

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/331018555>

Diabetes-induced hypomagnesemia is not modulated by metformin treatment in mice

Article in *Scientific Reports* · February 2019

DOI: 10.1038/s41598-018-38351-3

CITATIONS

0

READS

342

7 authors, including:



Caro Bos

Radboud University Medical Centre (Radboudumc)

7 PUBLICATIONS 7 CITATIONS

[SEE PROFILE](#)



Mohammed KEBIECHE

The University of Batna2

68 PUBLICATIONS 278 CITATIONS

[SEE PROFILE](#)



Jeroen H.F. de Baaij

Radboud University Medical Centre (Radboudumc)

57 PUBLICATIONS 836 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



effet de sélénium et les huiles essentielle sur la toxicité du nickel [View project](#)



XENOBOTIQUE ET STRESS OXYDATIF [View project](#)

SCIENTIFIC REPORTS

OPEN

Diabetes-induced hypomagnesemia is not modulated by metformin treatment in mice

Steeff Kurstjens¹, Hacene Bouras^{1,2}, Caro Overmars-Bos¹, Mohamed Kebieche³, René J. M. Bindels¹, Joost G. J. Hoenderop¹ & Jeroen H. F. de Baaij¹

Approximately 30% of patients with type 2 diabetes mellitus (T2D) have hypomagnesemia (blood magnesium (Mg^{2+}) concentration <0.7 mmol/L). In T2D patients, treatment with metformin is associated with reduced blood Mg^{2+} levels. To investigate how T2D and metformin affect Mg^{2+} homeostasis db/m and db/db mice were treated with metformin or placebo. Mice were housed in metabolic cages to measure food and water intake, and to collect urine and feces. Serum and urinary Mg^{2+} concentrations were determined and mRNA expression of magnesiotropic genes was determined in kidney and distal colon using RT-qPCR. Db/db mice had significantly lower serum Mg^{2+} levels than db/m mice. Mild hypermagnesuria was observed in the db/db mice at two weeks, but not at four weeks. Metformin-treatment had no effect on the serum Mg^{2+} concentration and on the urinary Mg^{2+} excretion. Both in kidney and distal colon of db/db mice, there was a compensatory upregulation in the mRNA expression of magnesiotropic genes, such as transient receptor potential melastatin 6 (*Trpm6*), whereas metformin treatment did not affect gene expression levels. In conclusion, we show that T2D causes hypomagnesemia and that metformin treatment has no effect on Mg^{2+} homeostasis in mice.

Approximately 30% of patients with type 2 diabetes mellitus (T2D) have hypomagnesemia (blood magnesium (Mg^{2+}) <0.7 mmol/L)^{1,2}. Hypomagnesemia has serious clinical consequences as it increases the risk of complications such as retinopathy, nephropathy, micro and macrovascular disease and foot ulceration^{3,4}. Moreover, Mg^{2+} deficiency is correlated with insulin resistance, abrogated glucose metabolism and an increased risk of developing T2D⁵⁻⁷. However, the etiology and underlying mechanisms of hypomagnesemia in T2D patients remains largely unknown⁸.

As Mg^{2+} is necessary for the activity of over 600 enzymes, it plays numerous vital physiological functions including macromolecule synthesis, energy balance and DNA transcription⁹. Moreover, Mg^{2+} stabilizes ATP and is required for its phosphor transfer reactions¹⁰.

The intestine and kidney collaboratively regulate Mg^{2+} balance and maintain its blood concentrations within a narrow range^{11,12}. In the gut, the bulk of Mg^{2+} absorption occurs in the small intestine *via* paracellular (passive) transport¹¹. In the colon, the final absorption of Mg^{2+} takes place by an active transcellular mechanism through transient receptor potential melastatin type 6/7 (TRPM6/TRPM7) cation channels⁹. In the kidney, 95–99% of filtered Mg^{2+} is reabsorbed under physiological circumstances⁹. Approximately 85% of the filtered Mg^{2+} is reabsorbed paracellularly by the proximal tubule and the thick ascending loop of Henle (TAL), where transport relies on tight junction permeability^{13,14}. Active transport in the distal convoluted tubule (DCT) determines the final urinary Mg^{2+} concentration, as this is the final segment where Mg^{2+} is reabsorbed¹⁵. In physiological conditions, the DCT reclaims 5–10% of filtered Mg^{2+} transcellularly via TRPM6/7 channels^{14,16}. The expression and/or the activity of TRPM6 is affected by SNPs, dietary Mg^{2+} intake, drugs and hormones, such as insulin and epidermal growth factor (EGF)^{14,17-20}. SNPs in TRPM6 that impair its response to insulin have been associated with an increased risk of developing T2D and gestational diabetes^{7,19}.

Metformin, the first-line pharmacotherapy in T2D²¹, suppresses hepatic gluconeogenesis and improves insulin sensitivity²². Therefore, its major clinical benefit is reducing blood glucose levels with only a minimal risk of

¹Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands. ²Faculty of Nature and Life Sciences, University of Mohammed Seddik Ben Yahia, Jijel, Algeria. ³Faculty of Nature and Life Sciences, University of Batna 2, Batna, Algeria. Steeff Kurstjens and Hacene Bouras contributed equally. Correspondence and requests for materials should be addressed to J.H.F.d.B. (email: jeroen.debaaij@radboudumc.nl)

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>Cldn10b</i>	GGAGTTCCCCTCCATGCT	GCAAAAATGGAACCGAAAAA
<i>Cldn14</i>	GTCCAGCTCCTAGGCTTCCT	CATCCACAGTCCCTTCAGGT
<i>Cldn16</i>	GTTGCAGGGACCACATTAC	GAGGAGCGTTCGACGTAAAC
<i>Cldn19</i>	GGTTCCTTTCTCTGCTGCAC	CGGGCAACTTAACAACAGG
<i>Cnm4</i>	TCTGGGCCAGTATGTCTCTG	CACAGCCATCGAAGGTAGG
<i>Fxyd2</i>	TCAGCCTTTCTTGACTGG	GGTCTTCTGTGGCCTCTACT
<i>Hprt</i>	TTGCTGACCTGCTGGATTAC	AGTTGAGAGATCATCTCCAC
<i>Slc12a1</i>	CACATGGTCTTCCACTGTGGTT	GGCTCTCCACACAGGCTC
<i>Slc12a3</i>	CTTCGGCCACTGGCATTCTG	GATGGCAAGGTAGGAGATGG
<i>Slc41a1</i>	CATCCACACGCCTTCCTGC	CGGCTGGCCTGCACAGCCAC
<i>Slc41a3</i>	TGAAGGGAAACCTGGAAATG	GGTTGTCTGCTGATGATTTTG
<i>Trpm6</i>	CTTCACAATGAAAACCTGCC	AAAGCCATGCGAGTTATCAGC
<i>Trpm7</i>	GGTTCCTCTGTGGTGCCTT	CCCCATGTCGTCTCTGTCTG

Table 1. Primer sequences used for RT-qPCR. *Cldn10b*, claudin 10b; *Cldn14*, claudin 14; *Cldn16*, claudin 16; *Cldn19*, Claudin 19; *Cnm4*, cyclin M4; *Fxyd2*, FXYD-domain containing 2; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; *Slc12a1*, solute carrier family 12 member 1; *Slc12a3*, solute carrier family 12 member 3; *Slc41a1*, solute carrier family 41 member 1; *Slc41a3*, solute carrier family 41 member 3; *Trpm6*, transient receptor potential melastatin type 6; *Trpm7*, transient receptor potential melastatin type 7.

hypoglycemia^{23,24}. The most common side effects of metformin treatment are lactic acidosis, nausea and diarrhea²⁵. Recent cohort studies showed that metformin use in T2D patients is associated with reduced blood Mg²⁺ levels^{1,26}. However, the mechanism that underlies this correlation has not yet been elucidated. To investigate how T2D and metformin affect Mg²⁺ homeostasis, control (db/m) and diabetic (db/db) mice were treated with placebo or metformin for four weeks. Serum and urinary electrolytes were measured and mRNA expression of magnesium-tropic genes was evaluated in kidney and distal colon.

Methods

Animal study. The animal study was approved by the animal ethics board of the Radboud University Nijmegen (RU DEC 2015-0073) and by the Dutch Central Commission for Animal Experiments (CCD, AVD103002015239). Experimental procedures were conducted in accordance with the institutional guidelines and in compliance with Dutch and European laws and policies. Twenty diabetic (db/db) and twenty control (db/m) male mice (Charles River, Germany), aged 8–10 weeks, were acclimated for two weeks in a temperature- and light-controlled room two mice per cage (Eurostandard Type IIL), with *ad libitum* access to tap water and standard pellet chow. At day 0, diets were changed to a diet containing 0.05% (w/w) MgO (#S9074-E1107, Ssniff Spezialdiäten, GmbH, Germany) and drinking water to demineralized water. At days -2, 12 and 26 mice were housed individually in metabolic cages for 48 hours (24 hours adaptation, 24 hours collection) to measure food and water intake and to collect urine and feces. Mice were weighed twice weekly and blood was collected *via* the submandibular vein at days -2 and 15. Mice were randomly divided into four experimental groups of ten mice per group, of which half received metformin hydrochloride (0.5 mg/ml, Sigma Aldrich, MI, USA), dispersed in the drinking water. Researchers and animal caretakers were blinded for the metformin treatment. After 28 days of treatment, mice were anaesthetized by 4% (v/v) isoflurane and exsanguinated by orbital sinus bleeding, and death was confirmed by cervical dislocation. Colon and kidney tissues were cleaned with ice-cold PBS and snap-frozen in liquid nitrogen.

RT-qPCR. TRIzol reagent (Invitrogen, Bleiswijk, the Netherlands) was used to extract total RNA from kidney and distal colon according to the manufacturer's protocol. RNA was subjected to DNase (Promega, the Netherlands) treatment at 37 °C for 30 min and then to DNase stop buffer at 65 °C for 10 min. The RNA concentration was measured using the Nanodrop 2000c (Thermoscientific, Wilmington, DE). To synthesize cDNA, 1.5 µg of total RNA was reverse transcribed for 1 hour at 37 °C using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Bleiswijk, the Netherlands). SYBR Green Supermix (BioRad, Veenendaal, the Netherlands) was used to analyze the gene expression levels on a BioRad (Hercules, CA, USA) analyzer. After normalizing to housekeeping gene expression (*Hprt*), the relative gene expression was calculated by the Livak method ($2^{-\Delta\Delta Ct}$). Primers sequences are provided in Table 1.

Analytical measurements. Serum and urinary Mg²⁺ concentrations were determined using a spectrophotometric assay (Roche/Hitachi, Tokyo, Japan), according to manufacturer's protocol. Ca²⁺ concentrations were determined by the o-cresolphthalein complexone method. Absorbance for the Mg²⁺ and Ca²⁺ assays was measured at 600 nm and 570 nm, respectively, on a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories, CA, USA). Serum and urinary Na⁺ and K⁺ concentrations were measured at the clinical chemistry department applying standardized methods¹. Serum and urinary glucose concentrations were determined by a spectrophotometric assay according to the manufacturer's protocol (Instruchemie, Delfzijl, the Netherlands).

Statistical analyses. Interaction between the two main variables (genotype and treatment) was investigated using a two-way ANOVA test. If there was a significant interaction effect, an unpaired multiple *t* test, with

the Holm–Sidak method for multiple comparisons, was used. In the absence of a significant interaction effect, a two-way ANOVA approach with a Tukey's multiple comparisons test was used. Statistical significance was assessed using Graphpad Prism v7 (La Jolla, CA, USA, RRID: SCR_002798). A p -value of ≤ 0.05 was considered statistically significant. Results are presented as mean \pm SEM.

Results

Metformin reduces food intake of db/db mice without affecting body weight. To investigate how T2D and its first-line treatment metformin affect Mg^{2+} homeostasis, control (db/m) and diabetic (db/db) mice were treated with metformin or placebo for four weeks. Db/db mice were significantly heavier than db/m mice (Fig. 1a,b; 27.0 ± 0.3 vs. 45.6 ± 0.6 gr. for db/m and db/db mice at four weeks, respectively, $p \leq 0.05$). Metformin treatment had no effect on body weight in both db/m and db/db mice (Fig. 1a,b). Metformin treatment reduced the food intake only in the db/db mice (Fig. 1c). The lower food intake was accompanied by a decreased feces weight, water intake and urinary volume in the metformin-treated db/db mice (Fig. 1d–f). Metformin did not influence non-fasting serum glucose levels in both genotypes (Fig. 1g). However, the glycosuria of the db/db mice was attenuated by metformin treatment (Fig. 1h).

Db/db mice have reduced serum Mg^{2+} concentrations. Serum Mg^{2+} concentrations were lower in db/db than db/m mice at two weeks (Fig. 2a, 1.17 ± 0.04 vs. 0.88 ± 0.04 mmol/L in db/m vs. db/db placebo-treated mice, respectively, Holm-Sidak's multiple comparison $p \leq 0.05$) and four weeks (Fig. 2b, 1.10 ± 0.05 vs. 0.95 ± 0.04 mmol/L in db/m vs. db/db placebo-treated mice, respectively, Holm-Sidak's multiple comparison $p \leq 0.05$). At two weeks, there was a significant genotype effect on urinary Mg^{2+} excretion, demonstrating an increased urinary Mg^{2+} loss in db/db mice (Fig. 2c, 6.8 ± 0.6 vs. 8.6 ± 0.6 μ mol/24h in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$), whereas no significant difference was observed at four weeks (Fig. 2d). At four weeks, the serum Ca^{2+} concentration was higher in db/db compared to db/m mice, indicated by a significant genotype effect (Fig. 2e, 1.28 ± 0.05 vs. 1.46 ± 0.06 mmol/L Ca^{2+} in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$). There were no significant differences on urinary Ca^{2+} excretion (Fig. 2f). Despite the higher food intake of db/db animals, a significant genotype effect demonstrated lower serum Na^+ levels in db/db compared to db/m mice (Fig. 2g, 167 ± 2 vs. 159 ± 3 mmol/L Na^+ in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$). Urinary excretion of Na^+ and K^+ was higher in db/db than db/m mice, and metformin treatment reduced Na^+ and K^+ excretion only in db/db mice (Fig. 2h,j). Serum K^+ concentrations were not different between all experimental groups (Fig. 2i).

Db/db mice have an enhanced colonic expression of *Trpm6*. When serum Mg^{2+} levels decrease, intestinal uptake of Mg^{2+} is enhanced¹⁵. Colonic mRNA expression of *Trpm6*, the major channel for regulated Mg^{2+} absorption, was elevated in db/db compared to db/m mice (Fig. 3a). There was no difference in mRNA expression of the ubiquitous Mg^{2+} channel *Trpm7* and of the Mg^{2+} transport regulator Cyclin m4 (*Cnnm4*) (Fig. 3b,c). The colonic gene expression of the basolateral Mg^{2+} transporter solute carrier family 41 (*Slc41a1*) was lower in both db/db groups (Fig. 3d).

Db/db mice have an elevated renal expression of genes involved in Mg^{2+} handling. Db/db mice had an enhanced gene expression of the DCT-specific apical Mg^{2+} channel *Trpm6*, and the basolateral Mg^{2+} extruder *Slc41a3* (Fig. 4a,b). While both db/db groups showed a higher expression of *Slc12a3*, encoding for NCC, metformin further enhanced the expression of this gene in db/db mice (Fig. 4c). The driving force for paracellular Mg^{2+} uptake in the TAL is generated by NKCC2, encoded by *Slc12a1*, which is expressed higher in db/db mice (Fig. 4d). A significant genotype effect indicated a decreased expression of *Claudin 10b* (*Cldn10b*) in db/db mice (Fig. 4e, 1.00 ± 0.05 vs. 0.83 ± 0.02 relative gene expression in db/m vs. db/db mice, two-way ANOVA $p \leq 0.05$). In contrast, the mRNA expression of *Cldn14*, *Cldn16* and *Cldn19* was enhanced in db/db mice (Fig. 4f–h). The gene expression of the ubiquitous Mg^{2+} channel *Trpm7* was elevated in the placebo-treated db/db mice and the expression of *Fxyd2*, encoding for the gamma subunit of the Na^+ - K^+ -ATPase, was enhanced in both db/db groups (Fig. 4i,j).

Discussion

Hypomagnesemia is a common clinical feature in T2D patients^{1,3}. Metformin use is associated with a lower blood Mg^{2+} concentration in these patients^{1,26}. In this study, db/db mice developed hypomagnesemia with compensatory upregulation of key renal and colonic magnesiotropic genes. Metformin treatment had no effect on Mg^{2+} homeostasis in either control or diabetic mice. Our data demonstrate that hypomagnesemia is a consequence of T2D and is not modulated by metformin treatment in mice. INSERT ENTER Metformin is the first-line therapy for T2D²⁷. In large-scale observational cohort studies metformin-use is associated with lower serum Mg^{2+} levels and reduced renal Mg^{2+} wasting in T2D patients^{1,26,28–30}. In a small intervention study in T2D patients, metformin treatment resulted in a minor reduction in the serum Mg^{2+} concentration (from 0.72 to 0.70 mmol/L), despite major improvements in the blood glucose concentration²⁹. In our study, metformin treatment did not affect the serum Mg^{2+} concentration and urinary Mg^{2+} excretion in db/db and db/m mice. In addition, metformin did not alter gene expression of colonic and renal Mg^{2+} transporters. This is in line with a study that observed no effect of a two-week metformin treatment on serum Mg^{2+} levels in streptozotocin-induced diabetic rats³¹. Possibly, a two- to four-week treatment duration is too short to detect effects on Mg^{2+} homeostasis. The association between metformin and lower serum Mg^{2+} levels in T2D patients could also be caused by other factors that were not included in the analyses. For instance, a well-known side effect of metformin-treatment is chronic diarrhea, leading to intestinal malabsorption and hypomagnesemia²⁸.

Hypomagnesemia is prevalent in over 30% of T2D patients^{32–35}. A remaining question is whether hypomagnesemia is the cause or the consequence of T2D⁸. In the present study, db/db mice developed hypomagnesemia,

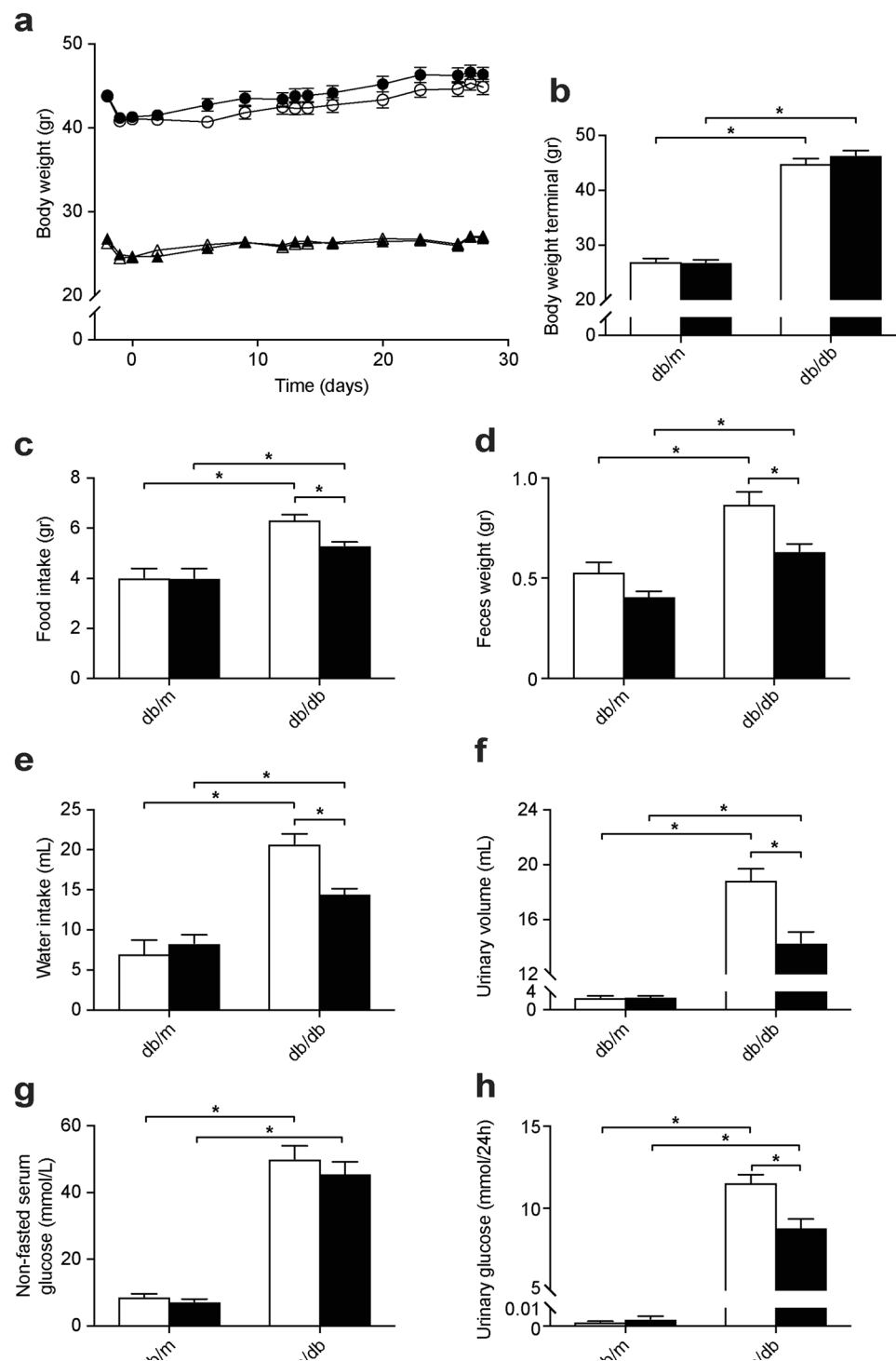


Figure 1. Metformin treatment does not affect body weight, but reduces food intake and urinary glucose excretion in db/db mice. Db/m and db/db mice were treated with metformin for four weeks. **(a)** Body weight of the animals, measured twice weekly and on the days of the metabolic cage experiments. Triangles, db/m mice; circles, db/db mice; open symbols, placebo-treated mice; closed symbols, metformin-treated mice. **(b)** Body weight at the end of the experiment, after four weeks of treatment. **(c)** Food intake, **(d)** total feces weight, **(e)** water intake and **(f)** urinary volume determined over a period of 24 hours, using metabolic cages, after four weeks of treatment. **(g)** Non-fasted serum glucose concentration and **(h)** 24-hour urinary glucose excretion after four weeks of treatment. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean \pm SEM. Depending on the absence or presence of a significant interaction effect between genotype and treatment, either a two-way ANOVA (Tukey's multiple comparison test) or an unpaired multiple *t* test (Holm-Sidak multiple comparison test) approach, respectively, was used to determine statistical significance. *Indicates a $p \leq 0.05$.

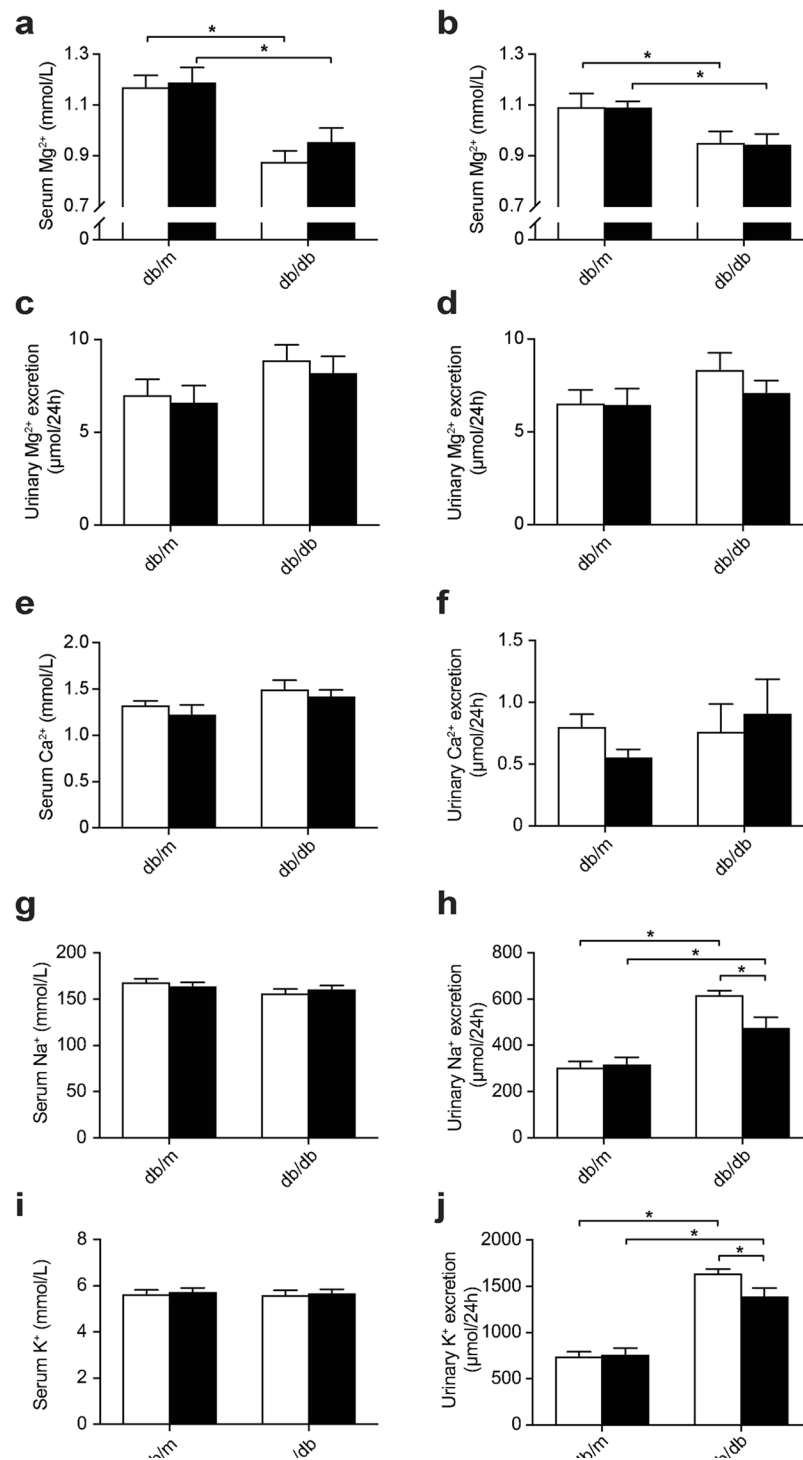


Figure 2. Db/db mice have a lower serum Mg^{2+} concentration which is not modulated by metformin treatment. **(a)** Serum Mg^{2+} concentration after two weeks of treatment and **(b)** after four weeks of treatment. **(c)** 24-Hour urinary Mg^{2+} excretion after two weeks of treatment (6.8 ± 0.6 vs. $8.6 \pm 0.6 \mu\text{mol}/24\text{h}$ in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$) and **(d)** after four weeks of treatment. **(e)** Serum Ca^{2+} concentration (1.28 ± 0.05 vs. $1.46 \pm 0.06 \text{mmol/L}$ Ca^{2+} in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$) and **(f)** 24-hour urinary Ca^{2+} excretion, after four weeks of treatment. **(g)** Serum Na^+ concentration (167 ± 2 vs. $159 \pm 3 \text{mmol/L}$ Na^+ in db/m vs. db/db mice, respectively, two-way ANOVA $p \leq 0.05$) and **(h)** 24-hour urinary Na^+ excretion, after four weeks of treatment. **(i)** Serum K^+ concentration and **(j)** 24-hour urinary K^+ excretion, after four weeks of treatment. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean \pm SEM. Depending on the absence or presence of a significant interaction effect between genotype and treatment, either a two-way ANOVA (Tukey's multiple comparison test) or an unpaired multiple t test (Holm-Sidak multiple comparison test) approach, respectively, was used to determine statistical significance. *Indicates a $p \leq 0.05$.

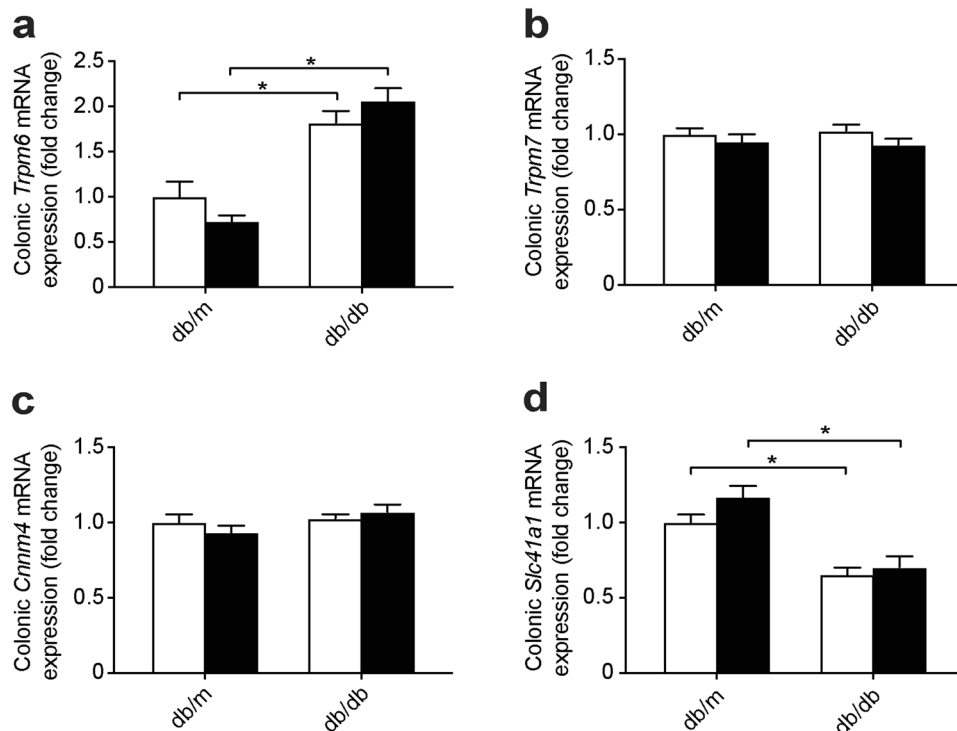


Figure 3. Upregulation of *Trpm6* mRNA expression in the colon of db/db mice. mRNA expression of key magnesiotropic genes in distal colon (a) *Trpm6*, (b) *Trpm7*, (c) *Cnm4* and (d) *Slc41a1*. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean \pm SEM. Depending on the absence or presence of a significant interaction effect between genotype and treatment, either a two-way ANOVA (Tukey's multiple comparison test) or an unpaired multiple *t* test (Holm-Sidak multiple comparison test) approach, respectively, was used to determine statistical significance. *Indicates a $p \leq 0.05$.

indicating that hypomagnesemia is a consequence of T2D. At the fourth week of the experiment, db/db mice developed massive glycosuria but no renal Mg^{2+} wasting. This finding is against the leading hypothesis that renal Mg^{2+} wasting in T2D patients is a result of glycosuria^{2,3,36}. Indeed, metformin treatment noticeably decreased glycosuria in db/db mice but did not modify the urinary Mg^{2+} excretion. This is in line with recent observations that glycosuria-causing SGLT2 inhibitors, lead to a mild increase in serum Mg^{2+} levels^{37,38}. Therefore, it is unlikely that glycosuria underlies hypermagnesuria-induced hypomagnesemia in T2D. As db/db mice develop severe hyperinsulinemia, the observed hypomagnesemia could be a consequence of a Mg^{2+} -shift towards the intracellular compartment, induced by insulin^{39–41}. Future studies should focus on measuring intracellular Mg^{2+} concentrations in diabetic mice.

The kidneys are essential in maintaining the serum Mg^{2+} concentration within the physiological range¹⁵. The DCT is the final segment where Mg^{2+} can be reabsorbed⁹. In the DCT, regulated Mg^{2+} reabsorption takes place transcellularly via TRPM6¹⁸. Mg^{2+} uptake by TRPM6 is dependent on NCC, although the underlying mechanism remains largely unknown^{42,43}. Gene expression levels of *Trpm6* and *Slc12a3*, encoding for NCC, were enhanced in db/db mice, indicative of compensation in the DCT. As only a minor hypermagnesuria is observed at two-weeks, and no hypermagnesuria at four-weeks, there appears to be proper renal compensation in the db/db mice. The TAL is responsible for the bulk of renal Mg^{2+} reabsorption⁹. In the TAL, paracellular Mg^{2+} and Ca^{2+} reabsorption is regulated by the *Cldn14/16/19* complex^{44,45}. *Cldn14* mRNA expression is strongly regulated by dietary Ca^{2+} intake^{46,47}. The high food intake, and therefore high Ca^{2+} intake, of db/db mice is likely the underlying cause of the extensive upregulation of *Cldn14* expression. The high expression of *Cldn14* will have a negative effect on Mg^{2+} reabsorption in the TAL, leading to a compensatory increase in *Cldn16/19* expression⁴⁸. In contrast, gene expression of *Cldn10b* was decreased. *Cldn10b* enhances the Na^{+} -permeability of the TAL, and thereby indirectly increases uptake of Mg^{2+} and Ca^{2+} in the TAL. Therefore, *Cldn10b*-deficient mice develop hypermagnesemia and hypomagnesuria. Likely, the observed reduction in *Cldn10b* expression in the db/db mice is a response to the high osmolality of the pro-urine. INSERT ENTER The strength of this study is that using oral metformin treatment in diabetic mice closely resembles the human situation. Db/db mice developed hypomagnesemia making them an excellent model to study the mechanisms of hypomagnesemia in T2D. Moreover, this study extensively investigated differences in expression of all known genes involved in Mg^{2+} transport, in both kidney and colon. Some limitations have to be considered. The fact that metformin treatment did not affect Mg^{2+} homeostasis raises the question whether the dose and duration of metformin treatment were sufficient. However, the metformin treatment reduced the food intake of db/db mice, a known positive effect of metformin. Moreover, the dosage of metformin that the db/db received (0.5 mg/ml, equivalent to a daily intake of approximately 165 mg/kg bodyweight) is similar to previous studies investigating the metabolic effects of metformin in mice^{49–52}. A second

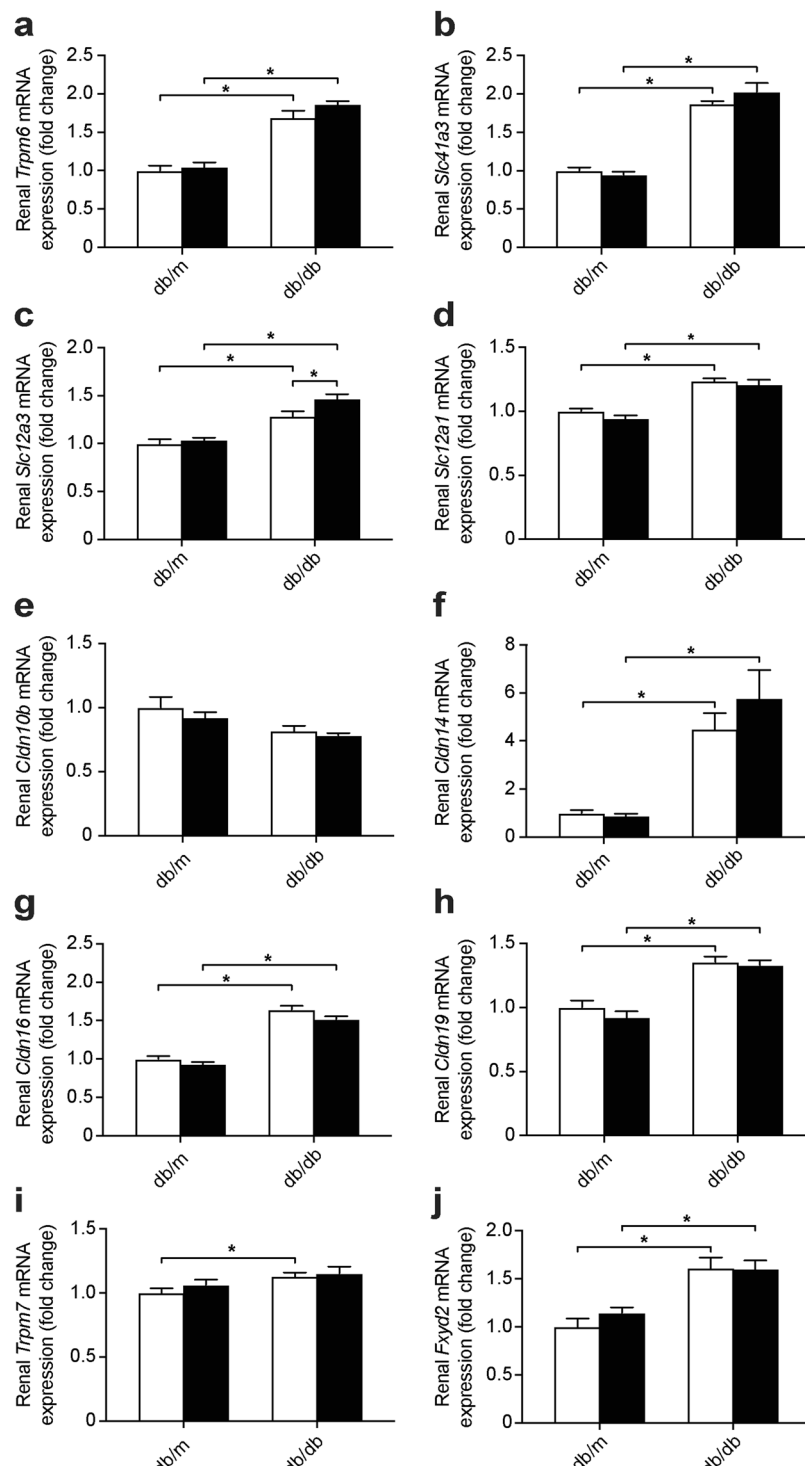


Figure 4. Upregulation in the expression of essential renal magnesiotropic genes in db/db mice. mRNA expression of genes involved in renal electrolyte handling (a) *Trpm6*, (b) *Slc41a3*, (c) *Slc12a3*, (d) *Slc12a1*, (e) *Cldn10b* (1.00 ± 0.05 vs. 0.83 ± 0.02 relative gene expression in db/m vs. db/db mice, two-way ANOVA $p \leq 0.05$), (f) *Cldn14*, (g) *Cldn16*, (h) *Cldn19*, (i) *Trpm7* and (j) *Fxyd2*. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean \pm SEM. Depending on the absence or presence of a significant interaction effect between genotype and treatment, either a two-way ANOVA (Tukey's multiple comparison test) or an unpaired multiple *t* test (Holm-Sidak multiple comparison test) approach, respectively, was used to determine statistical significance. *Indicates a $p \leq 0.05$.

limitation is that the expression of genes such as *Cldn10b/14*, *Slc12a1* and *Slc12a3* is regulated by both dietary intake and serum levels of K^+ , Na^+ and Ca^{2+} ^{43,44,53}. As db/db mice have hyperphagia, their dietary intake of ions is also increased. Despite the higher food intake, db/db mice still develop hypomagnesemia. However, for other

differences between db/m and db/db mice it is difficult to differentiate whether they are caused by T2D-related factors or by a higher food intake.

In conclusion, hypomagnesaemia is a consequence of T2D, which is not affected by metformin treatment. The reason that metformin-users have lower serum Mg^{2+} concentrations is likely mediated by other factors, and not by a direct effect of metformin on Mg^{2+} (re)absorption.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

- Kurstjens, S. *et al.* Determinants of hypomagnesaemia in patients with type 2 diabetes mellitus. *European journal of endocrinology* **176**, 11–19, <https://doi.org/10.1530/EJE-16-0517> (2017).
- Mather, H. M. *et al.* Hypomagnesaemia in diabetes. *Clinica chimica acta; international journal of clinical chemistry* **95**, 235–242 (1979).
- Pham, P. C., Pham, P. M., Pham, S. V., Miller, J. M. & Pham, P. T. Hypomagnesaemia in patients with type 2 diabetes. *Clinical journal of the American Society of Nephrology: CJASN* **2**, 366–373, <https://doi.org/10.2215/CJN.02960906> (2007).
- Sakaguchi, Y. *et al.* Hypomagnesaemia in type 2 diabetic nephropathy: a novel predictor of end-stage renal disease. *Diabetes care* **35**, 1591–1597, <https://doi.org/10.2337/dc12-0226> (2012).
- Kandee, F. R., Balon, E., Scott, S. & Nadler, J. L. Magnesium deficiency and glucose metabolism in rat adipocytes. *Metabolism* **45**, 838–843 (1996).
- Huerta, M. G. *et al.* Magnesium deficiency is associated with insulin resistance in obese children. *Diabetes care* **28**, 1175–1181 (2005).
- Kieboom, B. C. T. *et al.* Serum magnesium and the risk of prediabetes: a population-based cohort study. *Diabetologia* **60**, 843–853, <https://doi.org/10.1007/s00125-017-4224-4> (2017).
- Gommers, L. M., Hoenderop, J. G., Bindels, R. J. & de Baaij, J. H. Hypomagnesaemia in Type 2 Diabetes: A Vicious Circle? *Diabetes* **65**, 3–13, <https://doi.org/10.2337/db15-1028> (2016).
- de Baaij, J. H., Hoenderop, J. G. & Bindels, R. J. Magnesium in man: implications for health and disease. *Physiol Rev* **95**, 1–46 (2015).
- Wilson, J. E. & Chin, A. Chelation of Divalent-Cations by Atp, Studied by Titration Calorimetry. *Anal Biochem* **193**, 16–19, [https://doi.org/10.1016/0003-2697\(91\)90036-S](https://doi.org/10.1016/0003-2697(91)90036-S) (1991).
- Konrad, M., Schlingmann, K. P. & Gudermann, T. Insights into the molecular nature of magnesium homeostasis. *American journal of physiology. Renal physiology* **286**, F599–605, <https://doi.org/10.1152/ajprenal.00312.2003> (2004).
- Chubanov, V., Gudermann, T. & Schlingmann, K. P. Essential role for TRPM6 in epithelial magnesium transport and body magnesium homeostasis. *Pflugers Archiv: European journal of physiology* **451**, 228–234, <https://doi.org/10.1007/s00424-005-1470-y> (2005).
- de Baaij, J. H., Hoenderop, J. G. & Bindels, R. J. Regulation of magnesium balance: lessons learned from human genetic disease. *Clinical kidney journal* **5**, i15–i24, <https://doi.org/10.1093/ndtplus/sfr164> (2012).
- Groenestege, W. M., Hoenderop, J. G., van den Heuvel, L., Knoers, N. & Bindels, R. J. The epithelial Mg^{2+} channel transient receptor potential melastatin 6 is regulated by dietary Mg^{2+} content and estrogens. *Journal of the American Society of Nephrology: JASN* **17**, 1035–1043, <https://doi.org/10.1681/ASN.2005070700> (2006).
- Rondon, L. J., Groenestege, W. M. T., Rayssiguier, Y. & Mazur, A. Relationship between low magnesium status and TRPM6 expression in the kidney and large intestine. *Am J Physiol-Reg I* **294**, R2001–R2007, <https://doi.org/10.1152/ajpregu.00153.2007> (2008).
- Schlingmann, K. P., Waldegger, S., Konrad, M., Chubanov, V. & Gudermann, T. TRPM6 and TRPM7—Gatekeepers of human magnesium metabolism. *Biochimica et biophysica acta* **1772**, 813–821, <https://doi.org/10.1016/j.bbadis.2007.03.009> (2007).
- Quamme, G. A. & de Rouffignac, C. Epithelial magnesium transport and regulation by the kidney. *Frontiers in bioscience: a journal and virtual library* **5**, D694–711 (2000).
- Thebault, S., Alexander, R. T., Tiel Groenestege, W. M., Hoenderop, J. G. & Bindels, R. J. EGF increases TRPM6 activity and surface expression. *Journal of the American Society of Nephrology: JASN* **20**, 78–85, <https://doi.org/10.1681/ASN.2008030327> (2009).
- Nair, A. V. *et al.* Loss of insulin-induced activation of TRPM6 magnesium channels results in impaired glucose tolerance during pregnancy. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 11324–11329, <https://doi.org/10.1073/pnas.1113811109> (2012).
- Cao, G. *et al.* Regulation of the epithelial Mg^{2+} channel TRPM6 by estrogen and the associated repressor protein of estrogen receptor activity (REA). *J Biol Chem* **284**, 14788–14795 (2009).
- Ben Sahra, I. *et al.* The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin D1 level. *Oncogene* **27**, 3576–3586, <https://doi.org/10.1038/sj.onc.1211024> (2008).
- Rena, G., Hardie, D. G. & Pearson, E. R. The mechanisms of action of metformin. *Diabetologia* **60**, 1577–1585, <https://doi.org/10.1007/s00125-017-4342-z> (2017).
- Foretz, M., Guigas, B., Bertrand, L., Pollak, M. & Viollet, B. Metformin: from mechanisms of action to therapies. *Cell metabolism* **20**, 953–966, <https://doi.org/10.1016/j.cmet.2014.09.018> (2014).
- Song, R. Mechanism of Metformin: A Tale of Two Sites. *Diabetes care* **39**, 187–189, <https://doi.org/10.2337/dci15-0013> (2016).
- Scheen, A. J. & Paquot, N. Metformin revisited: a critical review of the benefit-risk balance in at-risk patients with type 2 diabetes. *Diabetes Metab* **39**, 179–190, <https://doi.org/10.1016/j.diabet.2013.02.006> (2013).
- Peters, K. E., Chubb, S. A., Davis, W. A. & Davis, T. M. The relationship between hypomagnesaemia, metformin therapy and cardiovascular disease complicating type 2 diabetes: the Fremantle Diabetes Study. *PLoS one* **8**, e74355, <https://doi.org/10.1371/journal.pone.0074355> (2013).
- Bailey, C. J. Metformin: historical overview. *Diabetologia* **60**, 1566–1576, <https://doi.org/10.1007/s00125-017-4318-z> (2017).
- Svare, A. A patient presenting with symptomatic hypomagnesaemia caused by metformin-induced diarrhoea: a case report. *Cases journal* **2**, 156, <https://doi.org/10.1186/1757-1626-2-156> (2009).
- McBain, A. M., Brown, I. R., Menzies, D. G. & Campbell, I. W. Effects of improved glycaemic control on calcium and magnesium homeostasis in type II diabetes. *Journal of clinical pathology* **41**, 933–935 (1988).
- Dosa, M. D., Hangan, L. T., Crauciuc, E., Gales, C. & Nechifor, M. Influence of therapy with metformin on the concentration of certain divalent cations in patients with non-insulin-dependent diabetes mellitus. *Biological trace element research* **142**, 36–46, <https://doi.org/10.1007/s12011-010-8751-9> (2011).
- Ewis, S. A. & Abdel-Rahman, M. S. Influence of atenolol and/or metformin on glutathione and magnesium levels in diabetic rats. *Journal of applied toxicology: JAT* **17**, 409–413 (1997).
- Topf, J. M. & Murray, P. T. Hypomagnesaemia and hypermagnesaemia. *Reviews in endocrine & metabolic disorders* **4**, 195–206 (2003).

33. Simmons, D., Joshi, S. & Shaw, J. Hypomagnesaemia is associated with diabetes: Not pre-diabetes, obesity or the metabolic syndrome. *Diabetes Res Clin Pr* **87**, 261–266, <https://doi.org/10.1016/j.diabres.2009.11.003> (2010).
34. Kao, W. H. *et al.* Serum and dietary magnesium and the risk for type 2 diabetes mellitus: the Atherosclerosis Risk in Communities Study. *Archives of internal medicine* **159**, 2151–2159 (1999).
35. Guerrero-Romero, F., Rascon-Pacheco, R. A., Rodriguez-Moran, M., de la Pena, J. E. & Wacher, N. Hypomagnesaemia and risk for metabolic glucose disorders: a 10-year follow-up study. *European journal of clinical investigation* **38**, 389–396, <https://doi.org/10.1111/j.1365-2362.2008.01957.x> (2008).
36. Sheehan, J. P. Magnesium deficiency and diabetes mellitus. *Magnes Trace Elem* **10**, 215–219 (1991).
37. Gilbert, R. E. *et al.* Effects of Canagliflozin on Serum Magnesium in Patients With Type 2 Diabetes Mellitus: A Post Hoc Analysis of Randomized Controlled Trials. *Diabetes therapy: research, treatment and education of diabetes and related disorders* **8**, 451–458, <https://doi.org/10.1007/s13300-017-0232-0> (2017).
38. Tang, H. L. *et al.* Elevated serum magnesium associated with SGLT2 inhibitor use in type 2 diabetes patients: a meta-analysis of randomised controlled trials. *Diabetologia* **59**, 2546–2551, <https://doi.org/10.1007/s00125-016-4101-6> (2016).
39. Hwang, D. L., Yen, C. F. & Nadler, J. L. Insulin increases intracellular magnesium transport in human platelets. *J Clin Endocrinol Metab* **76**, 549–553, <https://doi.org/10.1210/jcem.76.3.8445010> (1993).
40. Kobayashi, K. *et al.* The db/db mouse, a model for diabetic dyslipidemia: molecular characterization and effects of Western diet feeding. *Metabolism* **49**, 22–31 (2000).
41. Koranyi, L., James, D., Mueckler, M. & Permutt, M. A. Glucose transporter levels in spontaneously obese (db/db) insulin-resistant mice. *The Journal of clinical investigation* **85**, 962–967, <https://doi.org/10.1172/JCI114526> (1990).
42. Viering, D., de Baaij, J. H. F., Walsh, S. B., Kleta, R. & Bockenhauer, D. Genetic causes of hypomagnesemia, a clinical overview. *Pediatric nephrology* **32**, 1123–1135, <https://doi.org/10.1007/s00467-016-3416-3> (2017).
43. Nijenhuis, T. *et al.* Enhanced passive Ca²⁺ reabsorption and reduced Mg²⁺ channel abundance explains thiazide-induced hypocalciuria and hypomagnesemia. *The Journal of clinical investigation* **115**, 1651–1658, <https://doi.org/10.1172/JCI24134> (2005).
44. Milatz, S. *et al.* Mosaic expression of claudins in thick ascending limbs of Henle results in spatial separation of paracellular Na⁺ and Mg²⁺ transport. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E219–E227, <https://doi.org/10.1073/pnas.1611684114> (2017).
45. Hou, J. Claudins and mineral metabolism. *Current opinion in nephrology and hypertension* **25**, 308–313, <https://doi.org/10.1097/MNH.0000000000000239> (2016).
46. Plain, A. *et al.* Corticomedullary difference in the effects of dietary Ca²⁺ on tight junction properties in thick ascending limbs of Henle's loop. *Pflug Arch Eur J Phy* **468**, 293–303, <https://doi.org/10.1007/s00424-015-1748-7> (2016).
47. Dimke, H. *et al.* Activation of the Ca(2+)-sensing receptor increases renal claudin-14 expression and urinary Ca(2+) excretion. *American journal of physiology. Renal physiology* **304**, F761–769, <https://doi.org/10.1152/ajprenal.00263.2012> (2013).
48. Gong, Y. F. *et al.* Claudin-14 regulates renal Ca⁺⁺ transport in response to CaSR signalling via a novel microRNA pathway. *Embo J* **31**, 1999–2012, <https://doi.org/10.1038/emboj.2012.49> (2012).
49. Eskens, B. J., Zuurbier, C. J., van Haare, J., Vink, H. & van Teeffelen, J. W. Effects of two weeks of metformin treatment on whole-body glycocalyx barrier properties in db/db mice. *Cardiovasc Diabetol* **12**, 175, <https://doi.org/10.1186/1475-2840-12-175> (2013).
50. Hou, M. *et al.* Protective effect of metformin in CD1 mice placed on a high carbohydrate-high fat diet. *Biochem Biophys Res Commun* **397**, 537–542, <https://doi.org/10.1016/j.bbrc.2010.05.152> (2010).
51. Foretz, M. *et al.* Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *The Journal of clinical investigation* **120**, 2355–2369, <https://doi.org/10.1172/JCI40671> (2010).
52. Anisimov, V. N. *et al.* Metformin extends life span of HER-2/neu transgenic mice and in combination with melatonin inhibits growth of transplantable tumors *in vivo*. *Cell Cycle* **9**, 188–197, <https://doi.org/10.4161/cc.9.1.10407> (2010).
53. Haque, M. Z., Ares, G. R., Caceres, P. S. & Ortiz, P. A. High salt differentially regulates surface NKCC2 expression in thick ascending limbs of Dahl salt-sensitive and salt-resistant rats. *American journal of physiology. Renal physiology* **300**, F1096–1104, <https://doi.org/10.1152/ajprenal.00600.2010> (2011).

Acknowledgements

The authors thank Maikel School, Janneke Mulders and Jeroen Mooren (Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, the Netherlands) for their excellent technical support. This work was supported by funding from the Radboud Institute for Molecular Life Sciences and by grants from the Netherlands Organization for Scientific Research (NWO VICI 016.130.668 to Joost Hoenderop). Jeroen de Baaij is supported by a grant from the NWO (Veni 016.186.012) and the Dutch Diabetes Research Foundation (2017.81.014). Hacene Bouras is supported by a research fellowship from the Islamic Development Bank (IsDB).

Author Contributions

S.K., H.B., M.K., R.B., J.H. and J.H.F.d.B. conceived and designed the study. S.K., H.B. and C.O.-B. contributed to data acquisition. S.K. and H.B. analyzed the data. All authors interpreted data, drafted the article, revised it and approved the final version.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019