The following full text is a publisher’s version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/201333

Please be advised that this information was generated on 2019-08-30 and may be subject to change.
Diabetes-induced hypomagnesemia is not modulated by metformin treatment in mice

Steef Kurstjens1, Hacene Bouras1,2, Caro Overmars-Bos3, Mohamed Kebieche3, René J. M. Bindels1, Joost G. J. Hoenderop1 & Jeroen H. F. de Baaij1,4

Approximately 30% of patients with type 2 diabetes mellitus (T2D) have hypomagnesemia (blood magnesium (Mg2+) concentration < 0.7 mmol/L). In T2D patients, treatment with metformin is associated with reduced blood Mg2+ levels. To investigate how T2D and metformin affect Mg2+ 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 Mg2+ 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 Mg2+ 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 Mg2+ concentration and on the urinary Mg2+ 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 Mg2+ homeostasis in mice.

Approximately 30% of patients with type 2 diabetes mellitus (T2D) have hypomagnesemia (blood magnesium (Mg2+) < 0.7 mmol/L). Hypomagnesemia has serious clinical consequences as it increases the risk of complications such as retinopathy, nephropathy, micro and macrovascular disease and foot ulceration. Moreover, Mg2+ deficiency is correlated with insulin resistance, abrogated glucose metabolism and an increased risk of developing T2D. However, the etiology and underlying mechanisms of hypomagnesemia in T2D patients remains largely unknown.

As Mg2+ is necessary for the activity of over 600 enzymes, it plays numerous vital physiological functions including macromolecule synthesis, energy balance and DNA transcription. Moreover, Mg2+ stabilizes ATP and is required for its phosphor transfer reactions. The intestine and kidney collaboratively regulate Mg2+ balance and maintain its blood concentrations within a narrow range. In the gut, the bulk of Mg2+ absorption occurs in the small intestine via paracellular (passive) transport. In the colon, the final absorption of Mg2+ takes place by an active transcellular mechanism through transient receptor potential melastatin type 6/7 (TRPM6/TRPM7) cation channels. In the kidney, 95–99% of filtered Mg2+ is reabsorbed under physiological circumstances. Approximately 85% of the filtered Mg2+ is reabsorbed paracellularly by the proximal tubule and the thick ascending loop of Henle (TAL), where transport relies on tight junction permeability. Active transport in the distal convoluted tubule (DCT) determines the final urinary Mg2+ concentration, as this is the final segment where Mg2+ is reabsorbed. In physiological conditions, the DCT reclaims 5–10% of filtered Mg2+ transcellularly via TRPM6/7 channels. The expression and/or the activity of TRPM6 is affected by SNPs, dietary Mg2+ intake, drugs and hormones, such as insulin and epidermal growth factor (EGF). SNPs in TRPM6 that impair its response to insulin have been associated with an increased risk of developing T2D and gestational diabetes.

Metformin, the first-line pharmacotherapy in T2D, suppresses hepatic gluconeogenesis and improves insulin sensitivity. Therefore, its major clinical benefit is reducing blood glucose levels with only a minimal risk of complications.
hypeglycemiat,23,24. The most common side effects of metformin treatment are lactic acidosis, nausea and diar-
hy. Recent cohort studies showed that metformin use in T2D patients is associated with reduced blood Mg2+
levels.25 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
magnesiotropic 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 acclimatized for two weeks in a temperature-
and light-controlled room two mice per cage (Eurostandard Type III), 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
consumption, 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 kid-
ney 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 con-
centration was measured using the Nanodrop 2000c (Thermoscientific, Wilmington, DE). To synthetize cDNA,
1.5µg 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 (Hprrt), the relative gene expression was calculated by the Livak
method (2^−∆∆cθ). Primers sequences are provided in Table 1.

Analytical measurements. Serum and urinary Mg2+ concentrations were determined using a spectropho-
tometric assay (Roche/Hitachi, Tokyo, Japan), according to manufacturer's protocol. Ca2+ concentrations were
determined by the o-cresophthalein complexone method. Absorbance for the Mg2+ and Ca2+ assays was mea-
ured 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 methods.1 Serum and urinary glucose concentrations were determined by a
spectrophotometric assay according to the manufacturer's protocol (Instruchemie, Delfzijl, the Netherlands).

Table 1. Primer sequences used for RT-qPCR. Cldn10b, claudin 10b; Cldn14, claudin 14; Cldn16, claudin
16; Cldn19, Claudin 19; Connm4, cyclin M4; Fxyd2, FXYD-domain containing 2; Hprt, hypoxanthine-guanine
phosphoribosyltransferase; Slc12a1, solute carrier family 12 member 1; Slc12a3, solute carrier family 12 member 2;
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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cldn10b</td>
<td>GGAGTTCCCTCCCTCATGCT</td>
<td>GCAAAATGGAACGAAAAA</td>
</tr>
<tr>
<td>Cldn14</td>
<td>GTCCAGCCTGCTAGGCTCCT</td>
<td>CATCCACAGTCCCTTCAGG</td>
</tr>
<tr>
<td>Cldn16</td>
<td>GTTCAGAGGAGGCACACATTAC</td>
<td>GAGGAGGTTTCGAGTAAAC</td>
</tr>
<tr>
<td>Cldn19</td>
<td>GGTCTCTTTCTGCTGTCAC</td>
<td>CCGGCAACTAAACGAGG</td>
</tr>
<tr>
<td>Connm4</td>
<td>TCGGCGGACGTAGTCTCTG</td>
<td>CGACGCGATGAAAGTGGG</td>
</tr>
<tr>
<td>Fxyd2</td>
<td>TCACGGCTCTTCTGACTGG</td>
<td>GGTTCTTCGTCGGCTACT</td>
</tr>
<tr>
<td>Hprt</td>
<td>TGTGCTGAGCTGTGATTAC</td>
<td>AGTTGAGAGATCCTCCAC</td>
</tr>
<tr>
<td>Slc12a1</td>
<td>CACATGGTCTCCACATGTTG</td>
<td>GGCCTCTCCACACAGGCTC</td>
</tr>
<tr>
<td>Slc12a3</td>
<td>CTTGCGGCACTGGCATCTCG</td>
<td>GAGTGCAGAGTAGGAGTG</td>
</tr>
<tr>
<td>Slc41a1</td>
<td>CATCCCCAGGCCCCTTCGTG</td>
<td>CGGCTGGCTGCACAGCCAC</td>
</tr>
<tr>
<td>Slc41a3</td>
<td>TGAAGGGAACCTGGAAATG</td>
<td>GGTTGCTGCTGATGTATTGG</td>
</tr>
<tr>
<td>Trpm6</td>
<td>TTTCACATGAAACCTGGCCC</td>
<td>AAAGGCAATGGAAGTTACAGC</td>
</tr>
<tr>
<td>Trpm7</td>
<td>GTTCTCTCTCTGCTGTCGT</td>
<td>CCCATGCTGCTCTGCTG</td>
</tr>
</tbody>
</table>

Statistical analyses. Interaction between the two main variables (genotype and treatment) was investi-
gated using a two-way ANOVA test. If there was a significant interaction effect, an unpaired multiple t test, with
Gene expression of the ubiquitous Mg\(^{2+}\). In contrast, the mRNA expression of Cldn14 was elevated in db/db mice at two weeks, the serum Ca\(^{2+}\) concentration and urinary 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 μmol/24 h 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 reduced serum Mg\(^{2+}\) concentrations. Serum Mg\(^{2+}\) concentrations were lower in db/db mice, respectively, two-way ANOVA \(p \leq 0.05\)). At four weeks, there was a significant genotype effect on urinary Mg\(^{2+}\) excretion, demonstrating an increased urinary Mg\(^{2+}\) loss in db/db mice (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 \(p \leq 0.05\)). 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 μmol/24 h 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 enhanced. 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\(^{2+}\) transporter regulator Cyclin m4 (Cnm4) (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\(^{2+}\) exchanger Scl41a3 (Fig. 4a,b). While both db/db groups showed a higher expression of Scl12a3, 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 Scl12a1, 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. Metformin use is associated with a lower blood Mg\(^{2+}\) concentration in these patients. 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 T2D. 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. 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 concentration. 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 rats. 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 hypomagnesemia.

Hypomagnesemia is prevalent in over 30% of T2D patients. A remaining question is whether hypomagnesemia is the cause or the consequence of T2D. 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 ± 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 $p < 0.05$. 


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 ± 0.6 µmol/24 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 ± 0.06 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 ± 3 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 ± 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$^{2+}$ levels37,38. 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 insulin39–41. 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 unknown42,43. 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$^{2+}$ intake46,47. 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 mice49–52. A second

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) Cnnm4 and (d) Slc41a1. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean ± 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 $p \leq 0.05$. 

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 mice49–52. A second
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 Ca²⁺. 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

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 ≤ 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 ± 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 ≤ 0.05.
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, hypomagnesemia is a consequence of T2D, which is not affected by metformin treatment. The reason that metformin-users have lower serum Mg²⁺ concentrations is likely mediated by other factors, and not by a direct effect of metformin on Mg²⁺ (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**


6. Huerta, M. G.


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