However, such a discussion about iatrogenic adynamic bone disease remains speculative in the absence of bone histomorphometry and serial bone metabolism data. Both BSAP and TRAP5b markedly exceeded the normal range in our cohort, but such levels cannot reliably exclude the presence of localized transient osteoporosis. Our notion of overall predominantly 'low' PTH levels in the CUA cohort is not in contrast with findings from the Evaluation of Cinacalcet Hydrochloride Therapy to Lower Cardiovascular Events (EVOLVE) trial,⁹ indicating that cinacalcet treatment was associated with a significant decrease of CUA incidence compared with the control arm. In the EVOLVE trial, the median PTH levels prior to CUA development were 796 pg/ml in the placebo arm and 410 pg/ml in the cinacalcet arm. Of note, the EVOLVE trial included preselected patients with advanced hyperparathyroidism at baseline, while a nationwide approach such as the registry is unlimited by any exclusion criteria. So, the EVOLVE design presumably missed the predominant PTH range for calciophylaxis development and fuels speculation about an

optimal intermediate (protective) PTH range. Taken together, for all classical CKD-MBD parameters (calcium, phosphate, calcium-phosphate product and PTH), further CUA studies should investigate the time course (trend analysis) in the months prior to CUA. Interpretation of the innovative research biomarkers is limited due to the absence of a control group. Remarkably, fetuin-A levels were much below the normal range in our CUA dialysis patients. Deficiency of fetuin-A may be expected in a dialysis cohort with high CRP levels and overall low protein and albumin levels. Cause or effect of low fetuin-A levels cannot be established from our data, and both interpretations are possible. Interestingly, we also observed median sclerostin levels above the normal range in our CUA patients. The potential role of sclerostin as a marker or mediator of vascular calcification is a matter of ongoing debate.¹⁰ If sclerostin alone or in combination with the various alternative bone markers, which proved to be above the normal range in our calciophylaxis cohort (OPG, BSAP and TRAP5b), might help in identifying patients at particular risk for developing calciophylaxis remains undetermined.

The high prevalence of VKA treatment is striking. Vitamin K activates matrix Gla protein (MGP) via gamma-carboxylation.¹ Fully active MGP is a prerequisite for maintaining vascular wall integrity by avoiding calcification. Hence, the application of a VKA such as warfarin and phenprocoumon (most often Marcumar® in Germany) is thought to interfere with calcification defence mechanisms and promote vascular wall calcification.¹¹ There are no confirmed data available about the exact prevalence of a VKA application in dialysis patients in Germany, but valid estimates point towards about 10% of patients. This suggests that our 50% VKA treatment prevalence might be a factor of 5-fold increased prevalence of VKA in CUA patients compared with non-CUA dialysis patients. This finding is in line with a previous report from Japan that calculated a factor (hazard ratio) of 10 according to a case-control approach.¹² We should point out that the assumption of VKA being causally linked to CUA development has already influenced treatment strategies (see below). A recent state-of-the-art review by Nigwekar et al.¹³ nicely summarized current treatment strategies in CUA patients, showing several analogies, but also discrepancies, with our registry data—the latter reflecting the real-world treatment situation in Germany. Overall, our data confirm the clinical experience that treatment is multimodal. In terms of homologues, wound care (noninvasively and surgically) is a mainstay of therapy, accompanied by systemic antibiotic therapy. Real-world care considers a reduction in calcium supply via reduced oral intake, less active vitamin dosages and lowering dialysis bath calcium as effective. PTH-lowering interventions (cinacalcet and parathyroidectomy) are a rarely used treatment option in Germany. German nephrologists increased the frequency and duration of dialysis sessions in a substantial proportion of cases, although CKD-MBD parameters were formally ‘controlled’ (with the exception of highly prevalent hyperphosphatemia). Modification of the anticoagulation regime by stopping VKA and/or vitamin K replenishment was a common treatment option.¹⁴ Pain management and other supportive care measures such as nutritional consulting have not been regularly recorded in the German registry. We speculate about ‘false-negative’ results here because physicians might not have regarded this as specific CUA interventions.

The issue of sodium thiosulphate (STS) application warrants some additional comments. Case series published, e.g. by Nigwekar et al.,¹⁵ have fuelled the discussion about

potentially positive effects of STS in CUA patients. Accordingly, STS is part of the treatment summary provided by Nigwekar et al.¹³ However, evidence regarding a positive risk: benefit ratio of STS in CUA is low, given the fact that no controlled, prospective data are available.¹ STS is indeed applied in about a fifth of patients in Germany. We speculate that this percentage is a reliable estimate of the real-world situation (meaning a low number of false-negative recordings), as physicians might be especially prepared and motivated to communicate STS as specific CUA treatment. Regarding CUA treatment, we cannot draw conclusions about any therapeutic effect of any intervention, since systematic follow-up visits were not part of the registry protocol. Hence, it remains speculative if any single intervention (such as the application of STS) or any multimodal treatment approach influences outcomes in these patients. A relevant limitation of our registry is the cross-sectional nature with single-spot recording of data—so, we cannot comment on biomarker trends and upcoming warning signs prior to CUA outbreak. The latter is of particular interest in terms of average low PTH levels, which we recorded at the time of diagnosis. Moreover, the registry did not record long-term outcomes in terms of potential treatment effects or survival. The international European Calciphylaxis Network initiative (<http://www.eucalnet.net>)—a multinational European calciphylaxis registry—is aimed at biochemistry trend analyses and outcome assessment to overcome this limitation [16]. In summary, we consider three issues raised by the registry as important. Our data confirm that CUA qualifies as a truly rare disease. A novel finding is that median PTH levels among dialysis patients are low. VKA usage is highly prevalent in CUA patients, pointing towards the need to investigate the potential beneficial effects of early VKA withdrawal and vitamin K replenishment.

5. Supplementary data

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

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b)
Calciophylaxis (IF 55)

Calciophylaxis.

Nigwekar SU, Thadhani R, Brandenburg VM. N Engl J Med. 2018 May 3; 378(18): 1704-1714.

Calciphylaxis is a rare, life-threatening syndrome of vascular calcification characterized by occlusion of microvessels in the subcutaneous adipose tissue and dermis that results in intensely painful, ischemic skin lesions. Once calciphylaxis has been diagnosed, the prognosis is generally poor (survival, <1 year).¹⁻³ The disorder, which is underrecognized,⁴ typically affects patients with end-stage renal disease (ESRD),^{2,5} a population with a high prevalence of extraskeletal calcifications. A clear majority of such calcifications do not represent calciphylaxis, which cannot be placed on a simple continuum of vascular calcification. However, an improved understanding of vascular calcification has helped to elucidate the pathogenesis of calciphylaxis and promising approaches to treatment. Calciphylaxis also occurs in patients with earlier stages of chronic kidney disease,^{2,5} acute kidney injury,⁶ or prior receipt of a kidney transplant,⁷ and in rare cases, it occurs in patients with normal kidney function.^{2,5,8,9} We therefore prefer the name calciphylaxis to calcific uremic arteriolopathy, which is another name for this entity.¹⁰ This review presents the current understanding of calciphylaxis and provides a framework for its interdisciplinary management.

Clinical Manifestations

Calciphylaxis causes painful skin lesions (Figures 1A through 1H). The pain is somatic and may precede the appearance of skin lesions.¹¹ Although the pain may initially fluctuate in intensity,¹² it characteristically remains severe during the course of the disease and is frequently accompanied by tactile hyperesthesia.¹¹ The initial skin manifestations may include induration, plaques, nodules, livedo, or purpura.^{12,13} A dusky discoloration of the skin indicates an area of imminent necrosis. Because an arteriole supplies a 1-to-3-cm conical area of microvasculature in a funnel shape, there are frequently reticulate (netlike) areas of erythema (Figure 1C) and livedo. Patients typically have multiple, bilateral lesions, with surrounding skin showing a leatherlike texture. The initial lesions rapidly progress to stellate, malodorous ulcers with black eschars.^{12,13} Sepsis originating from the resultant wounds is considered the most common cause of death.^{8,14,15} Approximately 50% of patients are bedridden or wheelchair-bound, and more than 70% require hospitalization for severe ulcers.¹⁴ Ongoing pain, anorexia, insomnia, and depression further compromise the quality of life.¹⁶ Extraskeletal calcifications (Figures 1I and 1J) are often found on imaging studies, but the sequelae of cutaneous involvement are the predominant clinical manifestations. On rare occasions, extracutaneous vascular calcifications lead to skeletal myopathy, intestinal bleeding, or visual impairment.¹⁷

Figure 1: Clinical Manifestations, Radiographic Features, and Histologic Characteristics of Calciphylaxis. Panels A through E show the early manifestations of calciphylaxis: a violaceous patch with surrounding retiform purpura (Panel A), a palpable subcutaneous nodule with overlying erythema (Panel B), a reticulate pattern of erythema (Panel C), an induration with dusky discoloration (Panel D), and multiple plaques with livedo (Panel E). Panels F, G, and H show late manifestations: a necrotic ulcer partially covered with a black eschar (Panel F), a leg with a mummified appearance (Panel G), and gangrenous fingers (Panel H). Panels A, B, E, and F show central involvement, and Panels C, D, G, and H show peripheral involvement. In Panel I, a mammogram from a patient with calciphylaxis involving the breast shows microvascular calcifications (arrows) and fat necrosis (oval). In Panel J, microvascular calcifications in a netlike pattern (arrow), subcutaneous extravascular calcifications, and a calcified femoral artery are evident on a radiograph from a patient with ulcerated calciphylaxis involving the thigh. A skin-biopsy section stained with hematoxylin and eosin (Panel K) shows coarse basophilic calcification, fibrointimal hyperplasia, and fibrin thrombus in dermal and subcutaneous microvessels with septal panniculitis. A skin-biopsy section with von Kossa stain (Panel L) shows fine arteriolar and interstitial calcifications. Pathological and radiographic findings by themselves are not diagnostic of calciphylaxis and should be clinically correlated.

Clinical Classification

Calciphylaxis can be classified as uremic (in patients with ESRD) or nonuremic (in patients with normal renal function or earlier stages of chronic kidney disease). The lesions in affected patients can be classified as central (involving central areas within subcutaneous adipose tissue such as the abdomen or thighs) or peripheral (restricted to peripheral sites that have limited adipose tissue, such as the digits). In addition, lesions can be nonulcerated (in the earlier stages of the disease) or ulcerated (in later stages). The lesions are similar whether or not patients have kidney disease; however, 70 to 80% of lesions in patients with ESRD have a central distribution,^{1,14} as compared with approximately 50% of lesions in patients who do not have ESRD.^{8,14} Patients without ESRD have a better prognosis (1-year mortality, 25 to 45%)^{8,9} than those who have ESRD (1-year mortality, 45 to 80%)¹⁻³, probably because of the differences in coexisting conditions and the location of lesions. Patients with central lesions are more likely to have a high body-mass index than patients without central lesions,¹ are more likely to be women,¹ and have a higher risk of death.¹⁸ The presence of ulcerated (late) lesions reduces the 6-month survival rate to 20%.³ Histopathological Features Characteristic histologic features of calciphylaxis (Figures 1K and 1L) include calcification, fibrointimal hyperplasia, and thrombosis in microvessels in the subcutaneous adipose tissue and dermis, often accompanied by necrosis of epidermal and adipose tissue, dermal-epidermal separation, panniculitis, proliferation of dermal endothelial cells, and extravascular calcifications.^{19,20} The mean diameter of the involved microvessels is 100 μm (range, 40 to 600).¹⁵ Calcified lesions consist of calcium and phosphate in a molar ratio of 1.7, which matches that of hydroxyapatite.²¹

Epidemiology

Calciphylaxis, although rare, has been reported worldwide, with an estimated annualized incidence of 35 cases per 10,000 patients undergoing hemodialysis in the United States,¹ 4 per 10,000 in Germany,⁵ and less than 1 per 10,000 in Japan.²² A report from the United States suggests an increasing incidence,²³ which may be due to an actual increase, heightened awareness of the disorder, or both. The interval from the initiation of dialysis to the appearance of calciphylaxis ranges from 30 months in the United States¹ and Germany⁵ to 105 months in Japan.²⁴ Patients treated with peritoneal dialysis have a higher incidence of calciphylaxis than those treated with hemodialysis;^{25,26} however, the underlying mechanisms are unclear. The incidence in kidney-transplant recipients and in patients with earlier stages of chronic kidney disease is unknown. Several reports suggest that the mean age at the time of diagnosis is 50 to 70 years;^{2,5,14,25} very few patients are children.²⁷ Approximately 60 to 70% of patients with calciphylaxis are women.^{3,5,14} In a U.S. study involving 1030 patients with calciphylaxis who were dependent on hemodialysis, 49% were white and 28% were black,¹ a racial distribution approximating that of patients undergoing hemodialysis in the United States.

Risk Factors

Table 1 lists risk factors for calciphylaxis,^{1,8,17,25,28-31} which include obesity, diabetes mellitus, female sex, and dependence on dialysis for more than 2 years. Obesity and diabetes mellitus are also risk factors for calciphylaxis in patients without ESRD.^{8,9} Elevations in phosphate and calcium levels increase the risk of subsequent calciphylaxis in patients undergoing dialysis.¹ Patients with calciphylaxis have high parathyroid hormone (PTH) levels at the start of treatment with dialysis,¹ and primary hyperparathyroidism⁸ and administration of recombinant PTH³¹ are risk factors for calciphylaxis in patients who do not have ESRD. At clinical presentation, however, approximately 45% of dialysis-dependent patients with calciphylaxis have PTH levels below the recommended target range.⁵ These observations suggest overuse of calcium and vitamin D supplements, leading to PTH suppression and adynamic bone (low bone turnover), which may exacerbate extraskelatal calcium depositions.³² Fibroblast growth factor 23 (FGF-23), a phosphaturic hormone, is increased in patients with ESRD and calciphylaxis;⁵ however, the role of FGF-23 in vascular calcification remains controversial.^{33,34} The use of warfarin, a vitamin K antagonist, increases the risk of calciphylaxis by a factor of 3 to 13.^{1,24,25,35} About 40 to 50% of patients with ESRD and calciphylaxis^{5,36} and 25% of those with calciphylaxis in the absence of ESRD have been treated with warfarin,⁸ and warfarin use is also linked with increased mortality among patients with this disease.³⁶ Even in the absence of warfarin use, vitamin K deficiency from malabsorption or other causes develops in 80% of patients with ESRD and calciphylaxis.³⁷ Most patients with one or even multiple risk factors do not have calciphylaxis. A triggering event in patients with underlying risk factors probably induces calciphylaxis. Cutaneous calcifications develop in rats that have been sensitized with PTH extract, high-dose vitamin D, a high-phosphate diet, or nephrotoxic insults and then challenged with physical trauma or metal injections.³⁸ Repetitive trauma from subcutaneous injections is an example of a trigger reported in some cases of human calciphylaxis.¹ Most often, however, no obvious trigger is identified.

End-stage renal disease
Female sex
Obesity
Diabetes mellitus
Hypercalcemia
Hyperphosphatemia
Hyperparathyroidism (both primary and secondary)
Oversuppressed PTH with adynamic bone disease (low bone turnover)
Elevated alkaline phosphatase
Vitamin K deficiency
Hepatobiliary disease
Thrombophilia (e.g., antithrombin deficiency, protein C deficiency, or lupus anticoagulant)
Autoimmune disorders (e.g., systemic lupus erythematosus)
Hypoalbuminemia
Metastatic cancers (e.g., colon or lung cancer)
POEMS syndrome
Genetic polymorphisms (e.g., rs4431401 and rs9444348)
Skin trauma (e.g., from subcutaneous injections)
Recurrent hypotension
Rapid weight loss
Exposure to ultraviolet light
Exposure to aluminum
Medications (e.g., warfarin, calcium, vitamin D, iron, and recombinant PTH)

Table 1: Risk Factors for Calciphylaxis.*

Pathogenesis

The pathogenesis of calciphylaxis is uncertain. Histologic analysis suggests that calcified, narrowed microvessels lead to chronic, low-grade ischemia, and further occlusion of vessels induced by endothelial injury and microthrombosis results in infarction.^{20,21} Thrombosis could be due to a local prothrombotic state,²¹ with or without systemic hypercoagulability.³⁹ The similarity of histologic features in patients with calciphylaxis, irrespective of their status with respect to ESRD, suggests a common final pathway.²⁰ The development of microvascular calcifications in patients with calciphylaxis (and in patients with other vascular calcifications) is likely to be an active, cell-mediated process that

depends on the balance between the promoters and inhibitors of calcification (Figure 2).^{21,40,41} Serum samples from patients with calciphylaxis show impaired inhibition of calcium phosphate precipitation,⁴² which is probably the result of a deficiency of calcification inhibitors. Matrix Gla protein (MGP) is an extracellular matrix protein synthesized by vascular smooth-muscle and endothelial cells. Carboxylated MGP is a potent inhibitor of calcification, with carboxylation dependent on vitamin K.⁴³ A deficiency of carboxylated MGP accelerates spontaneous arterial calcifications in mice.⁴³ Calciphylaxis in humans is characterized by relative reductions of carboxylated MGP in cutaneous tissue²¹ and the circulation.³⁷ Moreover, reduced circulating levels predict more extensive lesions and ulcer formation.³⁷ Carboxylated MGP, in addition to being a direct calcification inhibitor, inhibits the procalcifying factors bone morphogenetic protein 2 (BMP-2) and bone morphogenetic protein 4 (BMP-4).⁴⁴ As shown in Figure 2, in calciphylaxis, the deficiency of carboxylated MGP probably promotes increased cutaneous expression of BMP-2²¹ and BMP-4⁴⁵ and further osteogenic transcription, as evidenced by increased runt-related transcription factor 2 (RUNX2).^{21,46} Another calcification inhibitor is fetuin-A, which is involved in the formation of calciprotein particles that transport mineral nanocrystals.⁴² Fetuin-A is down-regulated in chronic inflammatory conditions, including chronic kidney disease.^{42,47} Calciphylaxis is characterized by a pronounced burden of circulating calciprotein particles, indicative of severe, functional fetuin-A deficiency.^{47,48} The discovery of causal genes for mendelian traits with extensive calcifications may provide clues to the pathogenesis of calciphylaxis. Mutations of NTSE⁴⁹ and the gene encoding ectonucleotide pyrophosphatase and phosphodiesterase (ENPP1),⁵⁰ which are involved in the pathways that regulate the metabolism of pyrophosphate (a critical inhibitor of calcification),⁵¹ lead to arterial calcifications in humans. Polymorphisms in NTSE (rs4431401 and rs9444348) are overrepresented in patients with calciphylaxis.³⁰ In murine models, administration of an ENPP1-Fc fusion protein⁵⁰ or a small-molecule ENPP1 inhibitor⁵² prevents cardiovascular calcifications — findings that highlight a promising pathway for the treatment of calcification disorders, including calciphylaxis. Calcifications of the aorta, coronary artery, and femoral artery are common in patients with ESRD.³⁴ Nonetheless, cutaneous microvascular calcifications leading to clinical manifestations of calciphylaxis remain rare. Why calciphylaxis occurs primarily in the cutaneous microcirculation is unclear. Predominant involvement of subcutaneous panniculus and aggregation of lesions in body areas with abundant adipose tissue, such as the abdomen and thighs, suggest a role of adipocytes in the pathogenesis of calciphylaxis. Mature adipocytes exposed to high phosphate levels calcify and unidirectionally induce calcification of vascular smooth-muscle cells, probably through the release of adipokines.⁵³ Vascular endothelial growth factor A (VEGF-A) is a potential adipokine that has been shown to produce a procalcific response through BMP-4.⁵⁴ The relatively high prevalence of calciphylaxis (4%) among patients with the polyneuropathy, organomegaly, endocrinopathy, M component, and skin changes (POEMS) syndrome underscores the possible role of VEGF-A in calciphylaxis, since the POEMS syndrome is characterized by marked elevations in VEGF-A.⁵⁵ Adipocytes also secrete MGP,⁵⁶ which may be inadequate or ineffective in patients with calciphylaxis. Whether differences in susceptibility to calciphylaxis are due to genetic or environmental variations in adipose tissue is unknown.

Figure 2: Proposed Pathogenesis of Calciphylaxis. A representative arteriole from a skin section is magnified. The top left quarter of the arteriole represents normal histologic features, including an intact endothelial lining, internal elastic lamina, vascular smooth-muscle cells (VSMCs), and elastin fibers. In the presence of chronic kidney disease (CKD) and toxins from other sources, VSMCs are probably transdifferentiated from a contractile phenotype to an osteochondrogenic phenotype with up-regulated transcription factors such as runt-related transcription factor 2 (RUNX2). The transdifferentiated cells elaborate matrix vesicles containing calcium (Ca) and phosphate (PO₄), which nucleate crystalline hydroxyapatite in the extracellular matrix. Eventually, the balance between calcification promoters (e.g., bone morphogenetic protein 2 and 4 [BMP-2 and BMP-4]) and inhibitors (e.g., carboxylated matrix Gla protein [c-MGP], fetuin-A, and inorganic pyrophosphate [PPi]) determines whether the arteriole will calcify. Carboxylated MGP inhibits BMP-driven VSMC transdifferentiation and, by loading into matrix vesicles, prevents mineralization. Vitamin K deficiency or antagonism blocks MGP carboxylation, which then promotes VSMC transdifferentiation and matrix mineralization. The transdifferentiated VSMCs produce less MGP, resulting in a cascade effect. Adipocytes may influence this process by releasing vascular endothelial growth factor A (VEGF-A). Arteriolar calcification combined with endothelial destruction and thrombosis ultimately leads to clinical manifestations.

Interdisciplinary Management

Although there is no approved therapy for calciphylaxis, several approaches may be effective (see Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). However, the evidence in support of these approaches comes from uncontrolled, observational studies and case reports; for this reason, prospective clinical trials of repurposed and novel therapies are now under way (Table S2 in the Supplementary Appendix). Nonetheless, on the basis of expert opinion, we recommend an interdisciplinary approach (including specialists in dermatology, nephrology, nutrition, pain and palliative medicine, plastic surgery, and wound care) with the aim of expediting the diagnosis and treatment (Figure 3).

Sodium thiosulfate ²⁹⁻³²
Bisphosphonate ^{33,34}
Non-calcium based phosphate binder ³⁵
Calcimimetic agent ³⁵
Opioid analgesic ³⁶
Neuropathic analgesic ³⁶
Spinal anesthetic ³⁶
Non-vitamin K anticoagulant ³⁷
Tissue plasminogen activator ³⁸
High flow oxygen ³⁹
Vitamin K ⁴
Pentoxifylline ^{32,40}
Intensification of dialysis duration and or frequency ^{4,39}
Transition from hemodialysis to peritoneal dialysis ⁴¹
LDL-apheresis ⁴²
Sterile maggot therapy ⁴⁰
Recombinant platelet-derived growth factor ⁴³

Table S1: Examples of the reported medical therapies for calciphylaxis

Study Name	Study ID	Study Type	Sponsor	Comments
A clinical trial with Sodium Thiosulfate for the treatment of Calciphylaxis	ISRCTN number, ISRCTN73380053	Phase 2/3, clinical trial	Dr. F. Köhler Chemie GmbH (Germany)	Multicenter, open-label clinical trial to evaluate the efficacy of intravenous Sodium Thiosulfate for treatment of calciphylaxis wounds in hemodialysis patients
CALISTA Trial	Clinicaltrials.gov number, NCT03150420	Phase 3, clinical trial	Hope Pharmaceuticals	Multicenter, randomized, double-blind, placebo-controlled clinical trial to evaluate the efficacy and safety of intravenous Sodium Thiosulfate Injection for treatment of acute calciphylaxis-associated pain in hemodialysis patients
EuCalNet Registry	Clinicaltrials.gov number, NCT02635373	Prospective registry	RWTH Aachen University	Observational, prospective, non-interventional collection of data and samples from patients with uremic and non-uremic calciphylaxis
Partners Calciphylaxis Biobank	Clinicaltrials.gov number, NCT03032835	Prospective registry	Massachusetts General Hospital	Observational, prospective, non-interventional collection of data and samples from patients with uremic and non-uremic calciphylaxis
Phase 2 Study with SNF472 in Calciphylaxis Patients	Clinicaltrials.gov number, NCT02790073	Phase 2, clinical trial	Laboratoris Sanifit	Multicenter, open-label clinical trial to evaluate the efficacy of SNF472 for treatment of calciphylaxis wounds in hemodialysis patients
The UK Calciphylaxis Study	ISRCTN number, ISRCTN34171966	Prospective registry	Salford Royal NHS Foundation Trust (UK)	SNF472 is an intravenous formulation of phytate or myoinositol-1,2,3,4,5,6-hexakis dihydrogen phosphate (InsP6) Observational, prospective, non-interventional collection of data and samples from patients with calciphylaxis in the setting of chronic kidney disease

Table S2: Prospective registries and clinical trials for calciphylaxis⁴⁴

Figure 3: An Interdisciplinary Approach to the Management of Calciphylaxis. A stepwise process for the diagnosis and therapy of calciphylaxis underscores the potential benefits of interdisciplinary collaboration.

Diagnosis

Clinical suspicion is important for an early diagnosis. Elevations in serum calcium or phosphate levels are not specific. A recent analysis of data from the German Calciphylaxis Registry showed that 86% of dialysis-dependent patients with calciphylaxis had either normal or low plasma calcium levels, and 40% had either normal or low plasma phosphate levels.⁵ Many disorders mimic calciphylaxis (Table 2). These diagnoses can be eliminated by performing a meticulous clinical examination and evaluating histopathological features, laboratory findings, and imaging results (Table S3 in the Supplementary Appendix). Skin biopsy is the standard method for confirmation of clinically suspected calciphylaxis; however, its role in practice is debated, given the risk of provoking new, nonhealing ulcers and infection.⁴ A biopsy is not needed for a patient with ESRD and the classic presentation of a painful necrotic ulcer covered with a black eschar. However, a biopsy should be strongly considered if a patient has early, atypical lesions or if calciphylaxis is suspected in a patient without ESRD. A biopsy is contraindicated for acral, penile, or infected lesions. A punch biopsy is safer than a large, excisional biopsy but has limited depth and can be nondiagnostic. The yield on biopsy can be improved with a double-punch technique, wherein a second punch is inserted through the center of the first to retrieve deeper subcutaneous tissue, and by biopsy of an active lesion margin rather than a central or necrotic area. Specialized stains (e.g., the von Kossa stain, which uncovers phosphate within hydroxyapatite) are necessary to avoid missing subtle calcifications. Patients with ESRD may have cutaneous microvascular calcifications from causes other than calciphylaxis (e.g., metastatic calcinosis cutis or Mönckeberg's arteriosclerosis).^{19,20,57} The following features, combined with clinical manifestations, help to establish the diagnosis of calciphylaxis: a stippled appearance of calcifications, involvement of capillaries, internal elastic lamina or perieccrine glands, or the presence of fibrointimal hyperplasia or thrombosis.^{15,19,20,57} Although imaging studies (e.g., plain radiography, mammography, or nuclear bone scanning) are not routinely recommended for the diagnosis, they may support the diagnosis when a biopsy is inconclusive or contraindicated. In small, retrospective studies, a netlike pattern of subcutaneous calcification on plain radiographs (Figure 1J) and increased heterogeneous radiotracer uptake in soft tissues on nuclear bone scanning have robust specificity (Figure S1 in the Supplementary Appendix).^{58,59}

Warfarin-induced skin necrosis
Atherosclerotic vascular disease
Venous stasis ulcer
Cellulitis
Cholesterol embolization
Dystrophic calcinosis cutis
Livedoid vasculopathy
Nephrogenic systemic fibrosis
Oxalosis
Pyoderma gangrenosum
Purpura fulminans
Necrotizing vasculitis
Martorell's ulcer

Table 2: Differential Diagnosis of Calciphylaxis.

Analgesia and Wound Management

A clinician should first consider analgesic agents to address the severe pain that accompanies calciphylaxis. Adequate pain relief can be challenging because the pain may be unresponsive to high-dose opioids, and opioid toxicity may develop.¹¹ Concomitant treatment with gabapentin, ketamine, or the application of spinal anesthetic agents is used in refractory cases.¹¹ The primary goals of wound care are to remove exudate and necrotic tissue and prevent infection. Calciphylaxis wounds are particularly complicated because the ischemic tissue bed often heals poorly. Moreover, the lesions are extremely painful, making débridement difficult; thus, collaboration with a plastic surgeon and a pain specialist is beneficial. Retrospective studies have suggested improved survival among patients who underwent operative débridement.^{2,14} However, patients who can tolerate débridement may be healthier than those who cannot, possibly accounting for the difference in survival. Corroborating data from prospective, controlled studies are lacking. Operative débridement accompanied by negative-pressure wound therapy is recommended for infected wounds and large necrotic areas with drainage, but such therapy can result in defective soft tissue lined by marginally viable tissue, requiring further excision. Once the wound has stabilized and granulation tissue is present, split-thickness

skin grafting may be performed to close the wound. Transcutaneous oxygen tension is reduced at lesion sites.⁶⁰ In a retrospective study involving 34 patients with predominantly peripheral disease, hyperbaric oxygen therapy was associated with complete healing in half the patients after 44 sessions of therapy administered over a period of 2 months.⁶¹ These findings provide support for the use of hyperbaric oxygen, particularly in patients with peripheral lesions.⁶¹ Attention to nutritional status is necessary to prevent or treat protein-energy wasting.⁶² There is no indication for prophylactic use of antibiotics.

Elimination of Risk Factors

Hypercalcemia and hyperphosphatemia should be corrected. Intake of vitamin D and calcium (including calcium-based phosphate binders) should be eliminated, and high levels of dialysate calcium should be avoided. The optimal PTH level for patients with calciphylaxis is unknown; however, extreme values (high and low) should be avoided. In a randomized trial involving more than 3500 patients being treated with hemodialysis, the administration of cinacalcet (a calcimimetic agent) decreased the median PTH level from approximately 690 pg per milliliter to 300 pg per milliliter.⁶³ Adverse-event analyses in that trial showed a reduced incidence of calciphylaxis (unadjusted relative hazard, 0.31; 95% confidence interval, 0.13 to 0.79) in the cinacalcet group. The low event rate for calciphylaxis in both study groups (0.3% [6 patients] in the cinacalcet group and 0.9% [18 patients] in the placebo group) precluded detailed analyses; furthermore, this trial did not address the role of cinacalcet once calciphylaxis is present. However, the possibility that a calcimimetic agent is helpful warrants further prospective examination in both preventive and treatment studies. Considering the risks associated with parathyroidectomy, including irreversible reduction of PTH levels and the hungry bone syndrome, requiring calcium and calcitriol, a calcimimetic agent is preferable to parathyroidectomy, the latter being reserved for refractory cases.⁶⁴ In patients with ESRD, increasing the length or frequency of dialysis sessions may accelerate wound healing.⁶⁵ Intensified hemodialysis (beyond the regular regimen of 4-hour sessions three times weekly) is warranted for treating bone and mineral abnormalities, including severe hyperphosphatemia, hypercalcemia, and hyperparathyroidism. A transition to hemodialysis is recommended for patients being treated with peritoneal dialysis, since hemodialysis may accelerate wound healing, presumably through better control of mineral metabolism.²⁶ A complete resolution of calciphylaxis in patients with ESRD has been achieved with kidney transplantation;⁶⁶ however, transplantation is not a feasible treatment option for all such patients. Iatrogenic factors, including treatment with warfarin, should be identified and discontinued, and best practices for the treatment of predisposing conditions should be instituted. If subcutaneous injections cannot be avoided, injection sites should be rotated to minimize repetitive trauma to one location.

Pharmacotherapeutic Agents

Sodium thiosulfate is an agent with antioxidant and vasodilatory properties that also inhibits adipocyte calcification and blocks the ability of adipocytes to induce calcification of vascular smooth-muscle cells.^{48,53} In a study involving 53 hemodialysis-dependent patients with calciphylaxis who were treated with intravenous sodium thiosulfate (three times per week [with each dialysis session] for approximately 3 months), calciphylaxis completely

resolved in 26% of the patients, and 19% had marked improvement in skin lesions.⁶⁷ In another study, involving 27 patients being treated with dialysis, complete remission was observed in 52% of the patients and partial remission in 19% after treatment with intravenous sodium thiosulfate.¹⁸ Lack of a control group and the retrospective nature of the study preclude definitive conclusions regarding the effectiveness of sodium thiosulfate. Although successful use of sodium thiosulfate for the treatment of calciphylaxis was first reported more than a decade ago, only now are two ongoing trials investigating its safety and efficacy (Current Controlled Trials number, ISRCTN73380053; and ClinicalTrials.gov number, NCT03150420). Nevertheless, sodium thiosulfate is frequently used to treat calciphylaxis. A typical dose is 25 g in 100 ml of solution given intravenously three times a week during the last 30 to 60 minutes of hemodialysis. The optimal duration of the treatment course is unclear. Pharmacokinetic simulations may be used to estimate the dose for hemodialysis at various levels of intensity.⁶⁸ The dosing for patients undergoing peritoneal dialysis, patients who are not being treated with dialysis, and children is less standardized. Adverse effects include volume overload, hypocalcemia, QT-interval prolongation, hypotension, and metabolic acidosis. These adverse effects can be avoided with intralesional administration, which may be an alternative approach for patients with early and limited disease.⁶⁹ Bisphosphonates are pyrophosphate analogues that have been used successfully to treat patients with genetic ENPP1 deficiency.⁷⁰ In a prospective series of 11 patients, bisphosphonates halted the progression of lesions in all the patients starting at 2 to 4 weeks after treatment initiation.⁷¹ Further investigation of bone-vascular cross-talk is needed to understand the role of bisphosphonates and other agents that modify bone (e.g., receptor activator of nuclear factor- κ B ligand inhibitor and antisclerostin antibody). Decisions regarding anticoagulation therapy should be individualized after an assessment of the risk of bleeding versus the risk of thrombosis. If ongoing anticoagulation therapy is favored, the selection of an agent should be based on the patient's kidney function and the indication for anticoagulation.⁷² In a study involving patients 60 to 80 years of age, vitamin K1 supplementation (phyloquinone at a dose of 500 μ g per day) slowed the progression of preexisting coronary-artery calcification by 6% at 3 years of follow-up.⁷³ In healthy postmenopausal menopausal women, vitamin K2 supplementation (menaquinone-7 administered at a dose of 180 μ g daily for 3 years) reduced aortic stiffness (measured as carotid-femoral pulse wave velocity with the use of mechanotransducers) and common carotid-artery stiffness (measured according to the stiffness index β with the use of echotracking and oscillometry).⁷⁴ Moreover, vitamin K1 supplementation (phytonadione administered at a dose of 2 mg daily for 12 months) slowed the progress of aortic-valve calcification (measured by means of computed tomography) by 57%, as compared with placebo, in patients with mild-to-moderate aortic stenosis.⁷⁵ The efficacy of vitamin K supplementation for calciphylaxis, however, is unclear, with no support for the superiority of a specific form of vitamin K (K1 or K2). A proof-of-concept study of vitamin K1 supplementation (10 mg administered orally three times weekly after hemodialysis) is currently under way (NCT02278692).

Summary and Future Directions

Calciphylaxis is a complex disorder of microvascular calcification that is typically manifested as painful cutaneous lesions and results in poor outcomes. Currently, there are no approved therapies for calciphylaxis; however, ongoing trials are examining end points that include pain intensity and lesion characteristics. Although some risk factors for calciphylaxis are now better understood, animal models that recapitulate this rare human condition would be helpful to gain a better understanding of pathogenesis and to test novel therapeutic agents.

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**Slower Progress of Aortic Valve Calcification
With Vitamin K Supplementation: Results From
a Prospective Interventional Proof-of-Concept
Study (IF 19)**

Slower Progress of Aortic Valve Calcification With Vitamin K Supplementation: Results From a Prospective Interventional Proof-of-Concept Study.

Brandenburg VM, Reinartz S, Kaesler N, Krüger T, Dirrichs T, Kramann R, Peeters F, Floege J, Keszei A, Marx N, Schurgers LJ, Koos R. Circulation. 2017 May 23; 135(21): 2081-2083.

1. Results

Calcific aortic stenosis is a common degenerative disease characterized by progressive aortic valve calcification (AVC).¹ Effective medical treatment options to retard the progression of AVC are sparse.¹ Epidemiological data point to vitamin K as a potential protective factor for cardiovascular health, particularly for protection against vascular calcification.^{2,3} Matrix Gla-protein (MGP), a potent inhibitor of cardiovascular calcification, requires vitamin K for posttranslational carboxylation and hence full bioactivity.⁴ Thus, vitamin K supplementation might retard the progression of AVC.^{1,2} Dephosphorylated undercarboxylated MGP (dp-ucMGP) serves as a circulating marker for vitamin K deficiency.^{2,3} We performed a 12-month prospective, single-center, open-label, randomized interventional trial in patients with asymptomatic or mildly symptomatic AVC. Written informed consent was obtained before inclusion in the trial (URL: <http://www.clinicaltrials.gov>. Unique identifier: NCT00785109; RWTH Aachen Institutional Review Board No. 165/08). Inclusion criterion was a peak flow velocity exceeding 2 m/s. The main exclusion criteria were chronic kidney disease (estimated glomerular filtration rate $<60 \text{ ml} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$), expected valve replacement within the next year, and anticoagulation with vitamin K antagonists. Patients were randomized 1:1 to receive 2 mg phytomenadione (vitamin K1, Ka-vit, INFECTOPHARM Arzneimittel CONSILIUM GmbH, Heppenheim, Germany) or matching placebo once daily orally. Patients underwent a baseline and end-of-study cardiac computed tomography (CT) scan for AVC quantification (volume calcification score). All CT examinations were performed on a 128-slice dual-source CT scanner (SOMATOM Definition Flash, Siemens, Germany) and were reanalyzed in a blinded fashion by 2 radiologists experienced in cardiac CT. The primary end point was the difference in progression of AVC volume score between vitamin K and placebo. We also assessed changes of dp-ucMGP plasma levels (IDS, Boldon, UK) as a secondary end point. Linear regression models for AVC change with treatment effect and baseline measures were used as independent variables, and 95% confidence intervals for treatment effects were calculated. The trial cohort included 99 patients (82% male; 35% with aortic sclerosis [$\leq 2.5 \text{ m/s}$], 38% with mild aortic stenosis [$2.6\text{--}2.9 \text{ m/s}$], and 27% with moderate aortic stenosis [$3.0\text{--}4.0 \text{ m/s}$]; 71% of each group received statins). Seventy-two participants also underwent an end-of-study cardiac CT scan (representing the per-protocol analysis cohort: $n = 38$ vitamin K, $n = 34$ placebo). Twenty-seven patients (12 vitamin K, 15 placebo) dropped out of the study. Reasons for discontinuation were initiation of oral anticoagulant treatment ($n = 3$ placebo, $n = 4$ vitamin K), loss to follow-up, withdrawal of consent ($n = 6$ placebo, $n = 3$ vitamin K), cardiac surgery ($n = 2$ each), death ($n = 1$ each), or other reasons ($n = 3$ placebo, $n = 2$ vitamin K). Over the 12-month period, the AVC volume score progressed by 10.0% in patients in the vitamin K group compared with 22.0% in the placebo group (Table), representing a significant attenuation of AVC progression by vitamin K compared with placebo. Linear regression with treatment group and baseline AVC as independent variables revealed an estimated difference in the change in AVC volume score between the vitamin K and placebo groups of -101 ml (95% confidence interval, -194 to -8.3 ; $p = 0.03$, adjusted $R^2 = 0.08$). Adding age to the model did not improve the model or change the

estimated difference. Baseline mean gradient and peak flow velocity were highly correlated ($r = 0.88$). After adjustment for mean gradient, the estimated difference in AVC volume progression was -65 (95% confidence interval, -147 to 17 ; $p = 0.12$; adjusted $R^2 = 0.26$). Similar results were obtained after AVC was indexed to body surface area. Plasma dpucMGP concentration significantly decreased in the vitamin K group by 45% ($p < 0.001$; Table). Statistically, the change in peak flow velocity was not significantly different between the 2 groups. No thromboembolic events occurred. The present study is the first randomized controlled trial in men to demonstrate that vitamin K supplementation might decelerate the progression of AVC. Our findings are clinically meaningful because a strong, significant correlation exists between the AVC volume score and functional valvular parameters such as mean gradient or peak flow velocity.⁵ Hence, deceleration of AVC progression, a direct precursor of hemodynamic impairment, might finally translate into a stabilization of valvular functionality in calcific aortic stenosis and a slowing of cardiac and clinical deterioration. In parallel, vitamin K treatment induced a marked reduction of plasma dpucMGP, indicating increased vitamin K bioactivity. We consider the present study results to represent the first proof of concept in the evaluation of the potential anticalcification effects of vitamin K treatment in human calcific aortic valvular disease. We acknowledge that our results need to be confirmed and should therefore be interpreted with caution. Limitations of our trial are the relatively small study size and the additional high dropout rate, resulting in missing data for primary end-point interpretation, as well as the short duration of follow-up, the open-label design, and the broad spectrum of severity of valvular disease at baseline. Moreover, the study was not powered to assess valve functionality using echocardiography, an important determinant for clinical end points. Despite these limitations, our data lay the basis for future intervention trials to investigate valvular hemodynamic parameters or patient outcomes in parallel to calcification parameters. In summary, vitamin K supplementation may represent an effective and safe therapy in cardiovascular disease related to ectopic calcification such as calcific aortic stenosis.

	Vitamin K Group (n=38)				Placebo Group (n=34)			
	t0		t12		t0		t12	
	Mean	SD	Mean	SD	Mean	SD	Mean	P Value
Age, y	69.3	10.1					68.9	7.9
Calcification volume, mL	793	742	871	791	836	856	1017	939
Mean valvular gradient, mm Hg	17	6	17	7	18	8	21	11
Peak flow velocity, m/s	2.70	0.44	2.63	0.56	2.82	0.62	2.95	0.70
dp-ucMGP, pmol/L	432	149	243	165	483	215	515	276
Delta calcification volume, mL			78	165			181	234
Delta calcification volume index, mL/m ² BSA			41	84			91	113
Δdp-ucMGP, pmol/L			-199	233			37	112
Delta peak flow velocity, m/s			-0.05	-0.38			0.09	0.40

BSA indicates body surface area; and dp-ucMGP, dephosphorylated undercarboxylated matrix Gla-protein.

*Nominal *P* values from paired comparison between baseline (t0) and 12 months (t12) in the vitamin K group.

†Nominal *P* values from paired comparison between t0 and t12 in the placebo group.

‡Nominal *P* values from unadjusted, unpaired comparison of delta values between treatment groups.

Table: Longitudinal Development of Valvular Calcification and Echocardiographic Parameters and Matrix Gla-Protein Levels, by Intervention Group

2. Acknowledgments

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4. Disclosures

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4.SECTION:

Additional documents

a)

Summary

The scientific work summarized in the present thesis was performed in order to improve our understanding of how CKD influences cardiovascular risk and ascertain which treatment options potentially exist to modify this risk. Two major CKD-related cardiovascular risk factors and biomarkers were specifically selected as research targets for the present thesis, i.e. FGF23 excess and vitamin K deficiency – the latter was presented as closely linked to the calcification disorder calciphylaxis.

The major results of the work are as follows:

We analyzed the potency to predict outcome of the FGF23-klotho axis in the LURIC cohort, a prospective observational cohort consisting of patients undergoing coronary angiography for acute coronary syndrome. Regarding FGF23, the present work confirms that FGF23 (in this case c-term FGF23) is an independent predictor of mortality and morbidity in patients undergoing coronary angiography. Hence, our work investigated the previously well-described association between FGF23 serum levels and outcome, now in a novel type of patient cohort. Interestingly, a closely associated second publication denied such an association for soluble klotho levels, which is important in order to establish a hierarchy within the biomarkers of the FGF23-klotho axis in terms of risk prediction. Our data clearly showed that soluble klotho serum levels failed as risk prediction tool, at least in the LURIC cohort.

On top of these cross-sectional data, we also investigated the influence of i.v. iron (specifically ferric carboxymaltose) on FGF23 and phosphate metabolism in a prospective interventional trial. Results from our study show that ferric carboxymaltose modifies levels of intact as well as c-terminal FGF23 substantially. Noteworthy, ferric carboxymaltose leads to the development of FGF23 serum levels in opposing directions (increase in iFGF23 and decrease in c-term FGF23). Most importantly, these first-in-man data clearly indicate that hypophosphatemia development due to transient increases in intact FGF23 needs to be taken into account when discussing the pros and cons of i.v. iron therapy in heart failure. Further studies are required to evaluate the pathophysiology behind this finding, e.g. down-regulation of the splicing process of intact FGF23. It is currently also unclear, if and to what extent this transient iFGF23 increase might impair myocardial function.

A second key aspect of the thesis is vitamin K deficiency and its clinical consequences with a specific focus on calciphylaxis. Regarding calciphylaxis, the present thesis summarizes ground-breaking clinical work in the area of calciphylaxis, which led to a better understanding of the disease and provided the basis to introduce this rare disease to a broader audience. For this reason, we collected and analyzed data from the German calciphylaxis registry. Noteworthy, our data underline the close link between vitamin K deficiency and the subsequent risk for vascular calcification induced by vitamin K

antagonist treatment. The presented study contributed significantly to the introduction of preventive measures, such as avoidance of VKA treatment in ESRD patients whenever alternatives appear appropriate. In consequence, the Aachen calciphylaxis team was involved in a recent state-of-the-art review about calciphylaxis with a specific focus on treatment options. Moreover, we support these descriptive data about potential benefits of vitamin K in cardiovascular health by publishing results from a randomized, controlled vitamin K interventional pilot study in patients with calcific aortic stenosis. This trial opens up a promising treatment area by showing that vitamin K replenishment slows the progression of valvular calcification in patients with calcific aortic stenosis compared to placebo.

b)

Samenvatting

De onderzoekprojecten die samengevat zijn in deze promotie gaan enerzijds over de invloed van chronische nierziekte (CKD) op het cardiovasculaire risico en anderzijds over de vraag in hoeverre er potentiële behandelopties zijn om dit risico te verminderen. Twee zeer belangrijke CKD gerelateerde risicofactoren en biomarkers werden gekozen om nader te onderzoeken middels deze promotie: FGF23 exces en vitamine K deficiëntie (ook ivm de verkalkingsstoornis calcifylaxie).

De meest belangrijke bevindingen zijn:

- 1) We onderzochten het vermogen van de FGF23-klotho om de prognose te voorspellen in het LURIC cohort. Dat is een prospectief, observationeel cohort m.b.t. patiënten, die een katheterisatie ondergingen voor diagnostiek i.v.m. een acuut coronair syndroom. In feite was deze studie bedoeld om het bekend verband van FGF23 serum spiegel en uitkomst in een verdere patiëntenpopulatie te onderzoeken. Inderdaad kon het onderzoek bevestigen dat FGF23 (in dit geval c-term FGF23) een onafhankelijke voorspeller van mortaliteit en morbiditeit was. In het verleden was er geen associatie gezien tussen de soluble klotho spiegel wat belangrijk is om meer inzicht te hebben in het hiërarchische verband tussen de FGF23-klotho als risico voorspelling. Uiteindelijk was er geen risicoschatting mogelijk in de LURIC studie o.b.v. soluble klotho spiegel.
Vervolgens werd de invloed van i.v. ijzer (namelijk ferric carboxymaltose) op FGF23 en het fosfaat metabolisme in een prospectieve studie onderzocht. Wij konden aantonen dat ferric carboxymaltose een duidelijke invloed op zowel het intacte alsook het c-terminale FGF23 heeft. Bovendien werd duidelijk dat het toedienen van ferric carboxymaltose tot averechtse effecten namelijk verhoging van iFGF23 en afname van het c-term FGF23 leidde. Het is belangrijk om te benadrukken dat deze eerste resultanten in patiënten duidelijk laten zien dat een hypofosfatemie zich kan ontwikkelen vanwege een passagère afname van het intacte FGF23. Deze bevindingen moeten afgewogen worden ten opzichte van de voordelen van een i.v. ijzerbehandelingen. Hoe dan ook, er zijn meer studies nodig om de pathofysiologie (bijvoorbeeld down-regulatie van het splicing proces van het intacte FGF23) beter boven water te halen. Tot op heden is het niet duidelijk in hoeverre het passagère intacte FGF23 de cardiale functie belemmert.
- 2) Het tweede aspect van de promotie was het onderzoek naar de klinische consequenties van een vitamine K deficiëntie met een klemtoon op calcifylaxie. Vooral m.b.t. calcifylaxie vat deze promotie baanbrekend klinisch werk samen wat

tot betere inzichten in deze zeldzame aandoening geleidt en tegelijkertijd deze ziekte in een groter publiek van artsen geïntroduceerd heeft. Data uit het Duitse calcifylaxie register was voor deze analyses toegankelijk. Onze resultaten laten duidelijk het verband tussen vitamine K deficiëntie en het risico op vasculaire verkalkingen o.b.v. behandeling met vitamine K antagonisten zien. Ons onderzoek heeft een belangrijke bijdrage geleverd om alternatieve strategieën en preventieve maatregelen te implementeren (bijvoorbeeld vermijden van vitamine K antagonisten in patiënten met eindstadium nierfalen). Vervolgens werd het calcifylaxie team uit Aken uitgenodigd om een state-of-the-art review over calcifylaxie en de behandelopties van dien te schrijven.

Bovendien hebben wij een prospectieve, gerandomiseerde studie gedaan om de positieve effecten van vitamine K m.b.t. cardiovasculaire gezondheid bij patiënten met een verkalkte aortaklepstenose te onderzoeken. Omdat er een afname van de snelheid van progressie van klepverkalking door de suppletie van vitamine K te zien was, wijzen deze resultaten op nieuwe, potentiële behandelopties.

c)

Discussion

Role of FGF23 as cardiorenal biomarker and risk factor

The canonical role of the osteocyte-derived circulating hormone fibroblast growth factor 23 (FGF23) is to augment renal phosphate excretion via blockade of tubular reabsorption and the down-regulation of 1,25 dihydroxyvitamin D synthesis (62). In states of primary FGF23 excess, such as oncogenic osteomalacia or x-linked hypophosphatemic rickets, where the overproduction or oversupply of FGF23 hits a normally functioning tubular system, a dramatic hypophosphatemia may occur (63). Longstanding hypophosphatemia poses a substantial risk to the skeleton due to the induction of demineralization which may lead to the well-known entity hypophosphatemic (vitamin D resistant) rickets (osteomalacia in the adult population). However, the so-called off-target (specifically cardiac) effects of FGF23 have reached a significant level of interest (39;64-66) and prompted FGF23 to leave the bone-niche more than a decade ago.

Numerous cohort and observational studies have been published, all pointing in the same direction. They all describe the association between increasing circulating levels of FGF23 in serum and the dismal outcome and prognosis in various patient populations, e.g. in patients with chronic kidney disease, heart failure, critically ill patients, and in the general population (45;67-78). These data have homogeneously postulated an independent association between increasing serum FGF23 levels and the risk of increased cardiovascular morbidity events (such as hospitalization for heart failure) and risk of mortality in all the investigated cohorts (46). Notably, this association was stable despite thorough adjustments to competing standard risk factors. Noteworthy, the link between FGF23 and heart failure-associated endpoints was reported to be especially strong, while the association to atherosclerosis-related cardiovascular endpoints such as myocardial infarction was modest (79). In other words, the myocardium rather than the arterial wall structures (endothelial or medial layer) is apparently the primary target for off-target effects of FGF23. After years of collecting such associative observational data, experimental data finally seemed to prove causality: Experimental *in vivo* data have supported these human cohort data by attributing a direct, independent and causal role for FGF23 to left-ventricular hypertrophy and dysfunction (47;80;81). FGF23 caused pathological hypertrophy of isolated rat cardiomyocytes via FGF receptor-dependent activation of the calcineurin-NFAT signaling pathway. Intramyocardial or intravenous injection of FGF23 in wild-type mice induced increases in left ventricular mass. On the other side, the authors showed that FGF23 blockade is preventive and helps to reduce hypertrophy development. In an established animal model of CKD, treatment with an FGF receptor blocker attenuated

LVH (47). Taken together, these findings point towards FGF23 as a promising candidate as a biomarker in heart failure and / or chronic kidney disease. In this respect, our findings, obtained from the LURIC cohort, pertaining to FGF23 as a risk factor also in patients with acute coronary syndrome, support a strong and independent predictive power for FGF23 and are in line with the vast majority of previous reports (49). Our data are of particular interest in this respect since the LURIC patients were representative for an all-comer study in a typical cardiology setting, i.e. with an average burden of CKD and a large variety of underlying diseases and comorbidities. Of note, the independent association between c-term FGF23 and mortality in LURIC remained stable even after adjustment for brain natriuretic peptide (BNP), a potent biomarker for heart failure (49). As a next step we sought to investigate whether soluble klotho, as the obligatory co-receptor for FGF23, exhibited comparable associations to the outcome in the same cohort. Our data, also obtained from the LURIC cohort, do not indicate that a comparably strong role in risk prediction can be attributed to soluble klotho (50). This negative finding might in part be attributable to an unclear reliability of the currently available soluble klotho assay (46).

As previously mentioned, FGF23 serum levels associate primarily with clinical signs of heart failure and finally with mortality rather than vascular disease end-points such as myocardial infarction (82). Accordingly, it is worth analyzing if differences can be detected between various subtypes of heart failure. The term heart failure summarizes a large variety of entities with e.g. substantial differences in terms left-ventricular ejection fraction. In a follow-up study of our LURIC analysis, we were able to analyze for the first time potential differences regarding FGF23's role as a biomarker in patients with heart failure with reduced ejection fraction versus those with preserved ejection fraction (83). Indeed, such differences apparently exist since FGF23 was independently associated with an increased risk of mortality only in patients with heart failure with reduced ejection fraction but not in those with HF with preserved ejection fraction, suggesting a different pathophysiological role for both entities (83).

Novel aspects regarding FGF23 as a biomarker

However, a critical reappraisal is necessary, since novel and very recent findings challenge the probably over-simplified view that extra-cardiac FGF23 damages the heart and does so only in a mono-directional way (66).

The vast majority of previous studies investigating the association of serum FGF23 with outcome relied upon measuring c-terminal (c-term) FGF23. ELISAs measuring c-term FGF23 identify both the biologically active hormone (intact FGF23, iFGF23) as well as degradation products. Early analysis performed in dialysis patients attributed a comparable value regarding risk assessment to both iFGF23 and c-term FGF23 (44), and a small cohort study in 100 heart failure patients recently confirmed this assumption (75). However, the biological role of iFGF23 differs substantially from that of c-term FGF23 (84) and actually counteracts some of the iFGF23 effects. Hence, relevant differences in the potency as biomarkers and risk predictors between the two forms are likely. Additionally, the

independent role of FGF23 as myocardial noxa has been put into question. Causality must clearly be questioned in case two biomarkers with biological divergent or even opposite modes of action point in the same direction of association. Moreover, while Faul reported a klotho-independent effect of FGF-23 on cardiomyocytes (47), other groups suggested that klotho deficiency rather than FGF23 excess causes cardiac hypertrophy (85). The major finding of the latter study was that in heterozygous klotho-deficient CKD mice, the development of LVH was not modified by interventions normalizing FGF23 and phosphate levels, but only via exogenous klotho application (85). In humans, it is currently impossible to separate clearly the effects of CKD-induced klotho deficiency, phosphate load, and FGF2 rise.

Is FGF23 really the bad guy?

While some of the above-mentioned epidemiological data and experimental findings convincingly accuse FGF23 (presumably from skeletal origin) of being directly noxious to myocardial cells and a causative agent for LVH induction and myocardial damage, some data point in a different or even opposite direction. Two major unsolved issues emerge in this respect: First, does the FGF23 that finally acts upon the myocardium originate only from the bone, and second, are these actions only deleterious?

Noteworthy, also some beneficial cardiovascular effects have been attributed to FGF23, which raises the hypothesis about the existence of a U-curve-shaped association between FGF23 and myocardial disease. Remarkably, acute elevations of FGF23 were shown to have positive inotropic: FGF23 induced acute elevations of intracellular calcium of primary cardiomyocytes, an effect that could be abrogated by the calcium channel blocker Verapamil. FGF23 treatment of a ventricular muscle strip led to increased contractility, which was blocked by inhibition of FGF receptors (86). Hypothetically, this finding allows speculation about a potential physiological role of FGF23 elevations in situations of acute cardiomyocyte stress, such as ischemia and myocardial infarction, when sudden release of FGF23 can support preservation of myocardial contractility. It appears counterintuitive that skeletal FGF23 is the sole source for such favorable cardiac actions, because this would require a fast myocardial-skeletal messenger.

In fact, recently Richter et al. found cultured cardiomyocytes to express FGF23 (87). In subsequent clinical studies, FGF23 was shown to be present in the explanted hearts of patients with ischemic or dilated cardiomyopathy undergoing heart transplantation, but not in healthy hearts (87). In another set of experiments, Andhrukova et al. found that, in the setting of experimental myocardial infarction in mice, circulating FGF23 is increased in the circulation (88). Additionally to increased FGF23 production in the bone, myocardial FGF23 was also increased on a protein and mRNA level, suggesting that increased circulating FGF23 post-myocardial infarction is at least partly derived from the myocardium itself (88).

Some clinical data also fuel the hypothesis that FGF23 release and myocardial damage is not a one-way route from the skeleton to the myocardium. Cross-sectional data derived from patients with acute cardiogenic shock or the need to undergo mechanical assist device implantation promote the hypothesis that the heart is an active player – not only a recipient - in FGF23 metabolism (89;90). In patients with acute myocardial infarction complicated by cardiogenic shock, increased levels of FGF23 on admission correlated with increased mortality at 30 days and 1 year (90). However, when stratifying patients according to their baseline creatinine levels, the negative prognostic association of increased FGF23 only held true for patients whose serum creatinine was above the median (90), suggesting that FGF23 may not cause cardiac damage but rather is an indicator for it. Considering the speed and magnitude of the FGF-23 rise, the authors speculated that the myocardial damage per se induces FGF23 (be it heart derived and/or bone derived). Hence, these researchers challenged the mono-directional hypothesis that external factors stimulate bone FGF23 release which in turn induces myocardial damage (89;90). Instead, the FGF23 signaling pathway is increasingly recognized as having a paracrine component with myocardial FGF23 production and release acting directly locally within the heart (66).

FGF23 participates in i.v. iron-induced hypophosphatemia

The articles in this thesis also addressed a very recent discussion in heart failure patients, i.e. the link between iron deficiency, iron replenishment and hypophosphatemia induction. Iron deficiency has been identified as a relevant comorbidity in patients with heart failure with reduced ejection fraction (59). Novel iron formulations allow single-shot high dosage iron infusions, which permit a long interval (several months) between applications. This fact together with a clear recommendation to fill iron stores adequately in heart failure patients led to a substantial recent increase in i.v. iron usage in this setting. However, a novel disease entity emerged, the i.v. iron-related osteomalacia (57). Speculations pointed towards a causal relationship between the application of some (not all) i.v. iron formulations and the occurrence of a transient hypophosphatemia. Preliminary work in this area attributed this hypophosphatemia induction to a potential transient rise in iFGF23 (91). In our work presented in this thesis, we present a very important extension to these preliminary data (61). We performed the first controlled prospective interventional study in the field of heart failure. We investigated for the first time whether the hypophosphatemia induction is also present in heart failure patients after i.v. iron and if this increase is indeed attributable to increases in iFGF23. Additionally, we addressed the issue of whether underlying CKD in heart failure (the so-called cardio-renal syndrome) modifies the induction of hypophosphatemia. The major results of our pilot trial were (Figure 3) that 1000 mg FCM in heart failure patients with reduced ejection fraction induced a transient decrease in serum phosphorus (Figure 3), which was more pronounced in heart failure patients with reduced ejection fraction without concomitant CKD.

These biochemical changes emerged < 1 week after infusion and lasted 2-3 weeks. Most strikingly, in heart failure with reduced ejection fraction patients without CKD, 5 out of 11

patients developed severe hypophosphatemia (defined as serum phosphate <0.65mol/L) at least once after 1000 i.v. FCM.

Figure 3: Mean serum phosphate levels after 1000 mg FCM (V. Brandenburg, R. Stöhr, data on file)

These findings have highly relevant implications for heart failure therapy in several ways: Physicians must determine if the observed derangements in FGF-23-phosphate metabolism may partly outweigh the beneficial effects of iron infusion in heart failure patients, particularly with repetitive dosing. These negative effects might emerge from noxious effects of high levels of intact FGF23 upon the myocardium (in the same way as hypothesized by Faul and coworkers (47)) and also from phosphate depletion which might impair energy metabolism via impaired ATP supply. Potential negative skeletal effects should also be assessed in case repetitive dosing has subsequent comparable effects. Based on our findings (61), we recommend monitoring of serum phosphate for 2 to 3 weeks after high-dosage FCM infusion in heart failure patients.

Vitamin K, calcification and calciphylaxis

A second core area of the present thesis was the issue of vitamin K and its role in vascular calcification with unique insights into the rare disease calciphylaxis and first-in-man controlled prospective data on the ability of vitamin K to decrease vascular calcification progression.

The rationale of vitamin K science in cardiovascular disease emerges from early genetic studies about MGP deficiency. The genetic deficiency model of the MGP null-mouse clearly shows how important a functioning matrix-Gla protein system is for the integrity of the arterial wall (20). The MGP deficiency results in an ultra-fast vascular calcification phenotype which ends up in premature, early death due to fracturing of the aorta (20). This very dominant phenotype points towards the overwhelming importance of MGP for the integrity of the arterial wall and prevention of vascular calcification (20). This phenotype can be mimicked by the titrated application of vitamin K antagonists in rodents (13) and potentially also in humans (92). However, the application of VKA might actually even induce further consequences since additional proteins have been identified as being vitamin K dependent such as osteocalcin, GAS6 or „Gla-rich protein“ (GRP, also known as cartilage matrix-associated protein (UCMA)) (93). There is obviously an important difference between the coagulation system and MGP in terms of amount of vitamin K needed to sufficiently activate these two systems. While bleeding disorders in adults virtually never occur due to a nutritional mal-supply of vitamin K (instead the most important causal factor for hemorrhage in adults is treatment with VKA) the MGP system is more susceptible. There is always a substantial amount of MGP circulating measurably in an un-carboxylated subfraction (ucMGP). This subfraction is suggestive of a suboptimal natural (nutritional) supply in patients since it can be easily diminished with oral vitamin K supply (23;37). This subfraction of ucMGP correlates positively with the amount of vascular calcification (94) fueling the hypothesis that circulating ucMGP levels actually reflect suboptimal local vitamin K activity in the vascular wall. In the last approximately 10 years, there has been growing evidence from animal experiments and human epidemiological and associative studies that vitamin K is important for vascular health (23). However, none of these studies could clearly prove causality in humans. Such proof can only be delivered by prospective interventional and randomized trials. It had previously been shown that vitamin K application can reduce the concentration of undercarboxylated MGP in the circulation (95), but the biological consequences had not yet been assessed. We were able to close this obvious gap in evidence via a prospective randomized trial. The study presented in this thesis assessed for the first time the potential biological consequences of vitamin K replenishment in humans (37). The study performed serial CT quantification of valvular calcification in patients with calcific aortic stenosis (37). This study can be considered the pilot first-in-man proof-of-concept study since it showed that the application of vitamin K over 12 months (2 mg phytomedadione per day) significantly slowed the progression of valvular calcification in patients with calcific aortic stenosis (37).

Figure 4 depicts the different progression of calcification levels in patients with vitamin K supply versus those with placebo.

Figure 4: Calcification progression in patients with 2 mg vitamin K daily versus placebo over 12 months (data from (37))

This result is of specific interest since the severity of stenosis in the aortic valve is directly correlated to the amount of calcification, meaning that a rapid progression of calcification translates directly into a progressive hemodynamic impairment (96).

Calciophylaxis

Our better understanding of the association between vitamin K deficiency and the development of vascular calcification has a direct clinical impact in the rare disease calciophylaxis. Calciophylaxis primarily occurs in patients with severe or end-stage renal failure. As such, prospective and systematic data collection is difficult since the occurrence in patients is unpredictable. Hence, it is of utmost importance to consider alternative methods of data collection, which we accomplished through the German internet-based online registry which was later transferred to a European registry “EucalNet” (14). In the field of vascular calcification research, calciophylaxis can be considered as a high-speed template for calcification processes, which also tend to occur in the elderly, diabetic, or CKD population. Due to the rapid occurrence of calciophylaxis, it is tempting to speculate that analysis of risk factors underlying calciophylaxis might ultimately lead to the identification of risk factors involved in other vascular calcification diseases. Indeed, our results from calciophylaxis research point in this direction. Based on data obtained from the German registry, we could identify two important key messages: the potential role of disturbances in bone metabolism and the deleterious role of VKA treatment regarding the induction of vascular calcification (16). An appropriate bone metabolism is crucial for providing a sufficient mineral buffering capacity in the skeleton. If mineral deposition or

release processes are deeply disturbed, such a buffering capacity might become inadequate, which renders the soft tissue susceptible for ectopic calcification. This is the theory behind the hypothesis that states of low bone turnover (a major portion of which are called adynamic bone disease) play a critical role in vascular calcification (9). Indeed, the German registry data point in the same direction. PTH levels were on average much lower in dialysis calciphylaxis patients than current dialysis patient guidelines recommend. Such low PTH levels might reflect suppressed bone turnover or even the co-existence of adynamic bone disease. Consequently, our recommendation, as summarized in (10), states that an intermediate PTH range (150 – 300 pg/ml) might be the optimal PTH range with regard to prevention of calciphylaxis.

A second dominant finding of our calciphylaxis registry is the confirmation of how strongly previous VKA usage associates with calciphylaxis. About 50% of calciphylaxis patients enlisted in our registry were on VKA prior to calciphylaxis development (16). These data underline previous findings of how prevalent VKA therapy in these patients is, and one study from Japan elaborated an odds ratio of around 10 for VKA usage inducing calciphylaxis compared to non-usage (97). In summary, merely observationally obtained calciphylaxis data strongly support experimental findings on the deleterious role of VKA and opposing beneficial effects of vitamin K in vascular calcification. These clinical and observational findings represent the rationale for ongoing controlled interventional trials in calciphylaxis patients testing the hypothesis that vitamin K application can support a viable cure for the disease (see above in (10)).

d)

Dankwoord

Science is nothing without teamplay. Numerous collaborators have supported my scientific efforts in the past with their valuable expertise and friendly support. Regarding the topics of this thesis, I'd like to thank especially those friends and collaborators who contributed to the projects in terms of planning, initiation and execution. I feel honored and pleased to have them all as co-authors in the corresponding publications!

Iris, you made it possible for me to spend endless hours with scientific work. I dedicate this thesis to you and our three wonderful children Franca, Lorenz, and Thea.

e)

Curriculum vitae

Vincent Brandenburg was born in 1970 in Monschau Germany.

He is nephrologist and head of the department of nephrology at the Rhein-Maas Klinikum in Würselen, Germany. He was associate professor and senior consultant at the Department of Cardiology, University Hospital of the RWTH Aachen, Germany until 2018. In this position he was Consultant for the Intermediate Care Unit and Heart Failure outpatient clinic. He gained his medical degree in 1998 at the RWTH Aachen and the Ludwig-Maximilians University Munich, Germany. In 2005, he completed a two-year postdoctoral research period at the IZKF BioMAT, also at the University Hospital Aachen on the subject of the mechanism of extraosseous calcification in uremia. Dr Brandenburg then became a nephrologist at the Department of Nephrology, University Hospital Aachen (Head: Professor Jürgen Floege, MD) until 2009 before he started completing his educational program at the cardiology department. Dr Brandenburg is conducting clinical research on the pathophysiology and treatment of renal osteodystrophy, bone disease after renal transplantation, and cardiovascular calcification in chronic kidney disease. His basic research interests are mechanisms of cardiovascular disease in uremia with particular focus on vitamin K and oral anticoagulation. He is currently also conducting research about the role of alkaline phosphatase, fetuin-A, FGF23, cardiovascular effects of vitamin D and Wnt/beta-catenin signaling in the bone-vascular axis in uremia.

A major research target is calciphylaxis. He was also board member of the ERA-EDTA scientific working group “CKD-MBD” until 2017. As such, he is currently initiating a multinational European calciphylaxis registry “EuCalNet” for which he is principal investigator (www.calciphylaxis.net). Prof. Brandenburg is also principal investigator in several prospective human intervention trials investigating interventions against cardiovascular calcification progression. The clinical focus is the chronic cardio-renal syndrome. Dr Brandenburg has authored or co-authored over 160 articles in peer-reviewed journals. He is member of the German and European societies of nephrology and cardiology.

f)

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g)

Valorization Addendum

The following chapter will briefly describe which impact the thesis will have in terms of novel products, processes or services that are relevant for the society.

1) Relevance: What are the economical and societal implications of the results?

The projects mentioned in the present thesis have significant implications for the present times regarding patient care. They lay the basis for future projects (see below) that have the potential to create data with also economical as well as societal implications.

2) Target groups: Are the results of interest to somebody beyond the medical world?

Obviously, results from medical trials do have the largest impact upon affected patients and their caregivers. However, I would like to mention specifically two issues from the thesis, where the projects itself and / or the results might influence a broader community or even societal structures. That is on one hand the issue of centralized data collection for patients with rare disease. We consider the internet-based structures implemented, tested and validated for the Germany calciphylaxis registry and later for the European Calciphylaxis network “EuCalNet” www.calciphylaxis.net as a template for other rare diseases which do not allow transferring affected patients to a central study site. Moreover, successfully addressing unmet medical needs in a prototypic rare disease such as calciphylaxis and sharing experiences about the project with other caregivers as well as societal structures might directly affect the motivation and the success to copy this approach in other rare diseases. Often patients with rare diseases suffer from a dismal lack of evidence-based treatment options since the hurdles for creating such evidence have been overwhelming.

On the other hand I would like to mention the potential impact of evaluating health benefits of vitamin K for broad parts of the society. Since vitamin K is an easily available component of nutrition studies pinpointing cardiovascular health benefits of high vitamin K intake can lay the basis for future projects evaluating the rationale to use high nutritional vitamin K intake as primary prevention. Such data could substantially influence future recommendations about healthy nutrition facing the fact that calcifying arteriosclerosis is an endemic disease.

3) Activities / products: Are there any specific products, processes, activities where the results are being applied?

The topics covered by the recent thesis have stimulated ongoing international research efforts. I would like to specifically point towards planned as well as already recruiting trials in the field of iron-induced osteomalacia (head-to-head randomized controlled trial scheduled in 2018) as well as vascular calcification (ClinicalTrials.gov Identifier: NCT02066662: "Rivaroxaban Compared to Vitamin K Antagonist Upon Development of Cardiovascular Calcification"). These trials have the potential to modify guideline recommendations regarding optimal treatment choice and need for monitoring which could increase both efficacy as well as safety.

4) Innovation: to what extent are those innovative in comparison to existing options?

In this regard the structures of "Eucalnet" can be considered as an innovative approach since the way data are collected and validated may be used as a template for future activities. Such an approach can help identifying patients, establishing experts' networks and optimizing patient care for many other rare diseases. Moreover, the innovations realized with EuCalNet (ClinicalTrials.gov Identifier: NCT02635373) have laid the foundation for performing prospective trials in the field (ClinicalTrials.gov Identifier: NCT02790073) which would be virtually impossible without a sufficient network basis.

5) Planning and realization: What are the actual plans for the valorization process, what are the risks, costs and chances?

We have not yet established a roadmap for a systematic valorization process. However, above mentioned implications for future projects e.g. via providing the rationale and facilitating the progress have the potential to add additional value.

h)

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