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Reference:

Neven Ellen, Vervaeke Benjamin, Brand Kerstin, Gottwald-Hostalek Ulrike, Opdebeeck Britt, De Maré Annelies, Verhulst Anja, Lalau Jean-Daniel, Kamel Said, de Broe Marc E.,- Metformin prevents the development of severe chronic kidney disease and its associated mineral and bone disorder
Kidney international / International Society of Nephrology - ISSN 0085-2538 - 94:1(2018), p. 102-113
Full text (Publisher's DOI): <https://doi.org/10.1016/J.KINT.2018.01.027>
To cite this reference: <https://hdl.handle.net/10067/1511010151162165141>

[QUERY TO AUTHOR: title and abstract rewritten by Editorial Office – not subject to change]

Metformin prevents the development of severe chronic kidney disease and its associated mineral and bone disorder.

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Running title: Metformin prevents severe CKD

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Abstract

Chronic kidney disease (CKD) causes dysregulation of mineral metabolism, vascular calcification and renal osteodystrophy, an entity called 'CKD-Mineral and Bone Disorder' (CKD-MBD). Here we determine whether metformin, an anti-diabetic drug, exerts favorable effects on progressive, severe CKD and concomitant mineral metabolism disturbances. Rats with CKD-MBD, induced by a 0.25% adenine diet for eight weeks, were treated with 200 mg/kg/day metformin or vehicle from one week after CKD induction onwards. Severe, stable CKD along with marked hyperphosphatemia and hypocalcemia developed in these rats which led to arterial calcification and high bone turnover disease. Metformin protected from development towards severe CKD. Metformin-treated rats did not develop hyperphosphatemia or hypocalcemia and this prevented the development of vascular calcification and inhibited the progression towards high bone turnover disease. Kidneys of the metformin group showed significantly less cellular infiltration, fibrosis and inflammation. To study a possible direct effect of metformin on the development of vascular calcification, independent of its effect on renal function, metformin (200 mg/kg/day) or vehicle was dosed for ten weeks to rats with warfarin-induced vascular calcification. The drug did not reduce aorta or small vessel calcification in this animal model. Thus, metformin protected against the development of severe CKD and preserved calcium phosphorus homeostasis. As a result of its beneficial impact on renal function, associated comorbidities such as vascular calcification and high bone turnover disease were also prevented.

Key words: chronic kidney disease – renal fibrosis - vascular calcification – high bone turnover – CKD-MBD - metformin

Introduction

Over the past decades, metformin is used as a first-line treatment for type 2 diabetes because of its effect on the glucose metabolism. This therapeutic improves insulin sensitivity and thus stimulates glucose uptake, increases glycolysis and decreases gluconeogenesis in the liver. However, it is becoming clear that metformin exerts multiple actions beyond its prescribed use as an anti-hyperglycemic agent including beneficial effects on the kidney¹. Since metformin is cleared by the kidney and thus, for the time being, is contraindicated in patients with advanced stages of renal disease due to the fear of lactic acidosis, studies on the effect of metformin on the kidney are scarce and limited to preclinical experiments. In a rat model of gentamicin-induced nephropathy, metformin treatment fully blocked the induction of acute renal failure, prevented the histological changes in the kidney and normalized oxidative stress². Furthermore, this anti-diabetic agent was able to ameliorate renal fibrosis in 5/6th nephrectomized rats³ and mice with unilateral ureteral obstruction⁴. Also with regard to the cardiovascular system, benefits of metformin are reported in diabetic patients^{3, 5-7}, although the mechanism underlying its action is not yet fully understood. Vasculoprotective effects by metformin might indirectly be ascribed to improvements in glucose and lipid metabolism, but also direct actions on the heart and vasculature are increasingly being reported⁸. Metformin has been shown to attenuate atherosclerosis and vascular senescence⁹, improve diabetic cardiomyopathy by stimulation of cardiac autophagy and inhibition of apoptosis of cardiomyocytes^{10, 11} and to protect against myocardial ischemia reperfusion injury¹²⁻¹⁴.

Patients with chronic kidney disease (CKD) suffer from disturbances in mineral homeostasis including dysregulation of calcium, phosphorus, parathyroid hormone and fibroblast growth factor 23 which ultimately entails ectopic calcification in the arteries and bone abnormalities. These complications of chronic renal impairment cover a broader syndrome and are defined as Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). In the current study we aimed to investigate whether metformin exerts protective effects on the kidney, blood vessels and the bone in a rat model of CKD-MBD, recently optimized by our group¹⁵ and characterized by the induction of severe, stable CKD

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3 along with the development of moderate vascular calcification and high bone turnover disease. To
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5 evaluate the isolated effect of metformin on arterial calcification, independently of its effect on renal
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7 function, an additional study was executed in a rat model of warfarin-induced vascular calcification.
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Results

(i) Adenine-induced CKD rat model: effect of metformin on CKD-MBD

Mortality was limited as only 1 animal of the metformin group died before the planned sacrifice which was not related to the metformin treatment. Food intake was slightly higher in the vehicle treated group in weeks 2, 3 and 4, and slightly higher in the metformin treated group in weeks 6, 7 and 8 (Supplemental file). No significant differences in body weight were noted between vehicle and metformin treated CKD rats throughout the study period (Supplemental file).

Renal function

Incessant adenine dosing resulted in severe, stable CKD in vehicle treated rats. Serum creatinine levels dramatically rose up to ca. 6 mg/dl, which is 10-fold higher than control values at the start of the experiment (Figure 1A). In line with this result, creatinine clearance significantly decreased over time in vehicle treated CKD rats (Figure 1B). Interestingly, metformin dosing protected adenine exposed rats from the evolution towards severe CKD. Serum creatinine concentrations in the metformin treated CKD rats remained relatively low and were increased only 2,5-fold at the end of the study as compared to baseline values. From week 4 onwards, serum creatinine levels were significantly lower and creatinine clearance was significantly higher in the metformin treated CKD rats as compared to the vehicle treated animals.

Renal histology, fibrosis and inflammation

Histological examination of PAS stained renal sections revealed that induction of CKD by adenine exposure caused dilatation of proximal and distal tubules as well as epithelial flattening, proximal brush border loss, basement membrane thickening and tubulointerstitial cellular infiltration, whereas glomeruli remained relatively intact (Figure 2C). Masson staining clearly confirmed the presence of excess extracellular matrix (ECM) in the expanded tubulointerstitium (Figure 2D). The

tubulointerstitial area percent, comprising both ECM and infiltrating cells, was 35% lower in metformin treated animals ($p < 0.05$, Figure 2A).

The 2,8 dihydroxyadenine crystals were observed both in the tubules and the interstitium. To investigate whether the beneficial impact of metformin on renal function was not due to interference with the adenine metabolism and thus with the formation of 2,8 dihydroxyadenine crystals in the kidney, the % surface covered by these crystals was measured. Results indicated that the % surface covered by 2,8 dihydroxyadenine crystals in the metformin exposed CKD rats did not differ significantly from the vehicle exposed animals (Figure 2B).

The mRNA expression of the pro-inflammatory cell adhesion molecule VCAM1 was markedly decreased in CKD rats exposed to metformin (Figure 3). Metformin treatment also led to a significantly decreased expression of the inflammatory cytokines TNF α and IL-1 β in the kidney. In addition, TGF- β and Collagen 1a1 expression was significantly reduced in CKD rats treated with metformin as compared to vehicles. No difference was observed in the bax/bcl2 ratio between both study groups, suggesting no effect of metformin on apoptosis.

Mineral homeostasis

In line with the increase in serum creatinine, and thus with the decrease in renal function, serum phosphorus levels significantly rose with the duration of CKD (Figure 1C), whereas serum total calcium (Figure 1D) was significantly decreased after 8 weeks of CKD in vehicle treated animals in comparison with baseline values, which is inherent to chronic renal impairment. Metformin treatment prevented the development of hyperphosphatemia and hypocalcemia in CKD rats. Serum ionized calcium levels were also significantly higher in CKD rats treated with metformin as compared to the vehicle group (1.23 ± 0.03 mmol/L in the metformin group versus 0.77 ± 0.07 mmol/L in the vehicle group). Hypocalcemia and hyperphosphatemia led to a tremendous rise in serum PTH (Figure 1E) and FGF-23 (Figure 1F) levels, respectively, in CKD rats exposed to vehicle treatment. Metformin treatment was also able to significantly attenuate the increase in both hormones.

Acid/base balance and anemia

To evaluate whether daily metformin dosing caused acidosis, serum pH and bicarbonate were measured at the end of the study. Both pH (7.40 ± 0.07 in the vehicle group versus 7.36 ± 0.12 in the metformin group) and bicarbonate (31.75 ± 2.65 mmol/L in the vehicle group versus 27.78 ± 6.97 mmol/L in the metformin group) levels in the serum did not significantly differ between vehicle and metformin treated CKD animals. Due to the beneficial effect of metformin on renal function, metformin treated rats displayed a significantly higher hematocrit (34.08 ± 1.47 % in the metformin group versus 26.18 ± 1.73 % in the vehicle group, $p=0.001$) and serum haemoglobin concentration (11.60 ± 0.50 g/dL in the metformin group versus 8.89 ± 0.59 g/dL in the vehicle group, $p=0.001$) as compared to vehicle treated CKD animals.

Vascular calcification

Adenine-induced CKD rats treated with vehicle developed calcification in the aorta and peripheral arteries as shown by the increased calcium content of each vessel type (Figure 4 A, B and C). The calcium content of the aorta, carotid and femoral arteries in the metformin treated CKD rats was very low and comparable with values of rats with normal renal function measured in the frame of previous studies of our group¹⁵, indicating that no vascular calcification had developed in this study group. Although total arterial calcium content was clearly higher in the vehicle treated CKD group as compared to the metformin exposed study group, a significant difference between both study groups was only reached in the femoral artery. Figure 4D shows a Von Kossa stained section of a calcified aorta of a CKD rat treated with vehicle versus an intact aorta of a metformin treated CKD rat. Calcification had developed in the medial layer of the vessel wall.

Bone histomorphometry

Figure 5 presents static and dynamic bone parameters of both study groups. Compared to rats with normal renal function (historical data from previous studies in our lab), static and dynamic bone parameters are disturbed in vehicle treated CKD rats. Increased osteoid, eroded, osteoblast and osteoclast perimeters as well as increased bone formation rate and mineral apposition rate are features of high bone turnover which is inherent to the adenine-induced CKD rat. Based on the TMV categorization system established for humans ¹⁶, the type of renal osteodystrophy should be classified as mixed uremic osteodystrophy, i. e. normal bone area/volume in combination with an impaired mineralization (evidenced by the increased mineralization lag time and increased amount of osteoid) and an increased bone formation rate. Treatment with metformin significantly reduced osteoid perimeter and area and osteoblast perimeter. Also the dynamic bone parameters, which include bone formation rate and mineral apposition rate, were significantly reduced in metformin treated CKD rats as compared to vehicle treated ones thus pointing to an evolution towards normal bone histology.

Representative Goldner stained sections of a vehicle treated CKD rat show dramatically increased osteoid and active osteoblasts as compared to a metformin treated animal (Figure 6 A). In the vehicle group, tetracycline labels visualized by fluorescence microscopy are widely separated from each other and incorporated chaotically at some sites indicating a high bone turnover, whereas both labels are clearly distinguishable and much closer to each other in CKD rats exposed to metformin (Figure 6B).

(ii) Rat model of warfarin-induced vascular calcification: effect of metformin on vascular calcification

One week before the planned sacrifice, all rats received 30 mg/kg tetracycline to allow later histomorphometric analysis of bone samples. However, animals exhibited clear signs of illness after iv. tetracycline injection which caused death of 2 rats in the vehicle group and 4 rats in the metformin group.

Food intake was similar between vehicle and metformin treated animals with slightly higher food consumption in the metformin treated group at weeks 3 and 5. No differences in body weight between both study groups were noted throughout the study.

Renal function and mineral homeostasis

Table 1 presents parameters measured to evaluate renal function and mineral metabolism during the study. Serum creatinine levels were slightly but significantly increased in warfarin exposed rats during the course of the study as compared to baseline values, however, no differences were noted between both study groups. Warfarin exposure significantly decreased serum phosphorus levels and increased serum calcium concentrations. Daily metformin treatment at a 200mg/kg dose did not alter serum phosphorus levels, but significantly increased serum calcium levels at week 6 as compared to vehicle treated rats.

Acid/base balance and glucose metabolism

To evaluate whether daily metformin dosing caused acidosis, serum pH and bicarbonate were measured (Table 1). Although serum pH and bicarbonate were significantly higher in the metformin treated animals as compared to its baseline values, no differences in both parameters were noted between the metformin and vehicle treated group. In line with these results, serum lactate levels were not affected by metformin treatment, indicating that this metformin dose did not cause lactic acidosis in the warfarin rat model.

At baseline and after 6 and 10 weeks of metformin treatment, no differences were noted in serum glucose levels in comparison with vehicle treated rats.

Cardiovascular calcification

To evaluate calcification in the arteries and the heart, the total calcium content of the heart, aorta, carotid, femoral and renal arteries was measured (Figure 7). Continuous warfarin exposure via the

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3 diet led to significant medial calcification of both the aorta and smaller arteries in all vehicle-treated
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5 rats. Metformin treatment did not significantly reduce the calcium content in the different vessels
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7 neither did it reduce the calcium content in the heart.
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Discussion

Vascular calcification and bone abnormalities are two detrimental complications of CKD which are related to each other and substantially contribute to high mortality rates in CKD patients. CKD-MBD is defined as a systemic disorder of the bone and mineral metabolism in CKD patients and encompasses the interplay between affected kidneys, the vasculature which becomes prone to calcification and the bone which shows abnormalities in bone turnover and mineralization. Since there is a growing body of literature arguing that metformin, the mostly prescribed anti-diabetic drug, has a beneficial effect on renal fibrosis and atherosclerosis, beyond its action in the glucose metabolism, we studied the effect of the glucose-lowering compound in a validated rat model of CKD-MBD¹⁵. We found that metformin was able to dramatically slow down the progression of severe CKD. Preservation of renal function led to an intact mineral homeostasis in the circulation which in turn prevented the development of vascular calcification and high bone turnover disease. This is the first study to show that metformin has the capacity to prevent the development of severe CKD in the adenine-induced CKD rat, which is a well-known model for end stage renal disease^{15, 17, 18}. Our findings extend the results of a previous experimental study reporting that daily metformin treatment suppressed the progressive loss of kidney function, as assessed by glomerular filtration rate, in 5/6th nephrectomized rats, which represents a mild to moderate model of CKD³. The significant impact of metformin on renal function in the adenine rat model is striking and is further confirmed by the normalization of the calcium and phosphorus homeostasis as the induction of CKD-related hyperphosphatemia and hypocalcemia was prevented and the rise in serum FGF-23 and PTH levels was pronounced to a significantly lesser extent. Furthermore, exposure to metformin led to improvement of anemia as a significant rise in hematocrit and serum hemoglobin versus the vehicle group was found, indicating that the kidneys were capable to preserve normal erythropoiesis. Despite the significant effect of metformin on renal function, strongly dilated tubules were histologically detected and no effect on the surface taken by the 2,8 dihydroxyadenine crystals was found in metformin treated animals, indicating that this agent did not interfere with the formation of

renal crystals. Histologically, the difference between both study groups consisted of a decreased tubulointerstitial area in metformin treated rats, suggesting less cellular infiltration and deposition of extracellular matrix. It should be noted that the presence of the adenine crystals in the kidney impeded proper interpretation of immunohistological detection of inflammatory cells in the renal tissue due to nonspecific binding of the antibodies on the small adenine crystals. However, the significant reduction of the adhesion molecule VCAM1 and the inflammatory cytokines TNF α and IL-1 β by metformin treatment strongly suggests that the renoprotective effect of this antidiabetic drug consists of reducing the adhesion and infiltration of inflammatory cells in the interstitium. In mice with unilateral urether obstruction, a model for tubular injury and renal inflammation, metformin also antagonized renal inflammation by slowing down macrophage infiltration and normalizing TNF α mRNA expression in the kidney⁴. In addition, in our rat model with adenine-induced CKD, metformin also exerted anti-fibrotic effects. The renal expression of the fibrotic markers TGF- β and collagen 1a1 was markedly reduced in metformin exposed adenine rats which is consistent with previous findings in mice with unilateral ureteral obstruction⁴ and 5/6th nephrectomized rats³.

In a recent retrospective, observational cohort study, it was reported that metformin users were less likely to develop end stage renal disease than non-metformin users in a patient population with type 2 diabetes and CKD stage 5¹⁹. However, to date, there are no prospective clinical reports available investigating the effect of metformin on the progression of chronic renal impairment in different CKD stages, which is probably due to the fear for lactic acidosis, historically thought to be associated with the accumulation of metformin in renal failure patients. Metformin is eliminated by the kidney for 80-100% and thus in CKD patients metformin treatment might result in accumulation of the compound and ultimately lead to increased lactate production by stimulation of the glycolysis and decreased lactate uptake by the liver, thereby inducing lactic acidosis²⁰. However, no significant changes in serum pH and bicarbonate were found in metformin treated adenine rats in the current study, and neither in serum pH and lactate levels of metformin treated 5/6th nephrectomized rats³. Clinical studies exploring the effect of metformin on the incidence of lactic acidosis are performed in

diabetic patients with a varying degree of renal function and report conflicting results. Ekström and colleagues²¹ revealed that metformin use in the clinical trial setting showed no increased risk of all-cause mortality, acidosis and serious infection or cardiovascular disease in patients with eGFR 30-45 ml/min/1.73 m². Metformin use did not affect the incidence of lactic acidosis in patients with type 2 diabetes who had normal, mild, moderate or severe renal dysfunction²². However, Eppenga et al.²³ reported in a retrospective patient database analysis that the risk of lactic acidosis or elevated lactate concentrations in current metformin users was significantly associated with a renal function <60 mL/min/1.73 m² in diabetic patients. Because of the growing body of evidence on the multiple beneficial effects of this anti-diabetic drug, interest for the use of metformin in CKD patients is reinforced and the applicable contra-indication of its use in this patient population is currently challenged, which is discussed elaborately in a recent review by Lalau et al.²⁰. This review strongly encourages to perform clinical studies evaluating the feasibility to continue metformin therapy in CKD, even in severe cases. The positive results of metformin on the kidney of the current experimental study also support this view and may be the impetus for clinical trials in CKD patients. Also for patients with adenine phosphoribosyltransferase deficiency who suffer from 2,8 dihydroxyadenine urolithiasis, metformin treatment might be considered.

Administration of a diet containing 0.25% adenine and a low vitamin K content to the rat is a valid experimental model for CKD-induced vascular calcification and high bone turnover disease^{15, 24}. None of the animals treated with metformin had developed calcification in the aorta, femoral and carotid arteries. The beneficial effects of metformin on renal function and mineral metabolism presumably prevented the onset of vascular calcification, since calcifications in the arteries develop as a result of manifested hyperphosphatemia in the adenine-induced CKD rat model. However, from this experimental study it was not clear whether the protective effect of metformin against vascular calcification was entirely attributable to the renoprotective effect or whether a primary effect on the vasculature was additionally involved. As such, a second experiment was performed to investigate the isolated effect of metformin on vascular calcification beyond its favourable effect on renal

function using a rat model of warfarin-induced moderate arterial calcification. After 10 weeks of daily gavage with 200 mg/kg metformin, no significant effect was observed on the calcification in the aorta nor in the smaller arteries. These findings strongly support the hypothesis that metformin, in the adenine rat model, blocked arterial calcification because of its preventive effect on the kidneys and suggest that the antidiabetic compound could not directly reduce the development of moderate (warfarin-induced) vascular calcification. In contrast to our study, others have reported that metformin might protect from calcification in the arteries. One *in vitro* experiment revealed that metformin inhibited β -glycerophosphate-induced calcification in rat aortic smooth muscle cells along with the prevention of phenotypic reprogramming towards osteoblast-like cells via the AMPK-eNOS-NO pathway²⁵. Vascular smooth muscle cell-specific AMPK α 1 knockout promoted aortic calcification in apoE knockout mice further confirming the important role of this protein kinase in atherosclerotic calcification²⁶. Very recently, metformin has been shown to reduce aortic calcification induced by vitamin D3 and nicotine in rats through modulation of the AMPK/eNOS pathway²⁷. The discrepancy between these and our results might be explained by the administered metformin dose and the severity of the calcifications. In the study executed by Zhang et al.²⁷ a markedly higher daily metformin dose of 300 mg/kg was used whereas the severity of the calcification in the vitamin D3 + nicotine rat model was substantially less. Furthermore, the number of animals that survived until the study end and as such were used for calcium bulk analyses in the study of Zhang et al.²⁷ was low which might have significantly biased the outcome. Nevertheless, it would be important and relevant to execute additional studies in which the isolated effect of higher metformin doses on vascular calcification are tested in (alternative) animal models with rather mild calcified lesions in the arteries. A recent clinical study reported that metformin use was independently associated with a lower below-the-knee arterial calcification score in type 2 diabetic patients²⁸. Taken together all these data, further preclinical as well as clinical studies are required to establish the extent to which this anti-diabetic drug is able to modulate vascular calcification and to further explore the signalling mechanisms that are influenced by metformin treatment in this pathological setting.

Rather unexpected, warfarin exposure led to a slight but significant increase in serum creatinine values and an altered calcium and phosphorus metabolism in normal rats which in, contrast to the adenine and other models of renal failure, was not affected by metformin treatment. An increase in serum creatinine was also observed in 5/6th nephrectomized rats treated with brodifacoum ('superwarfarin') and was accompanied by glomerular hemorrhage, tubular red blood cell casts and acute tubular injury ²⁹. Warfarin-, or more general, anticoagulant-related nephropathy as a newly recognized syndrome has recently gained much attention ^{30, 31}. In a large-scale study, Brodsky's research team found that warfarin-related nephropathy manifests in patients with and without CKD and correlates with increased mortality rate ³². The molecular and cell biological mechanisms that promote acute kidney injury under warfarin treatment are currently being investigated in both clinical and preclinical studies. Our study is the first to report an increase in serum creatinine levels in warfarin exposed rats that have not been subjected to CKD induction. How and to which extent warfarin led to kidney damage and dysfunction is an interesting future research topic.

The bone of adenine-induced CKD rats is characterized by significantly increased osteoid parameters, higher amounts of osteoblasts and osteoclasts and markedly increased bone turnover, thereby reflecting CKD-induced high bone turnover disease, which according to the TMV classification could be defined as mixed uremic osteodystrophy, and is provoked by to the secondary hyperparathyroidism that had developed as indicated by the significant rise in serum PTH levels. In the current study, daily metformin treatment markedly attenuated the increase in serum PTH levels in CKD animals which resulted in a significant reduction of both static and dynamic bone parameters, approaching levels of rats with normal kidney function. In addition, an accompanying normalization of the calcium and phosphorus balance provided protection against disturbed bone metabolism. Given the remarkable metformin-induced preservation of renal function, the observed amelioration of high bone turnover disease might thus primarily be attributed to the renoprotective effect of metformin. It should be noted, however, that direct beneficial effects of metformin on osteoblasts and osteoclasts have also been reported. In vitro studies demonstrated that metformin promotes

osteoblast differentiation and the deposition of mineralized nodules³³⁻³⁵. Additionally, metformin reduced receptor activator of nuclear factor kappa-B ligand and stimulates osteoprotegerin expression in osteoblasts, inhibited osteoclast differentiation and prevented bone loss in ovariectomized rats³⁶. It is therefore not unlikely that the favourable effect of metformin observed in our study is the result of multiple organ dependent actions.

In conclusion, metformin was shown to strongly attenuate development of severe CKD in rats with adenine-induced CKD and to significantly reduce renal inflammation and fibrosis. The concomitant preservation of normal serum calcium and phosphorus levels and significantly lower levels of FGF-23 and PTH levels going along with metformin treatment inhibited vascular calcification to develop and markedly reduced high bone turnover disease, two main complications of CKD-MBD. An additional study revealed that metformin could not reduce the onset and progression of moderate vascular calcification by direct actions on the vessel wall, independently of its renoprotective effect. These experimental results require validation of the clinical effect of this anti-diabetic drug on renal failure progression and its accompanying mineral and bone complications among patients with different CKD stages. Clinical studies evaluating dose adjustment in the more severe stages of CKD to avoid lactic acidosis and establishment of a therapeutic window for metformin in CKD patients are promising³⁷.

Methods

Experimental design

Experimental procedures were conducted according the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethical Committee (Permit number: 2014-67).

(i) *Rat model of adenine-induced CKD, vascular calcification and bone disease* - Twenty eight male Wistar rats (Iffa Credo, Brussels, Belgium) were housed two per cage, exposed to a 12h light/dark cycle and had free access to food and water. A two week equilibration period with access to standard animalarium food preceded the induction of CKD in order to gain body weight. To induce stable CKD, rats were administered a 0.25% adenine enriched diet with a low vitamin K content (0.25% adenine, 0.2 mg/kg Vitamin K, 1% Ca, 1% P, 1 IU/g vitamin D and 6% protein) (SSNIFF Spezialdiäten, Soest, Germany) during the entire study period of 8 weeks, as described earlier by our group ¹⁵. The reduced dietary vitamin K content has as its aim to make the animals more sensitive to the development of CKD-related vascular calcification. CKD rats were randomly assigned to two treatment groups, (i) vehicle (1% carboxymethylcellulose) (n=14) or (ii) 200 mg/kg metformin, which corresponds with a daily dose of 65 mg (n=14). Assuming a daily food intake of 20 g in the rat and 1000 g in humans the ratio of the daily metformin dose /food intake (65mg/20g) corresponds to a ratio of 3250mg/1000g which is highly comparable to the daily therapeutic dose of 1500 – 3000 mg metformin in humans. From 1 week after CKD induction onwards until the end of the study at week 8, treatments were daily administered by oral gavage. Metformin was dissolved in 1% carboxymethylcellulose and a constant dose volume of 10 ml/kg was used. Before the start of CKD induction (week 0), before the start of treatment (week 1) and at weeks 4, 6 and 8 of the study, animals were housed in a metabolic cage for 24 hours to collect urine samples followed by blood sampling via the tail vein in restrained, conscious animals. At sacrifice, ionized calcium, pH, bicarbonate, hematocrit and hemoglobin were measured using i-STAT 1 Point-of-Care analyzer (Abbott, Berkshire, UK) immediately at the time of blood sampling. After 8 weeks of CKD, all animals

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3 were sacrificed by exsanguination through the retro-orbital plexus after anesthesia with 60mg/kg
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5 sodium pentobarbital (Nembutal, Ceva Sante Animale, France) via intraperitoneal injection.
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9 (ii) *Rat model of warfarin-induced vascular calcification* – Twenty eight male Wistar rats (Iffa Credo)
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11 were fed a 0.3% warfarin and 0.15% vitamin K₁ (to prevent bleeding from warfarin treatment)
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13 supplemented diet (17.6% protein, 1.0 % Ca and 0.7% P) according to Schurgers L et al.³⁸ for the
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15 entire study period of 10 weeks to induce vascular calcification. Animals were randomly assigned to
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17 two treatment groups, i.e. (i) vehicle (1% carboxymethylcellulose) (n=14) and (ii) 200 mg/kg
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19 metformin (n=14). Animals were daily gavaged with vehicle or 200 mg/kg metformin from the start
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21 of the study onwards until the end of the study at week 10. Metformin was dissolved in 1%
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23 carboxymethylcellulose and a constant dose volume of 10 ml/kg was used. At baseline, week 6 and
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25 before sacrifice, animals were placed in a metabolic cage for 24 hours to collect urine samples
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27 followed by blood sampling via the tail vein in restrained, conscious animals. Blood glucose
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29 (GlucoMen Lx Plus+ automated whole blood glucometer), pH, bicarbonate and lactate (i-STAT Point
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31 of Care) was determined to evaluate the effect of metformin on glucose metabolism and acid/base
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33 balance. After 10 weeks, animals of both study groups were sacrificed by exsanguination through the
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35 retro-orbital plexus after anesthesia with 60mg/kg sodium pentobarbital via intraperitoneal injection.
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40 Biochemical analyses

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42 Serum creatinine was determined according to the Jaffe method. Total calcium in serum and urine
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44 was measured with flame atomic absorption spectrometry (Perkin-Elmer, Wellesley, MA, USA) after
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46 appropriate dilution in 0.1% lanthanum nitrate La(NO₃)₃ to eliminate chemical interference. Serum
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48 and urinary phosphorus were measured using the Ecoline®S Phosphate kit (DiaSys, Germany). Serum
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50 iPTH (Immutopics, San Clemente, CA, USA) and FGF-23 (Kainos Laboratories, Tokyo, Japan) levels
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52 were determined by use of an ELISA kit.
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Adenine-induced CKD rat model - Renal histological analysis

Immediately after isolation of the left kidney, a transverse slice was fixed in neutral buffered formalin for 4 hours, rinsed with isopropanol and embedded in low-melting point paraffin (52°C; Kendall, Mansfield, MA, USA). Deparaffinized tissue sections (4 µm) were stained with Periodic Acid Schiff (PAS) for general morphological evaluation and quantitation of tubulointerstitial area. Nuclei were counterstained with methyl green.

A random picture (200x) of the renal cortex was obtained from each PAS-stained section and tubulointerstitial area was quantified histomorphometrically using Fiji image analysis software³⁹ by calculating the ratio between the number of pixels taken by the region that contains no tubules or glomeruli and the number of pixels taken by the total picture, resulting in the area percent tubulointerstitium. Additionally, the presence of excess extracellular matrix (i.e. fibrosis) was evaluated on neutral formalin fixed sections stained with Masson's trichrome after post-fixation in Bouin's fixative.

Adenine-induced CKD rat model - Quantification of renal crystals

Immediately after isolation of the left kidney, a transverse slice was fixed in neutral buffered formalin for 4 hours, rinsed with isopropanol and embedded in low-melting point paraffin (52°C; Kendall, Mansfield, MA, USA). Deparaffinized tissue sections (4 µm) were stained with haematoxylin and eosin. In each renal section 2, 8 dihydroxyadenine crystals were quantified histomorphometrically using Axiovision image analysis software (Release 4.5, Carl Zeiss, Oberkochen, Germany). Renal crystal content was determined by calculating the ratio between the number of pixels taken by the crystals and the number of pixels taken by the total renal tissue resulting in the area percent crystal deposition.

Adenine-induced CKD rat model - Quantitative real time PCR

Total mRNA of a snap-frozen section of the left kidney was extracted using the RNeasy Fibrous Tissue mini kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA by the High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Real time polymerase chain reaction (PCR) with an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) based on Taqman fluorescence methodology was used for mRNA quantification. Taqman probe and primers were purchased as Taqman® gene expression assays-on-demand from Applied Biosystems for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Rn99999916_s1), bax (Rn01480160_g1), bcl2 (Rn99999125_m1), tumor necrosis factor alpha (TNFα) (Rn00562055_m1), interleukin-1 beta (IL-1β) (Rn00580432_m1), transforming growth factor beta (TGF-β) (Rn00572010_m1), collagen type I, alpha 1 (Coll 1a1) (Rn00801649_g1) and vascular cell adhesion molecule 1 (VCAM1) (Rn00563627_m1). The expression of each tested gene was analyzed in triplicate for each sample and normalized to the expression of the house keeping reference gene GAPDH. Gene expression was calculated with the comparative Ct method using kidney samples of vehicle-treated CKD rats as calibrator samples.

Adenine-induced CKD rat model - Bone histomorphometry

The left tibia of each animal was isolated and cleared of surrounding tissue. After fixation in 70% ethanol overnight, the left tibia was further processed for bone histomorphometric analysis: tibias were dehydrated and embedded in 100% methylmetacrylate (Merck, Hohenbrunn, Germany). Five µm thick sections were Goldner stained for visualization and measurement of total bone area, mineralized bone area, osteoid width and area, osteoblast perimeter, eroded perimeter and osteoclast perimeter which were quantified with Axiovision image analysis software (Release 4.5, Carl Zeiss, Oberkochen, Germany). Ten µm thick unstained sections of the tibia were used to measure distance between and length of double tetracycline and demeclocycline labels by fluorescence microscopy. Out of these primary data, additional histological and dynamic bone parameters were calculated according to Parfitt et al⁴⁰.

Quantification of vascular calcification

The proximal part of the abdominal aorta and the left carotid and femoral artery were isolated and weighed on a precision balance. Subsequently, the samples were digested in 65% HNO₃ at 60°C for 5 hours. Tissue digests were adjusted to a final volume of 2.5 ml for the aorta or 1.5 ml for the peripheral arteries and appropriately diluted from this stock solution with 0.1% La(NO₃)₃ to eliminate chemical interference during measurement of the total calcium content. The calcium content of each artery was measured with flame atomic absorption spectrometry (FAAS) and expressed as mg calcium/g wet tissue. With regard to the warfarin rat model, the left renal artery and one slice of the heart was also taken to determine the calcium content by use of FAAS.

Statistics

Results are expressed as mean ± SEM unless otherwise indicated. Non-parametric statistical analyses were performed with SPSS 23.0 software. Statistical differences between groups were investigated with a Kruskal-Wallis unrelated samples test, followed a Mann Whitney-U test. Comparison of time-points was performed with a Friedman related samples test, followed by a Wilcoxon signed ranks test. Bonferroni correction was applied when appropriate. A P-value <0.05 is considered significant. Supplementary information is available at *Kidney International's* website.

Disclosure

This work was supported by a research grant from Merck KGaA, Darmstadt, Germany. Co-authors Kerstin Brand and Ulrike Gottwald-Hostalek are employees at Merck KGaA.

Acknowledgements

We especially thank Geert Dams, Hilde Geryl, Ludwig Lamberts, Rita Marynissen and Simonne Dauwe for their excellent technical assistance, and Dirk De Weerd for his help with the graphics. Ellen

Neven is a postdoctoral fellow of the Fund for Scientific Research-Flanders (FWO). Britt Opdebeeck and Annelies De Maré are PhD fellows of the FWO.

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Figure legends

Figure 1: Effect of metformin on renal function and mineral homeostasis. Serum creatinine (A), creatinine clearance (B), serum phosphorus (C), serum calcium (D), serum PTH (E) and serum FGF-23 (F) in CKD rats treated with vehicle (n=14) or metformin (n=13). * p< 0.05 vs. vehicle; # p< 0.05 vs. week 0 (vehicle group); # p< 0.05 vs. week 0 (metformin group).

Figure 2: Effect of metformin on the % of renal tissue surface covered by tubulointerstitium (A) and 2, 8 dihydroxyadenine crystals (B), renal histology (Periodic Acid Schiff staining) (C) and renal fibrosis (Trichome Masson staining) (D). *p< 0.05 vs. vehicle.

Figure 3: Effect of metformin on renal inflammation (TNF α and IL-1 β expression), apoptosis (bax/bcl2 expression), fibrosis (TGF β and collagen 1a1 expression) and cellular infiltration (VCAM1 expression). The mRNA expression of the various genes is presented relative to GAPDH expression. * p< 0.05 vs. vehicle.

Figure 4: Impact of metformin treatment on vascular calcification in adenine-induced CKD rats. Total calcium content in the aorta (A), femoral artery (B) and carotid artery (C) in vehicle (n=14) versus metformin (n=13) treated CKD rats. Von Kossa stained sections of (D) a calcified aorta of a vehicle treated rat and (E) an intact aorta of a metformin treated rat. * p< 0.05 vs. vehicle.

Figure 5: Impact of metformin treatment on static and dynamic bone parameters. Bone area, osteoid area, osteoid and eroded perimeter, osteoblast and osteoclast perimeter, mineralization lag time and bone formation rate in vehicle (n=14) versus metformin (n=13) treated CKD rats. The hatched zone represents data of rats with normal renal function (mean $\pm$ SD). * p< 0.05 vs. vehicle.

Figure 6: Goldner stained (A and C) and unstained (B and D) sections of the tibia of CKD rats treated with vehicle (A and B) or metformin (C and D).

Figure 7: Impact of metformin treatment on vascular calcification in warfarin rats. Total calcium content in the aorta (A), femoral artery (B) carotid artery (C) and renal artery (D) as well as in the heart (E) in vehicle (n=12) versus metformin (n=10) treated warfarin rats.

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Table 1: Serum parameters indicating renal function, mineral and glucose metabolism and acid/base balance in the warfarin rat model of vascular calcification . ° p<0.05 versus week 0. * p<0.05 versus metformin.

		week 0	week 6	Week 10
Creatinine (mg/dl)	Vehicle	0,41 ± 0,01	0,75 ± 0,01°	0,79 ± 0,02°
	Metformin	0,45 ± 0,02	0,75 ± 0,01°	0,77 ± 0,02°
Phosphorus (mg/dl)	Vehicle	10,5 ± 0,2	7,5 ± 0,2°	7,9 ± 0,7°
	Metformin	10,3 ± 0,3	7,1 ± 0,3°	9,6 ± 1,3
Calcium (mg/dl)	Vehicle	12,9 ± 0,1	13,9 ± 0,1°	16,8 ± 0,2°
	Metformin	12,7 ± 0,2	14,7 ± 0,1°*	17,1 ± 0,3°
Glucose (mg/dl)	Vehicle	141,8 ± 3,1	139,8 ± 4,6	156,5 ± 3,3°
	Metformin	142,3 ± 5,4	142,4 ± 3,0	172,1 ± 6,5°
pH	Vehicle	7,33 ± 0,03	7,39 ± 0,01	7,37 ± 0,03
	Metformin	7,32 ± 0,01	7,40 ± 0,02°	7,40 ± 0,03°
HCO ₃ (mM)	Vehicle	25,7 ± 0,6	26,7 ± 0,4	29,6 ± 1,0
	Metformin	25,7 ± 0,5	25,5 ± 0,6	30,8 ± 1,0°
Lactate (mM)	Vehicle	4,81 ± 0,49	4,50 ± 0,40	4,22 ± 0,84
	Metformin	5,24 ± 0,34	4,90 ± 0,62	3,83 ± 0,61

Figure 1

213x281mm (300 x 300 DPI)

Figure 2

157x197mm (300 x 300 DPI)

Figure 3

124x63mm (300 x 300 DPI)

Figure 4

131x106mm (300 x 300 DPI)

Figure 5

180x155mm (300 x 300 DPI)

Figure 6

83x58mm (300 x 300 DPI)

Figure 7

139x139mm (300 x 300 DPI)

69x29mm (300 x 300 DPI)