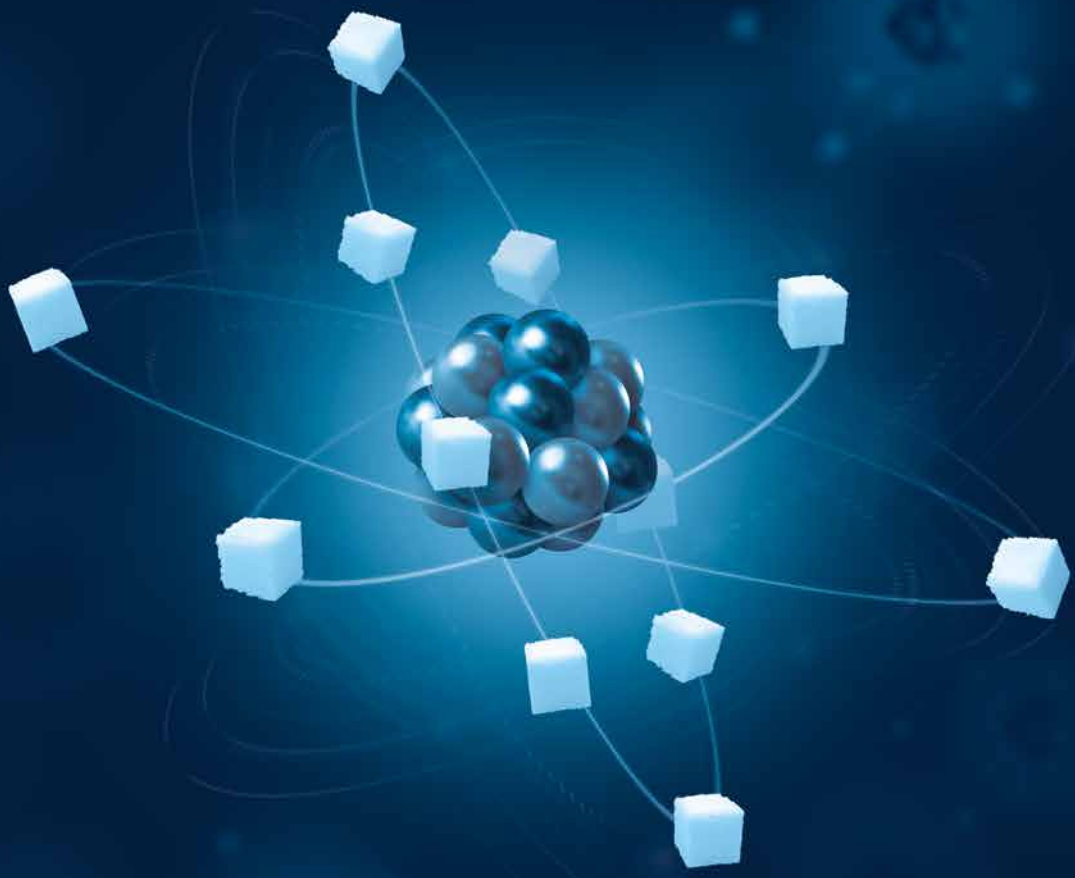

Hypomagnesemia in type 2 diabetes: cause or consequence?

Steef Kurstjens

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"If I have seen further it is by standing on the shoulders of Giants"

– Isaac Newton

"He who has a why to live can bear almost any how"

– Friedrich Nietzsche

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"If everyone is thinking alike, then somebody isn't thinking"

– George Patton

1

General introduction

General introduction

Magnesium (Mg^{2+}), a ubiquitous ion in nature, is a crucial mineral in the human body. The blood Mg^{2+} concentration is closely regulated by several transport systems, and abnormal concentrations of Mg^{2+} are associated with numerous diseases. Conversely, various disorders can result in abnormal Mg^{2+} concentrations. This thesis focuses on Mg^{2+} in type 2 diabetes mellitus (T2D).

Body magnesium homeostasis

Blood Mg^{2+} levels are maintained within the physiological range between 0.70 and 1.05 mmol/L (1). Only 1% of the body's Mg^{2+} is present in the circulating blood, while 46% is stored in soft tissue and 53% in bone (2). In blood, 27% of Mg^{2+} is bound to albumin, 8% to anions and 65% is ionized, which is the biologically active form of Mg^{2+} (3). Measurement of the blood Mg^{2+} concentration is the quickest and least invasive method of determining a patient's Mg^{2+} status and is widely used in clinical practice.

Hypomagnesemia (blood Mg^{2+} <0.7 mmol/L) can be a result of insufficient dietary intake, malabsorption in the intestine or excessive urinary Mg^{2+} loss (Figure 1) (4). Foods containing high amounts of Mg^{2+} include green leaf vegetables, grains, nuts, seeds and fish, whereas the Mg^{2+} content of processed foods is low (5). As the Western diet contains high amounts of processed foods and less unrefined grains and fresh vegetables dietary intake of Mg^{2+} in developed countries is reduced (6). Several oral Mg^{2+} supplementations are available to increase blood Mg^{2+} levels, but their effectiveness is hindered by the fact that high dose oral Mg^{2+} can result in diarrhea (7). Mg-citrate was found to be one of the most bioavailable Mg^{2+} preparations, while Mg-oxide is poorly reabsorbed (8, 9). Acute hypomagnesemia can also be treated by intravenous administration of a Mg-sulfate solution.

Three organs, namely the intestines, kidneys and bones, are involved in the regulation of the body's Mg^{2+} balance (6). The intestines regulate dietary Mg^{2+} uptake, the kidneys adjust the urinary excretion and the bones serve as dynamic storage compartments.

Physiological regulation of blood magnesium levels

Intestines

Approximately 30% of the variation in blood Mg^{2+} levels can be explained by dietary intake (10). Only 30-50% of dietary Mg^{2+} intake is absorbed in the intestines, which can vary based on the dietary Mg^{2+} availability (11, 12). Major causes for intestinal Mg^{2+} loss are the use of proton pump inhibitors or diarrhea, which can be caused by metformin use or diabetic neuropathy (4, 13-15). When dietary Mg^{2+} is high, the bulk of Mg^{2+} absorption takes place in the small intestine *via* the paracellular route by tight

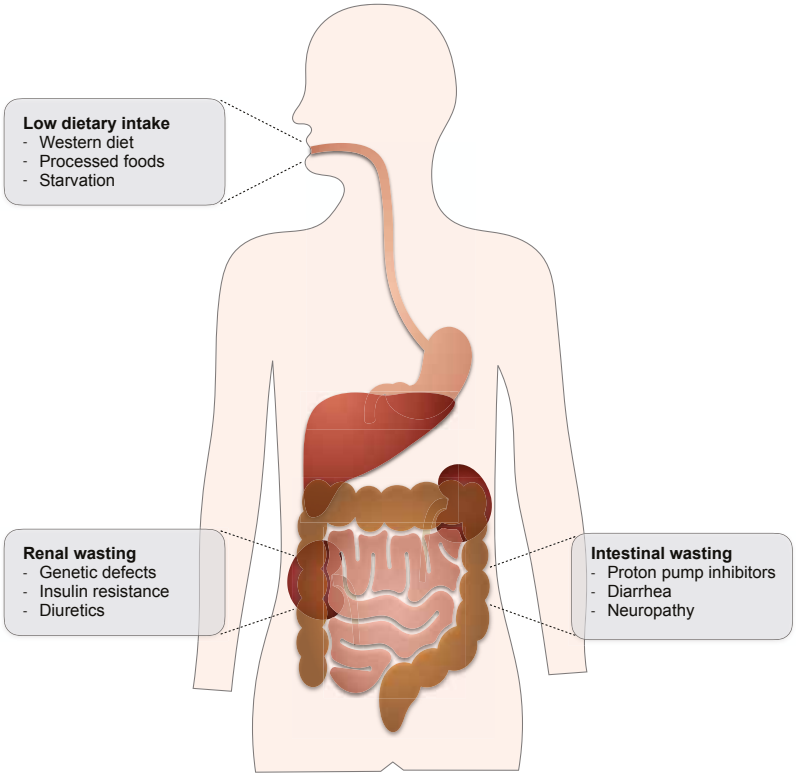

Figure 1 | Causes of hypomagnesemia

junction proteins, known as claudins (16). The rate of absorption in this segment is determined by the luminal concentration of Mg^{2+} (16). Little is known regarding the exact mechanisms and proteins involved in the transport of Mg^{2+} in this segment. Transcellular Mg^{2+} absorption takes place in the distal colon *via* the apical divalent cation channels transient receptor potential melastatin 6 and 7 (TRPM6 and TRPM7) and the basolateral transporter cyclin M4 (CNNM4) (17, 18). Intestine-specific *Trpm6* knockout mice develop severe hypomagnesemia, highlighting the essential role of colonic *Trpm6* in maintaining the body's Mg^{2+} homeostasis (19).

Kidneys

The kidneys are the key regulators of the body's Mg^{2+} homeostasis, as they can adjust the urinary excretion of Mg^{2+} based on changes in the blood Mg^{2+} concentration (20, 21). Each day the kidneys filter 2.4 g of Mg^{2+}, equivalent to 10% of

the total body Mg^{2+} content (6). Approximately 95% (2.4 g) of the filtered Mg^{2+} is reabsorbed, resulting in a total daily urinary excretion of 100 mg Mg^{2+} (22). Therefore, minor defects in renal Mg^{2+} reabsorption can already have large consequences on the body's Mg^{2+} balance.

In the proximal tubule, 10-25% of filtered Mg^{2+} is reabsorbed (23, 24). The reabsorption in this segment takes place *via* passive paracellular transport and is dependent on the active reabsorption of sodium (Na^+) salts and water (25, 26). Mg^{2+} reabsorption in the proximal tubule is increased when the osmolality or the Mg^{2+} concentration of the pro-urine rises (27). Downstream nephron segments can compensate for insufficient Mg^{2+} reabsorption in the proximal tubule. Therefore, changes in the reabsorption of Mg^{2+} in the proximal tubule do, in general, not affect the final urinary Mg^{2+} excretion.

The bulk of Mg^{2+} reabsorption in the nephron takes place in the thick ascending limb of Henle's loop (TAL, 55-70%) (28, 29). Also in this segment the reabsorption takes place *via* the paracellular route, facilitated by cation-specific tight junction proteins known as claudin 16 (CLDN16) and 19 (CLDN19) (30, 31). On the other hand, Mg^{2+} reabsorption in the TAL is negatively regulated by claudin 10 (CLDN10), as deletions in CLDN10 result in hypermagnesemia (32). The transepithelial voltage gradient required for the paracellular transport of Mg^{2+} in the TAL is achieved by the activity of the Na-K-2Cl cotransporter 2 (NKCC2), which reabsorbs one molecule of Na^+ and potassium (K^+) and two molecules of chloride (Cl^-) transcellular.

Fine-tuning of urinary Mg^{2+} excretion takes place in the distal convoluted tubule (DCT, 5-10%), the final tubular segment where Mg^{2+} can be reabsorbed, as the connecting tubule (CNT) and collecting duct (CD) are impermeable for Mg^{2+} (28, 33). In the DCT, Mg^{2+} is transported transcellularly *via* TRPM6 (34, 35). Reabsorption of Mg^{2+} by TRPM6 is dependent on the apical thiazide-sensitive sodium-chloride cotransporter (NCC), encoded by the gene *SLC12A3* (36). This is emphasized by the fact that hypomagnesemia is one of the main side effects of thiazide diuretics (37). Moreover, loss-of-function mutations in *SLC12A3* or genes regulating NCC activity (Gitelman syndrome) also cause hypomagnesemia (22, 38, 39). In which manner Na^+ reabsorption *via* NCC contributes to TRPM6-mediated Mg^{2+} uptake remains to be elucidated. Mutations in *TRPM6* lead to the recessive autosomal disease known as familial hypomagnesemia with secondary hypocalcemia (HSH). This disease is characterized by extreme hypomagnesemia (serum Mg^{2+} 0.1-0.3 mmol/L), underlining the critical importance of the DCT in Mg^{2+} homeostasis (17, 40). TRPM6 mRNA expression, membrane trafficking and activity are strongly regulated by several mechanisms, including epidermal growth factor (EGF), intracellular ATP levels and blood Mg^{2+} levels (6, 41, 42). Moreover, the activity of both TRPM6 and NCC is stimulated by insulin, but the exact mechanism and physiological relevance remain largely unknown (43, 44).

Hypomagnesemia in disease

A mild hypomagnesemia (blood Mg^{2+} 0.5-0.7 mmol/L) can result in symptoms such as fatigue, muscle ache and headache (45). As these complaints are aspecific, it is often difficult for physicians to attribute them to reduced blood Mg^{2+} levels. Therefore, hypomagnesemia is often overlooked in the clinic. The complications of hypomagnesemia get more pronounced in the case of severe hypomagnesemia (blood Mg^{2+} $<$0.5 mmol/L), where dangerous manifestations can arise including tetany, seizures, depression, cardiac arrhythmia, coma and potentially death (22). In the clinic, Mg^{2+} deficiencies are frequently observed in patients with metabolic disorders such as the metabolic syndrome and T2D (46, 47).

Type 2 diabetes

Diabetes, defined as a chronically elevated blood glucose concentration, is a global pandemic, with an estimated 422 million adults suffering from diabetes in 2014 (48). Since 1980 the prevalence of diabetes in adults has risen from 4.7% to 8.5% (49). T2D, also known as non-insulin-dependent diabetes, consists of the large majority ($\pm$ 90%) of diabetes cases (49, 50). In T2D, the pancreatic β-cells can no longer meet the increased demand for insulin due to insulin resistance, resulting in increased blood glucose levels. The major risk factors for T2D include obesity, sedentary lifestyle, on top of genetic susceptibility. The therapy of T2D consists of lifestyle changes, medication, of which metformin is the first-line treatment, and if necessary, insulin is added (51). Severe complications can occur when blood glucose levels remain poorly regulated. Cardiovascular disease, including hypertension, stroke, heart failure and coronary artery disease, is one of the major complications in T2D (52). Moreover, microvascular defects can lead to food ulcers, blindness and diabetic neuropathy. Neuronal dysfunction can hamper the enteric nervous system resulting in obstipation or diarrhea. Diabetic nephropathy is the main cause of end-stage renal disease (ESRD) in the developed world (53). These complications need specific individual treatments, making polypharmacy and drug-drug interactions a huge issue in the treatment of T2D (54).

Hypomagnesemia in type 2 diabetes

Hypomagnesemia is a common phenomenon in T2D patients, but remains poorly recognized in the clinics. The first study that suggested a relationship between Mg^{2+} and T2D stems from the 1920s, in which electrolyte changes in response to insulin usage and diabetic keto-acidosis were investigated (55). The direct reduction in serum Mg^{2+} and potassium (K^{+}) in response to insulin was discovered in the 1940s (56). In this article the authors advise Mg^{2+} salt supplementation when treating diabetes (57). The high prevalence of hypomagnesemia in diabetes patients first came to light in a cohort published in 1979, involving 582 diabetes patients and 140

control subjects (58). After this initial cohort study, more cohort studies showed hypomagnesemia specifically in T2D patients, with prevalence numbers ranging from 11% to 65% (Table 1). It remains unclear if hypomagnesemia is a cause or a consequence of T2D (59). Importantly, the definition of hypomagnesemia differs substantially among these population studies.

Table 1 | Prevalence of hypomagnesemia in cohorts of diabetes patients

First author	Year	Hypomagnesemia prevalence	Definition of hypomagnesemia	Patient population
Mather *et al*. (58)	1979	**25%**	Plasma Mg^{2+} <0.69 mmol/L	582 Diabetes patients
McNair *et al.* (60)	1982	**39%**	Serum Mg^{2+} <normal mean minus 2x SD	215 Insulin-treated diabetic outpatients
De Lordes Lima *et al.* (61)	1998	**48%**	Plasma Mg^{2+} <0.70 mmol/L	128 T2D patients
Wälti *et al.* (62)	2003	**38%**	Undefined	109 T2D patients
Corica *et al.* (46)	2006	**49%**	Serum ionized Mg^{2+} <0.46 mmol/L	290 T2D patients
Dasgupta *et al.* (63)	2012	**11%**	Serum Mg^{2+} <0.66 mmol/L	150 Non-critically ill T2D patients
Rasheed *et al.* (64)	2012	**65%**	Serum Mg^{2+} <0.70 mmol/L	219 Diabetes patients between 40-60 years old
Peters *et al*. (65)	2013	**19%**	Serum Mg^{2+} <0.70 mmol/L	920 Non-insulin-treated T2D patients
Ramadass *et al.* (66)	2015	**62%**	Serum Mg^{2+} <0.74 mmol/L	50 T2D patients in tertiary care
Umer Siddiqui *et al.* (67)	2016	**36%**	Serum Mg^{2+} <0.60 mmol/L	323 Uncontrolled T2D patients on oral hypoglycemic agents
Odusan *et al.* (68)	2017	**23%**	Serum Mg^{2+} <0.66 mmol/L	125 T2D patients, of which 62 with hypertension
Pokharel *et al.* (69)	2017	**50%**	Serum Mg^{2+} <0.70 mmol/L	150 T2D patients

When only 'diabetes' is mentioned, the article does not discriminate between types of diabetes.

Cause-effect relationship between hypomagnesemia and type 2 diabetes

From Table 1 it is clear that hypomagnesemia is a prominent issue in T2D patients. However, to better understand its clinical relevance, it is essential to understand the etiology of hypomagnesemia in T2D. The major question that arises is whether hypomagnesemia is merely a consequence of T2D, or if hypomagnesemia also contributes to the onset and development of T2D. The arguments for either side will be further elaborated in the following paragraphs.

Type 2 diabetes affects magnesium homeostasis

T2D has been shown to be an independent risk factor for hypomagnesemia (70). Besides the poor dietary habits of many T2D patients reducing their Mg^{2+} intake, several other mechanisms have been hypothesized on how T2D-related factors can induce hypomagnesemia (71). Firstly, high-dose insulin shots shift Mg^{2+} from the blood towards the intracellular compartment, thereby inducing a short-term decrease in the blood Mg^{2+} concentration (72-74). This is illustrated by the occurrence of a severe hypomagnesemia in a patient who took an insulin overdose (74). Secondly, other commonly used medications in T2D patients are known to cause hypomagnesemia, such as proton pump inhibitors (PPIs) and thiazides (6). Thirdly, as insulin stimulates TRPM6 activity, chronic insulin resistance can cause a reduction in renal and intestinal Mg^{2+} (re)absorption (75). Fourthly, metformin medication and diabetic neuropathy can cause diarrhea, leading to intestinal malabsorption (15, 76-78). Lastly, severe glucosuria and osmotic diuresis are hypothesized to result in renal Mg^{2+} loss (71). However, this hypothesis is in contradiction with the fact that SGLT2 inhibitors cause massive glucosuria and osmotic diuresis, but lead to an increase in blood Mg^{2+} levels (79). The contribution of each of these factors towards a decrease in blood Mg^{2+} levels, or if there are other unknown mechanisms that alter blood Mg^{2+}, remains unclear.

Hypomagnesemia as a risk factor for the development of type 2 diabetes

Hypomagnesemia may also be a causative factor for T2D. In large population studies, lower serum Mg^{2+} levels have been shown to increase the risk of developing pre-diabetes and T2D (80, 81). This increased risk is partially mediated through insulin resistance and may be conveyed by SNPs in important Mg^{2+}-regulating genes (80). Moreover, reduced Mg^{2+} levels are an independent predictor for the development of ESRD in diabetic nephropathy and are associated with albuminuria, a marker of renal damage (82, 83) In contrast, higher dietary Mg^{2+} intake improves glucose and insulin metabolism and reduces the risk to develop T2D (84-87). Elevating dietary Mg^{2+} intake could be an attractive method of reducing T2D

incidence in the general population. Oral Mg^{2+} supplementation has been shown to be a promising and low-priced treatment strategy for improving insulin sensitivity, β-cell function, metabolic control and lipid profile in T2D patients (88-91). However, the number of patients included in the clinical trials studying the beneficial effects of oral Mg^{2+} homeostasis on T2D-related parameters is limited, and the outcomes are contradicting (Table 2). In most studies no increase of blood Mg^{2+} levels by the supplementation was realized. Most of the beneficial effects are achieved in the trials enrolling hypomagnesemic patients and in which an increase in blood Mg^{2+} levels by the oral Mg^{2+} supplementation is realized (Table 2). The effect of dietary Mg^{2+} on the risk for developing T2D, and the consequences of dietary Mg^{2+} on metabolism and energy homeostasis remain unknown.

The role of magnesium in energy metabolism

These data implicate that a reduced dietary Mg^{2+} intake and lower blood Mg^{2+} levels could contribute to the development of T2D and its related complications. Moreover, Mg^{2+} supplementation potentially reduces the chance of developing T2D (Table 2). Little is known regarding the underlying molecular mechanism by which Mg^{2+} could influence glucose and energy homeostasis, which could contribute to T2D progression. However, a potential role for Mg^{2+} has been implicated in the regulation of glucose concentrations, insulin signaling and secretion, mitochondrial function and lipid metabolism.

Glucose homeostasis

Mg^{2+} plays an important role in the regulation of blood glucose levels. The enzymes in the glycolysis pathway require $MgATP^{2-}$ to function. Hexokinase, glucokinase, phosphofructokinase, and pyruvate kinase are the four main regulatory enzymes of the glycolysis pathway and require $MgATP^{2-}$. The K_m value for Mg^{2+} of some these glycolytic enzymes fall into the physiological range.

Hexokinase, the enzyme performing the first step in glycolysis, has a K_m for Mg^{2+} at 1.0-2.3 mmol/L and for $MgATP^{2-}$ at 1-2 mmol/L (108). Pyruvate kinase, catalyzing the last step in glycolysis, requires one K^+ and two Mg^{2+} ions for its catalytic function and has a K_m for Mg^{2+} of 1 mmol/L (108-110). However, in isolated human erythrocytes only extremely low intracellular Mg^{2+} levels (<0.2 mmol/L) negatively affect glycolysis rate. How a mild hypomagnesemia affects glycolysis rate or the functioning of enzymes involved in glycolysis remains unknown. Both in patients with T2D and subject with the metabolic syndrome, fasting blood glucose levels are inversely correlated with blood Mg^{2+} concentrations (58, 60, 111-113). Moreover, a higher dietary Mg^{2+} intake and oral Mg^{2+} supplementation have the potential to reduce fasting glucose levels (114-116).

Table 2 | Clinical trials that investigated the beneficial effect of oral Mg^{2+} supplementation on diabetes-related parameters

First author	Year	Study design	Patients
Gullestad *et al.* (92)	1994	Randomized double-blind placebo-controlled trial	56 T2D patients with >1 year disease duration
Eriksson *et al.* (93)	1995	Randomized double-blind cross-over trial	56 Diabetes patients
Eibl *et al.* (94)	1995	Randomized double-blind placebo-controlled trial	40 T2D patients with hypomagnesemia (undefined), treated with diet and oral hypoglycemic agents, (HbA1c <8%)
De Lourdes Lima *et al.* (95)	1998	Randomized double-blind placebo-controlled trial	128 T2D patients treated by diet or diet plus oral antidiabetic drugs
Rodriguez-Moran *et al.* (96)	2003	Randomized double-blind placebo-controlled trial	63 T2D patients with hypomagnesemia (serum Mg^{2+} <0.74 mmol/L) treated by glibenclamide
Barragán-Rodríguez *et al.* (97)	2008	Randomized active control equivalent trial	23 Elderly (>60 years) T2D patients with hypomagnesemia (<0.74 mmol/L) and newly diagnosed depression
Lee *et al.* (98)	2009	Randomized double-blind placebo-controlled trial	155 Participants with a BMI ≥23 kg/m^2
Barbagallo *et al.* (99)	2010	Placebo-controlled clinical trial (no randomization or blinding)	60 Elderly (≥ 65 years) diabetes patients
Chacko *et al.* (100)	2011	Randomized double-blind placebo-controlled crossover trial	14 Healthy overweight volunteers (BMI> 25 kg/m^2)
Mooren *et al.* (101)	2011	Randomized double-blind placebo-controlled trial	47 Non-diabetic subjects with BMI ≥25 kg/m^2 and decreased insulin sensitivity
Lima de Souza e Silva *et al.* (102)	2014	Randomized double-blind placebo-controlled trial	62 Patients with the metabolic syndrome as defined by the International Diabetes Federation

Intervention	Intervention increased blood Mg^{2+}?	Improved fasting glucose from baseline?	Other effects
Mg-lactatecitrate equivalent to approximately 266 g elemental Mg^{2+} daily for 4 months	No	No	No improvement on HbA1c, lipid profile and blood pressure
600 mg oral Mg^{2+} (unclear which form) or 2 g ascorbic acid daily for two 90-day periods	Not reported	No	No improvement on lipid profile and blood pressure in the T2D patients
Mg-citrate equivalent to 560 mg elemental Mg^{2+} daily for 3 months	Yes, serum Mg^{2+} from 0.73 to 0.81 mmol/L	No	No improvement on insulin resistance, HbA1c and lipid profile
Mg-oxide equivalent to 500 mg or 1 g elemental Mg^{2+} daily for 30 days	No	No	No effect on glycemia and HbA1c
2.5 g oral $MgCl_2$, equivalent to 450 mg elemental Mg^{2+} in 50 ml solution daily for 16 weeks	Yes, serum Mg^{2+} from 0.64 to 0.74 mmol/L	Yes, from 10.3 to 8.0 mmol/L	Improved HOMA-IR and HbA1c, no effect on blood pressure
2.5 g oral $MgCl_2$, equivalent to 450 mg elemental Mg^{2+}, or 50 mg Imipramine in 50 ml solution daily for 12 weeks	Yes, serum Mg^{2+} from 0.53 to 0.86 mmol/L	No	Improved triglyceride levels. No effect on HbA1c and blood pressure.
12.3 mmol Mg-oxide, equivalent to 300 mg of elemental Mg^{2+} daily for 12 weeks	No	No	No effect on blood pressure, lipid profile and HOMA-IR
2.25 g oral Mg-pidolate twice a day, equivalent to 368 mg of elemental Mg^{2+} daily for 1 month	Serum ionized fraction increased significantly but not the total level	No	No improvement on blood pressure
Mg-citrate equivalent to 500 mg elemental Mg^{2+} daily for 4 weeks (1-month washout period)	Increased by 0.1 mmol/L from baseline but not significantly different from placebo	No	No improvement on HbA1c and triglycerides concentrations but lower c-peptide levels
Mg-aspartate hydrochloride equivalent to 365 mg elemental Mg^{2+} daily for 6 months	Serum ionized fraction increased significantly but not the total level	No, from 5.07 to 4.75 ($p=0.07$)	Improved insulin sensitivity. Diastolic blood pressure borderline improved ($p=0.06$) but no effect on OGTT or lipid profile
400 mg of chelated Mg^{2+} (unspecified to which molecule) per day 12-week divided into 2 daily dosages	No	No	Improved blood pressure but no effects on lipid profile and HOMA-IR

Table 2 | Continued

First author	Year	Study design	Patients
Navarrete-Cortes *et al.* (103)	2014	Randomized double-blind placebo-controlled crossover trial	56 T2D patients with normomagnesemia (serum Mg^{2+} >0.74 mmol/L)
Cosaro *et al.* (104)	2014	Randomized double-blind placebo-controlled crossover trial	16 Healthy male subjects with positive family history of metabolic syndrome and/or T2D in at least one first-degree relative
Rodríguez-Moran *et al.* (105)	2014	Randomized double-blind placebo-controlled trial	47 Normal-weight individuals (BMI 20-25 kg/m^2) with hypomagnesemia (serum Mg^{2+} <0.74 mmol/L) and a fasting hyperglycemia, insulin resistance, hypertriglyceridemia and/or hypertension
Simental-Mendía *et al.* (106)	2014	Randomized double-blind placebo-controlled trial	62 Patients with newly-diagnosed prediabetes and hypomagnesemia (serum Mg^{2+} <0.74 mmol/L)
Razzaghi *et al*. (107)	2018	Randomized double-blind placebo-controlled trial	70 Diabetic patients with grade 3 diabetic foot ulcer

Articles were included if the study used oral Mg^{2+} treatment, specified the intervention, patient population and study design, and measured fasting glucose levels. BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; $MgCl_2$, magnesium chloride, OGTT, oral glucose tolerance test.

Insulin secretion

Blood glucose levels are strongly regulated by the islets of Langerhans in the pancreas, which secrete glucagon and insulin into the bloodstream (117). Limited studies have focused on the role of Mg^{2+} in insulin production and secretion. In small-scale human studies Mg^{2+} has been shown to be beneficial for insulin release and beta cell function (89, 118). These data are in contrast with *in vitro* data using insulin-producing cells, in which Mg^{2+}-deficient medium stimulated insulin secretion (119). Moreover, in *ex vivo* studies in which rat or dog pancreases were perfused with increasing concentrations of Mg^{2+}, glucose-induced insulin secretion was suppressed, also contradicting the results observed in human studies (120, 121). Therefore, it is unknown what effect changes in blood Mg^{2+} concentrations have on insulin secretion in T2D patients.

Intervention	Intervention increased blood Mg^{2+}?	Improved fasting glucose from baseline?	Other effects
Two tablets of 1.5 g Mg-lactate twice daily, equivalent to 360 mg elemental Mg^{2+} daily for 3 months (3-month washout period)	No	No	No effect on HbA1c, HOMA-IR and lipid profile
Mg-pidolate twice a day, equivalent to 368 mg of elementary Mg^{2+} per day for 8 weeks (4-week washout period)	No	No	No effect on lipid profile and HOMA-IR
30 mL 5% $MgCl_2$ orally, equivalent to 450 mg elemental Mg^{2+} daily for 16 weeks	Yes, serum Mg^{2+} from 0.55 to 0.76 mmol/L	Yes, from 6.1 to 5.3 mmol/L	Improved triglycerides, blood pressure and HOMA-IR
30 mL 5% $MgCl_2$ orally, equivalent to 450 mg elemental Mg^{2+} daily for 3 months	Yes, serum Mg^{2+} from 0.65 to 0.86 mmol/L	Yes, from 6.0 to 5.5 mmol/L	Improved C-reactive protein. No effect on blood pressure
250 mg Mg-oxide orally, equivalent to 150 mg elemental Mg^{2+}, daily for 12 weeks	Yes, serum Mg^{2+} from 0.86 to 0.95 mmol/L	Yes, from 12.6 to 10.1 mmol/L	Improved serum insulin, triglycerides and C-reactive protein

Insulin receptor sensitivity

When insulin is secreted by the pancreas and enters the bloodstream, it binds to the insulin receptor, which is expressed in almost all tissues (122). Insulin receptor activation stimulates cellular glucose uptake and/or breakdown (123, 124). Both dietary Mg^{2+} intake as well as oral Mg^{2+} supplementation have been shown to improve insulin sensitivity (96, 101, 125). In rats with streptozotocin-induced type 1 diabetes, oral Mg^{2+}-sulfate supplementation improved the intraperitoneal glucose tolerance test (IPGTT), while blood insulin levels were equal, showing an improved insulin sensitivity (126). Moreover, hypomagnesemic rats have increased insulin resistance (127). Insulin receptor signal transduction begins with the tyrosine kinase autophosphorylating tyrosine residues of the beta-subunit of the insulin receptor. Tyrosine kinase activity has been shown to be strongly Mg^{2+}-dependent, which

could be the underlying mechanism of the increased insulin sensitivity by Mg^{2+} (127-130). The most important downstream effect of the insulin receptor is the shuttling of vesicles, containing glucose transporter 4 (GLUT4), from the cytosol into the cell membrane, increasing cellular glucose uptake. In animal studies oral Mg^{2+} enhances *Glut4* mRNA expression levels, which could increase the effects induced by insulin (126, 131). It remains largely unknown how intracellular Mg^{2+} affects downstream insulin receptor signaling, finally leading to changes in GLUT4 membrane expression.

Lipid homeostasis

Besides serving as an important energy source, lipids are also components of the cell membrane and can act as signaling molecules (132). Dietary lipids are absorbed by the intestine in the form of free fatty acids (FFA) (133). In the blood, fatty acids are transported in two forms: as triacylglycerols, also known as triglycerides, the main component of lipoprotein particles or as FFAs (also known as non-esterified fatty acids). Due to the poor solubility of FFAs they require carrier proteins, predominantly albumin, to become soluble (134). FFAs are stored in soft tissue, mainly liver and white adipose tissue, in the form of triglycerides. When the energy demand increases, FFAs can be released from triglycerides in these tissues by lipolysis and subsequently secreted into the bloodstream for other tissues, e.g. skeletal muscle, to consume. Hyperlipidemia is present in over 75% of T2D patients (135). Low blood Mg^{2+} levels in T2D patients correlate with hyperlipidemia (136). Moreover, oral Mg^{2+} supplementation has beneficial effects on the lipid profile (91, 137, 138). Hypomagnesemia in rats increases blood triglyceride and cholesterol levels, and negatively influences the fatty acid composition in lipoprotein particles (139, 140). These findings demonstrate the detrimental effects of low Mg^{2+} levels on lipid homeostasis.

Mitochondrial function

Mitochondria are key organelles in regulating cellular energy homeostasis by producing ATP *via* aerobic respiration. Mitochondria are hypothesized to be stores for cellular Mg^{2+}, shown by fluorescent imaging (141). Culturing mitochondria in balanced deep-sea water with increasing Mg^{2+} concentrations enhances mitochondrial activity, indicated by higher mRNA expression of carnitine palmitoyltransferase 1 (*CPT1-L*), medium chain acyl dehydrogenase (*MCAD*) and Cytochrome C (*CYCS*) (142). This data is strengthened by the fact that chelating extracellular Mg^{2+} from mitochondria using EDTA reduced ATP production due to a disrupted utilization of octanoylcarnitine and palmitoylcarnitine, which could be rescued by re-administration of Mg^{2+} (19). More knowledge is needed regarding the effects of hypomagnesemia on mitochondrial function in T2D patients.

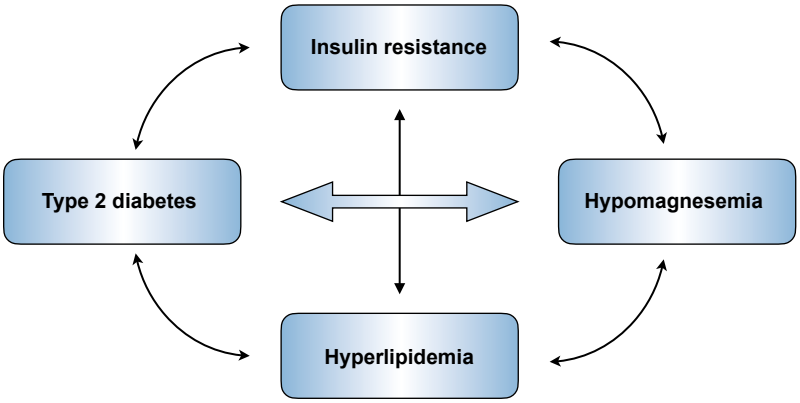

Figure 2 | The complex interconnection between hypomagnesemia and T2D

Hypomagnesemia could attribute to the onset of T2D by increasing insulin resistance and blood lipid levels. On the other hand, T2D could induce reductions in blood Mg^{2+} levels *via* insulin resistance and hyperlipidemia.

In summary, Mg^{2+} plays an important role in human physiology in general and energy metabolism in particular. Ample cohort studies have revealed that hypomagnesemia is a prominent issue in T2D patients. However, most of the current data is based on association studies, providing no insight into the causality and molecular mechanisms. This thesis aims to unravel the cause of the bidirectional relationship between T2D and hypomagnesemia, from molecule to population. On one hand, the effect of Mg^{2+} on energy homeostasis, glucose handling and lipid metabolism is investigated. On the other hand, this thesis will explore how T2D can lead to hypomagnesemia.

Outline of this thesis

This thesis aims to further disclose the causal relationship between hypomagnesemia and T2D (Figure 2). In **Chapter 2** the prevalence of hypomagnesemia and the extent of urinary Mg^{2+} wasting was studied in a cohort of patients with advanced T2D. Clinical and laboratory factors were associated to changes in plasma Mg^{2+} levels. **Chapter 3** aimed to study the metabolic consequences induced by hypomagnesemia in a T2D mouse model, using a Mg^{2+} deficient high fat diet. In **Chapter 4** the same dietary intervention was used to study the effects of a high fat diet on Mg^{2+} homeostasis**.** This chapter also studied the consequences of hypomagnesemia on the kidney. In **Chapter 5** we investigated the effect of metformin treatment on Mg^{2+} homeostasis in genetically modified mice (db/db mice), which suffer from T2D. The rationale for this study was based on the association between plasma Mg^{2+} and the use of metformin in T2D patients of the PARELSNOER cohort. In **Chapter 6** serum Mg^{2+} levels were correlated to alterations in the lipid profile of overweight individuals. This chapter further studied the binding of Mg^{2+} to negatively charged FFA molecules to unravel the inverse relationship between triglycerides and Mg^{2+}. Acute increases in blood lipid levels were achieved using oral loading of olive oil and cream in mice and human subjects, respectively. The main results of this thesis are summarized in **Chapter 7**. In **Chapter 8** the main findings are put into perspective. In this chapter we also discuss the clinical translation of the results and formulate recommendations for clinical practice. Finally, potential directions for further research are provided.

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"Not knowing the truth doesn't make you ignorant.
Not wanting to know the truth is what makes you ignorant"

– Joe Rogan

2

Determinants of hypomagnesemia in patients with type 2 diabetes mellitus

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Abstract

Hypomagnesemia (plasma magnesium (Mg^{2+}) concentration <0.7 mmol/L) has been described in patients with type 2 diabetes (T2D). Polypharmacy is inevitable when treating a complex disease such as T2D and could explain disturbances in the plasma Mg^{2+} concentration.

In this study, we aimed to establish the extent of hypomagnesemia in a cohort of 395 T2D patients and to identify determinants of plasma Mg^{2+} levels. Using Pearson correlation analyses, variables were correlated to plasma Mg^{2+} levels. After excluding confounding variables, all parameters correlating ($p<0.1$) with plasma Mg^{2+} were included in a stepwise backward regression model. The mean plasma Mg^{2+} concentration in this cohort was 0.74 ± 0.10 mmol/L. In total, 121 patients (30.6%) suffered from hypomagnesemia. Both plasma triglyceride (r=-0.273, $p<0.001$) and glucose levels (r=-0.231, $p<0.001$) negatively correlated with the plasma Mg^{2+} concentration. Patients using metformin (n=251, 62%), proton pump inhibitors (n=179, 45%) or β-adrenergic receptor agonists (n=31, 8%) displayed reduced plasma Mg^{2+} levels. Insulin use (n=299, 76%) positively correlated with plasma Mg^{2+} levels. The model predicted (R^2) 20% of all variance in the plasma Mg^{2+} concentration.

Hypomagnesemia is highly prevalent in T2D patients. Plasma triglycerides and glucose levels are major determinants of the plasma Mg^{2+} concentration, whereas only a minor part (<10%) of hypomagnesemia can be explained by drug intake, excluding polypharmacy as a major cause for hypomagnesemia in T2D.

Keywords: Glucose; hypomagnesemia; magnesium; medication; triglyceride; type 2 diabetes.

Introduction

Over the last decades, evidence is accumulating that hypomagnesemia (plasma M^{2+} concentration <0.7 mmol/L) is frequently present in patients with T2D [1]. Since its first report in the 1940s, hypomagnesemia has been shown in several cohort studies [2-4]. Although plasma Mg^{2+} levels are not regularly monitored in T2D patients, the presence of hypomagnesemia is of significant clinical importance [5] Oral Mg^{2+} supplementation has been shown to reduce the progression from pre-diabetes to diabetes and improve insulin sensitivity and glucose handling [3, 6-8]. Moreover, in T2D patients, hypomagnesemia results in a faster renal decline and is associated with a worse disease progression and outcome [9, 10]. Mg^{2+} also plays a key role in common comorbidities of T2D such as chronic kidney disease, atherosclerosis and hypertension [11-14].

Hypomagnesemia can have many underlying causes, related or unrelated to T2D [1, 15]. Firstly, hypomagnesemia can result from mutations in magnesiotropic genes, which has been extensively reviewed by Viering *et al.* [16]. Secondly, the processing of food leads to a marked depletion of Mg^{2+} in the Western diet, resulting in a reduced dietary Mg^{2+} intake [17]. Thirdly, hypomagnesemia can be a result of impaired intestinal Mg^{2+} uptake due to diarrhea that could be induced by diabetic autonomic neuropathy or metformin use [18-20]. Fourthly, the use of certain medication (i.e. diuretics, immunosuppressive drugs, proton pump inhibitors (PPIs)) has been associated with hypomagnesemia [21]. Lastly, metabolic acidosis and insulin resistance can decrease the expression of the renal Mg^{2+} channel transient receptor potential melastatin 6 (TRPM6), increasing urinary Mg^{2+} loss and thereby reducing the plasma Mg^{2+} concentration [22, 23].

The origin of hypomagnesemia in T2D is currently unknown. The contributing factors to disturbed Mg^{2+} homeostasis may be multiple and have been poorly studied. T2D patients suffer from a wide-range of clinical disturbances including increased plasma glucose concentrations, dyslipidemia (high triglyceride and low HDL-cholesterol) and insulin resistance [24].

In order to maintain proper glucose levels and blood pressure, polypharmacy is an inevitable consequence of effectively treating T2D. However, it also constitutes a growing risk factor as each drug carries its own side effects and drug-drug interactions [25]. Potentially, the use of medication can contribute to hypomagnesemia. Several regularly prescribed drugs in T2D are known to reduce the plasma Mg^{2+} levels, including PPIs and thiazide diuretics [26-30]. However, it is unclear to what extent the hypomagnesemia in T2D patients can be explained by the extensive use of medication.

In this study, we aimed to determine the prevalence of hypomagnesemia in a carefully phenotyped cohort of 402 T2D patients. Subsequently, the determinants of

plasma Mg^{2+} levels were analyzed using laboratory parameters and an extensive screening of drug use.

Results

Study population

In total, 395 T2D patients were included in the study cohort. Clinical characteristics and laboratory results are provided in Table 1.

Table 1 | Characteristics of T2D patients

Variable	Mean ± SD	Range	Reference value
Demographics			
Gender (m:f, %)	59:41	-	-
BMI (Kg/m^2)	32.6 ± 6.5	17 – 61	18.5-25
Age (years)	67 ± 10	34 – 90	-
Duration diabetes (years)	15.8 ± 9.5	0 – 56	-
Waist circumference (m:f, cm)	110 ± 16	65 – 152	<102:<88
SBP (mmHg)	143 ± 20	84 – 207	90 – 140
DBP (mmHg)	77 ± 11.	51 – 117	60 – 90
Heart rate (beats/min)	73 ± 12	45 – 116	60 – 100
Alcohol consumption (no:yes, %)	52:48	-	-
Laboratory analyses			
Total cholesterol (mmol/L)	4.5 ± 1.5	2.0 – 20.0	<5.2
Triglycerides (mmol/L)	2.5 ± 4.1	0.4 – 50.7	<1.7
HbA1c (mmol/mol)	63.1 ± 14.2	33.3 – 118.6	<42
Glucose (mmol/L)	9.4 ± 3.4	2.3 – 24.9	3.9 – 5.5
LDL (mmol/L)	2.3 ± 0.8	0.6 – 4.9	<2.6
HDL (m:f, mmol/L)	1.1 ± 0.3	0.5 – 2.2	>1.1:>1.3

m; male, f; female.

This is a group of patients with longstanding T2D, mostly on insulin treatment with an extensive use of medication (Table 2). The average plasma Mg^{2+} concentration in the cohort was 0.74 ± 0.10 mmol/L, and a total of 121 patients (30.6%) had levels below 0.70 mmol/L, indicating hypomagnesemia (Fig. 1A). Hypermagnesemia was found in 1 patient. Plasma sodium (Na^{+}) and (K^{+}) levels were normally distributed in the reference range (Fig. 1B,C).

Table 2 | Medication use of T2D patients

Medication	Number of patients (n=395)	Percentage
ACE inhibitor	187	47.3
ANGIIR antagonist	95	24.1
β-adrenergic agonist	31	7.8
β-adrenergic antagonist	209	52.9
Calcium channel blocker	88	22.3
Insulin	299	75.7
K^+-saving diuretic	48	12.2
Loop diuretic	75	19.0
Metformin	251	63.5
Nitrate	54	13.7
PPI	179	45.3
Statin	293	74.2
Sulfonylureum	77	19.5
Thiazide diuretic	128	32.4

Determinants of the plasma Mg^{2+} concentration

Using univariate regression analyses, a correlation between plasma Mg^{2+} concentration and several clinical characteristics was demonstrated: BMI (r=-0.162, p=0.001), waist circumference (r=-0.158, p=0.002), diastolic blood pressure (DBP, r=-0.144, p=0.004), heart rate (r=-0.104, p=0.039), eGFR (r=-0.168, p=0.001), HbA_{1c} (r=-0.123, p=0.015), and plasma levels of triglycerides (r=-0.273, p<0.001) and glucose (r=-0.231, p<0.001) negatively correlated with the plasma Mg^{2+} concentration. In contrast, the duration of diabetes (r=0.139, p=0.008) and the plasma level of HDL (r=0.156, p=0.002) and Na^+ (r=0.108, p=0.032) were correlated with an increased plasma Mg^{2+} concentration (Table 3). The effect of drug use was specifically analyzed. The use of metformin (r=-0.268, p<0.001) or β-adrenergic receptor agonists (r=-0.103, p=0.041) negatively correlated with plasma Mg^{2+}, whereas use of insulin (r=0.109, p=0.030) was associated with higher plasma Mg^{2+} levels. Confounding factors were identified using partial correlation analyses and excluded from subsequent analysis (Supplementary Tables 1-3). After correction for confounding, all parameters correlating (p<0.1) with plasma Mg^{2+} univariately, were included in the stepwise backward regression analysis model (Table 4). In this model, parameters with a p-value >0.1 were eliminated (BMI, DBP and angiotensin-converting-enzyme (ACE) inhibitors).

In the final model, plasma levels of glucose and triglycerides, as well as the use of PPIs, metformin or β-adrenergic receptor agonists negatively predicted plasma Mg^{2+} levels. Interestingly, patients on insulin had a trend (p=0.053) towards having higher plasma Mg^{2+} levels than people not on insulin. Altogether, the model predicted (R^2) 20% of the plasma Mg^{2+} concentration.

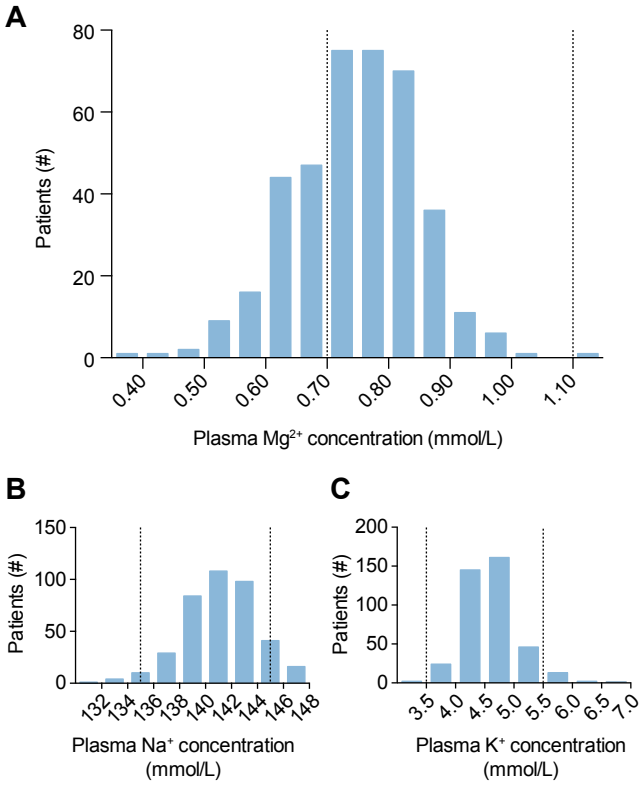

Figure 1 | Hypomagnesemia in T2D patients

The plasma (**A**) Mg^{2+}, (**B**) Na^{+} and (**C**) K^{+} concentration was determined in 395 type 2 diabetes patients. Hypomagnesemia was observed in 30.6% of the patients. Bars represent the number of patients. Dotted vertical lines indicate the normal range (Mg^{2+} 0.7-1.1 mmol/L, Na^{+} 135-145 mmol/L, K^{+} 3.5-5.5 mmol/L).

Increased urinary Mg^{2+} excretion

To investigate whether the hypomagnesemia is explained by renal Mg^{2+} loss, the fractional excretion of Mg^{2+} (FEMg) was determined. The mean FEMg in the cohort was 3.9 ± 2.7%. In total, 148 patients (40.8%) suffered from renal Mg^{2+} wasting, defined as FEMg >4%. Nevertheless, FEMg was not significantly different (p=0.75) between normomagnesemic patients (3.9 ± 2.8%) and hypomagnesemic patients (3.9 ± 2.5%) (Fig. 2A,B).

Table 3 | Univariate analyses for correlation of patient characteristics on the plasma Mg^{2+} concentration

Variable	Pearson's correlation coefficient	*p*-value	*n*
Demographics			
Gender	0.007	0.897	395
BMI (Kg/m^2)	-0.162	0.001	394
Age (years)	0.089	0.076	395
Duration diabetes (years)	0.139	0.008	365
Waist circumference (cm)	-0.158	0.002	392
SBP (mmHg)	-0.056	0.269	395
DBP (mmHg)	-0.144	0.004	395
Heart rate (beats/min)	-0.104	0.039	395
Alcohol consumption (no/yes)	-0.004	0.680	390
Laboratory analyses			
eGFR (ml/min)	-0.168	0.001	371
Log_{10} HDL (mmol/L)	0.156	0.002	387
Log_{10} Total cholesterol (mmol/L)	-0.059	0.247	388
Log_{10} HbA1c (mmol/mol)	-0.123	0.015	391
Log_{10} Triglycerides (mmol/L)	-0.273	<0.001	387
Log_{10} Glucose (mmol/L)	-0.231	<0.001	383
Log_{10} LDL (mmol/L)	0.077	0.145	365
Plasma Na^+ (mmol/L)	0.108	0.032	391
Plasma K^+ (mmol/L)	0.081	0.110	394
Medication			
ACE inhibitor	-0.094	0.063	
ANGIIR antagonist	0.037	0.469	
β-adrenergic agonist	-0.103	0.041	
β-blocker	-0.056	0.270	
Calcium channel blocker	-0.072	0.155	
Insulin	0.109	0.030	
K^+-saving diuretic	-0.023	0.685	
Loop diuretic	0.072	0.153	
Metformin	-0.268	<0.001	
Nitrate	-0.043	0.389	
PPI	-0.084	0.094	
Statin	0.002	0.962	
Sulfonylureum	-0.044	0.382	
Thiazide diuretic	-0.084	0.104	

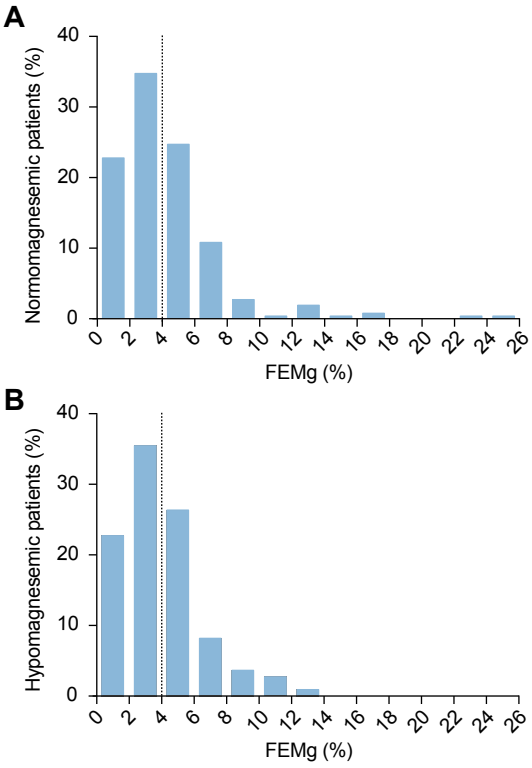

Figure 2 | Increased FEMg in T2D patients

The fractional excretion of Mg^{2+} (FEMg) was determined in 369 type 2 diabetes patients, of which 259 were normomagnesemic (**A**) and 110 were hypomagnesemic (**B**). Bars represent the percentage of the population. Dotted vertical lines indicate the threshold for urinary Mg^{2+} wasting (FEMg > 4%).

Table 4 | Stepwise backward regression analysis on the plasma Mg^{2+} concentration

Variable	Coefficient	*p*-value	Range
Log_{10} triglycerides (mmol/L)	-0.073	<0.001	-0.113 – -0.033
Log_{10} glucose (mmol/L)	-0.149	<0.001	-0.216 – -0.082
eGFR (mL/min)	0.000	0.022	-0.001 – 0.000
PPI*	-0.023	0.022	-0.043 – -0.003
Metformin*	-0.044	<0.001	-0.065 – -0.023
Insulin*	0.023	0.053	0.000 – 0.046
β-adrenergic agonist*	-0.047	0.009	-0.082 – -0.012

All variables with a $p>0.1$ were excluded from the model. *No; 0, Yes; 1.

Discussion

The present study further substantiates the high prevalence of hypomagnesemia in T2D. A negative correlation between plasma Mg^{2+} concentration and the plasma glucose and triglycerides levels was demonstrated. In addition, polypharmacy could be excluded as the main cause of hypomagnesemia in T2D patients, since only less than ten percent of changes in plasma Mg^{2+} could be attributed to medication use. These findings suggest that hypomagnesemia in T2D patients is intrinsic to the disease.

Hypomagnesemia was observed in 30.6% of the patients, corresponding to incidence numbers of 13.5-47.7% observed in previous cohort studies [3, 4, 31, 32]. In comparison, in a large study of 5179 subjects aged 55 and older the incidence of hypomagnesemia was only two percent, highlighting the extensive amount of hypomagnesemia in T2D patients [10]. Given that hypomagnesemia is related to a faster disease progression and an increased risk of diabetes-related complications, such as renal failure and cardiovascular disease, it is of great clinical importance to identify the factors that determine plasma Mg^{2+} concentrations [1, 9, 13, 33]. Therefore, we constructed a statistical model that included the factors influencing plasma Mg^{2+} levels. Twenty percent of the variation in the plasma Mg^{2+} concentration is explained by our model containing eGFR, the plasma concentrations of glucose and triglycerides and the use of PPIs, insulin, metformin and β-adrenergic receptor agonists. Although this is only a modest part of the total variation, it is comparable to the effect of dietary Mg^{2+} intake and higher than current genetic models, which explain 25-30% and 1-5% of changes in the plasma Mg^{2+} concentration respectively [34-36]. The finding that in our model most of the variance in plasma Mg^{2+} can be explained by factors that determine metabolic control underlines the importance of glucose and lipid homeostasis in the regulation of plasma Mg^{2+} levels.

Plasma glucose and triglyceride levels were the main determinants of plasma Mg^{2+} concentrations in our model. This correlation was independent of obesity-related factors such as waist circumference, BMI and cholesterol. Previous studies investigating the metabolic syndrome have shown an association between triglycerides and Mg^{2+} levels [37, 38]. However, these studies did not investigate the collinearity between triglycerides and HDL or were not based on the plasma Mg^{2+} concentration. Mg^{2+} supplementation is generally considered to improve the lipid profile in patients, however, studies addressing the effect of Mg^{2+} on plasma triglyceride concentrations are inconsistent [39, 40]. As Mg^{2+} increases the affinity of the insulin receptor tyrosine kinase for ATP, the decreased plasma Mg^{2+} levels could exacerbate insulin resistance in T2D patients, and thereby increase plasma glucose and triglyceride concentrations [41, 42].

Medication could only explain a minor part of the changes in plasma Mg^{2+} concentration in T2D patients (<10%), showing that polypharmacy is not the primary

cause of hypomagnesemia in T2D patients. Known hypomagnesemia-causing drugs β-adrenergic receptor agonists and PPIs, negatively correlated with the plasma Mg^{2+} concentration, although with minor effect sizes (<2%) [30, 43-45]. Of all drugs, the use of metformin was most strongly correlated (r=-0.268, p<0.001) with the plasma Mg^{2+} concentration, irrespective of eGFR or fasting glucose levels. Our study is the first large cohort study to identify the association between the use of metformin and plasma Mg^{2+} levels. A few studies with limited patient numbers from the 70s and 80s suggested that treating patients with metformin reduces plasma Mg^{2+} levels [4, 46]. How metformin affects Mg^{2+} handling remains to be elucidated. In contrast, patients taking insulin had a trend (p=0.053) towards higher plasma Mg^{2+} levels than those that did not require insulin treatment. This is in concordance with experimental studies showing that insulin stimulates the renal Mg^{2+} channel TRPM6, resulting in increased renal Mg^{2+} reabsorption [23]. Therefore, despite their worse glycemic control, patients on insulin treatment have slightly better plasma Mg^{2+} values than metformin-treated patients. These results suggest that the positive renal effect of insulin on the reabsorption of Mg^{2+} overrides the negative effect of poor glycemic control of insulin-dependent patients on their Mg^{2+} levels.

We observed that high renal Mg^{2+} excretion is prevalent among T2D patients, with 41% of the patients in the cohort having a FEMg >4%. While these findings suggest that impaired renal Mg^{2+} reabsorption contributes to hypomagnesemia in T2D patients, renal Mg^{2+} wasting was equally frequent in hypomagnesemic and normomagnesemic patients. Hypomagnesemia only arises when body Mg^{2+} stores, such as bone, are depleted [47]. Therefore, hypomagnesemia may only develop after several years, despite increased renal Mg^{2+} excretion. Our findings of overall high Mg^{2+} excretion may thus indicate that the complete diabetic population is at risk to develop hypomagnesemia. FEMg was not related to fasting glucose levels (data not shown), showing that T2D disease severity does not influence the amount of urinary Mg^{2+} loss.

The strength of our study is the thorough and extensive phenotyping of the T2D patients, allowing systematic investigation of the contribution of polypharmacy and metabolism-related parameters to changes in the plasma Mg^{2+} concentration of T2D patients. By carefully collecting data on drug use, this is the first cohort study that determined the contribution of medication usage to changes in plasma Mg^{2+} concentrations. The availability of urine samples enabled the determination of the FEMg in a large cohort of severe T2D patients for the first time. However, several limitations have to be considered. Firstly, data on the dietary habits of the participants was not collected, excluding the potential effects of diet from our analyses. However, the influence of diet on plasma Mg^{2+} levels will be minor, as samples were collected after an overnight fast. Secondly, all the samples were collected at a single point and no follow-up data are available. Therefore, the observed correlations will not provide

information about causality. A final limitation is the fact that the extent of insulin resistance was not directly determined. However, several studies have proposed that the product of fasting glucose and triglycerides can be used as a measure to estimate insulin resistance [48, 49]. The strong correlation of plasma Mg^{2+} with glucose and triglycerides in our study therefore provides an indirect link with insulin resistance.

In conclusion**,** this study shows that hypomagnesemia is a prominent feature of T2D, and is supported by excessive urinary Mg^{2+} loss. We excluded polypharmacy as the major cause of changes in plasma Mg^{2+} concentration in T2D patients. Given that metabolic factors such as glucose and triglyceride concentrations are main determinants of the plasma Mg^{2+} concentration, Mg^{2+} disturbances should be considered and, if required, corrected in T2D patients.

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Materials and methods

Subjects

Patients data and samples were taken from the Nijmegen-part of the Diabetes Pearl cohort [50]. In short, 402 T2D patients were included between June 2009 and February 2012 at the Radboud university medical center in Nijmegen, the Netherlands. Inclusion criteria were based on the WHO standards: a venous fasting plasma glucose concentration higher than 7.0 mmol/L, or a casual venous plasma glucose concentration higher than 11.1 mmol/L [51]. Personal information was obtained by dedicated patient questionnaires. This cohort consisted of patients under chronic secondary and tertiary care including many patients at an advanced disease stage.

BMI was calculated as the weight in kilograms divided by square of the height in meters. Blood pressure and heart rate were measured in triplicate, and the average value was used in subsequent analyses. Waist circumference was measured in duplicate after normal exhalation and if the difference between these two measurements was >1.0 cm, the measurement was repeated for a third time. Plasma

and urine samples were taken after an overnight fasting period. The samples were analyzed for laboratory parameters (glycated hemoglobin (HbA1c), plasma glucose, creatinine, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides) and immediately stored at -80˚C for further analyses. A detailed description of the patient characteristics is reported in Table 1.

Participants were requested to bring all their medication on the day of the visit or lists from pharmacists to score their medication use accurately. Medication was classed into groups according to the Anatomical Therapeutic Chemical Classification System (ATC) for statistical analysis (Table 2). The study was performed according to the declaration of Helsinki. All patients provided written informed consent and all study investigators had access to the study data.

Measures

This study was designed as an observational cohort study using samples from the existing Diabetes Pearl cohort biobank [50]. Laboratory values (fasting) and use of medication of 402 patients were examined at the inclusion date. Seven patients were excluded because of insufficient sample availability. Mg^{2+}, but also Na^{+} and potassium K^{+} concentrations were measured in the stored plasma and urine samples from the biobank. Fasting plasma Mg^{2+} concentrations were determined using a spectrophotometric assay (Roche/Hitachi, Tokyo, Japan), and measured at 600 nm on a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad laboratories, Hercules, USA), according to the manufacturer's protocol. Fasting plasma Na^{+}, K^{+} and creatinine, and urinary Mg^{2+}, Na^{+}, K^{+} and creatinine concentrations were measured at the clinical chemistry department using standardized methods. The fractional excretion of Mg^{2+} (FEMg) was calculated according to the formula $(U_{Mg} \times S_{crea})/(U_{crea} \times S_{Mg} \times 0.7) \times 100$ [52]. GFR was estimated (eGFR) using the 'Modification of Diet in Renal Disease (MDRD) formula: $186 \times \text{plasma creatinine}^{-1.154} \times \text{age}^{-0.203} \times 1.210$ (if black) $\times$ 0.742 (if female) [53]. Patients with an eGFR <30 mL/min were excluded from subsequent urinary analyses.

Statistical analyses

Data are presented as mean ± standard deviation (SD). Pearson's correlation tests were performed to determine the association between plasma Mg^{2+} concentration and clinical and anthropomorphic parameters. HbA_{1c} and plasma values of triglycerides, glucose, LDL, HDL and total cholesterol were $\log_{10}$ transformed prior to use in statistical analyses. All variables with a p-value <0.1 in the Pearson's correlation analyses, not corrected for multiple testing, were checked for confounding by performing partial regression analyses. Variables that correlated to plasma Mg^{2+} concentration with $p<0.1$, after correcting for confounding, were included in the stepwise multivariate backward elimination model. In this model variables with $p>0.1$ were eliminated. All

statistical analyses were performed using SPSS for Windows (V22.0.0.1 IBM, Armonk, NY). A *p*-value of <0.05 was considered statistically significant.

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Supplementary data

Supplementary table 1 | Stepwise backward regression analysis to investigate collinearity between correlating variables related to obesity and diabetes, with plasma Mg^{2+} as dependent variable

Variable	Coefficient	*p*-value
BMI (kg/m^2)	-0.002	0.300
Waist circumference (cm)	<0.001	0.847
Log_{10} glucose (mmol/L)	-0.133	0.001
Log_{10} triglycerides (mmol/L)	-0.067	0.006
Log_{10} HbA1c (mmol/mol)	0.025	0.686
Log_{10} HDL (mmol/L)	0.036	0.505
BMI (kg/m^2)	-0.001	0.094
Log_{10} glucose (mmol/L)	-0.132	0.001
Log_{10} triglycerides (mmol/L)	-0.067	0.006
Log_{10} HbA1c (mmol/mol)	0.025	0.684
Log_{10} HDL (mmol/L)	0.033	0.523
BMI (kg/m^2)	-0.001	0.102
Log_{10} glucose (mmol/L)	-0.125	<0.001
Log_{10} triglycerides (mmol/L)	-0.067	0.007
Log_{10} HDL (mmol/L)	0.034	0.511
BMI (kg/m^2)	-0.001	0.077
Log_{10} glucose (mmol/L)	-0.122	<0.001
Log_{10} triglycerides (mmol/L)	-0.074	0.001

Supplementary table 2 | Stepwise backward regression analysis to investigate collinearity between correlating variables related to blood pressure, with plasma Mg^{2+} as dependent variable

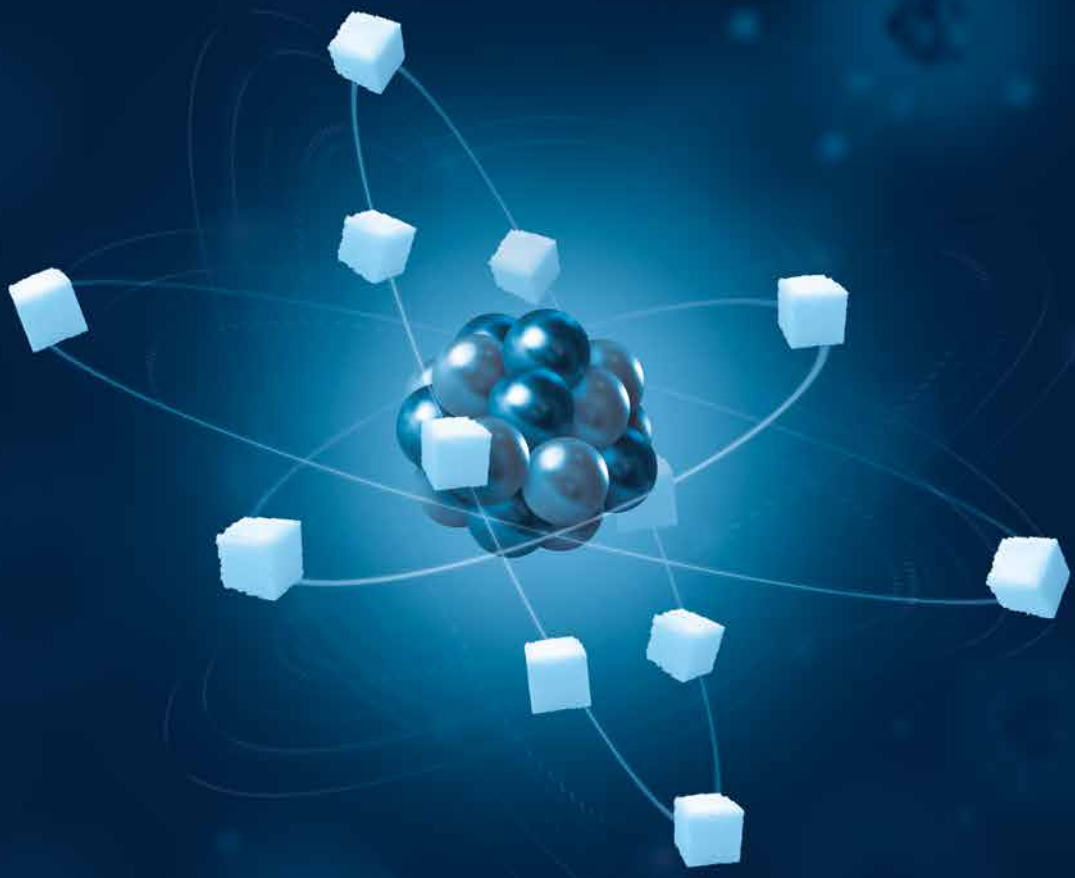
Variable	Coefficient	*p*-value
BMI (kg/m^2)	-0.003	0.002
DBP (mm Hg)	-0.001	0.082
Heart rate (beats/min)	<0.001	0.693
Plasma Na^+ (mmol/L)	0.002	0.421
Plasma K^+ (mmol/L)	0.017	0.168
eGFR (mL/min)	<0.001	0.085
ACE inhibitor*	-0.021	0.048
Log_{10} glucose (mmol/L)	-0.147	<0.001
BMI (kg/m^2)	-0.003	0.001
DBP (mm Hg)	-0.001	0.049
Plasma Na^+ (mmol/L)	0.002	0.423
Plasma K^+ (mmol/L)	0.017	0.169
eGFR (mL/min)	<0.001	0.084
ACE inhibitor*	-0.020	0.050
Log_{10} glucose (mmol/L)	-0.148	<0.001
BMI (kg/m^2)	-0.003	0.002
DBP (mm Hg)	-0.001	0.051
Plasma K^+ (mmol/L)	0.016	0.180
eGFR (mL/min)	<0.001	0.063
ACE inhibitor*	-0.021	0.043
Log_{10} glucose (mmol/L)	-0.157	<0.001
BMI (kg/m^2)	-0.003	0.002
DBP (mm Hg)	-0.001	0.040
eGFR (mL/min)	<0.001	0.022
ACE inhibitor*	-0.020	0.048
Log_{10} glucose (mmol/L)	-0.151	<0.001

*No; 0, Yes; 1.

Supplementary table 3 | Stepwise backward regression analysis to investigate collinearity between the use of metformin and the duration of diabetes.

Variable	Coefficient	*p*-value
Duration diabetes (years)	<0.001	0.154
Metformin*	-0.055	<0.001
Metformin*	-0.059	<0.001

*No; 0, Yes; 1.



"Compare yourself to who you were yesterday,
not to who someone else is today"

– Jordan Peterson | Twelve Rules for Life: an Antidote to Chaos

3

Magnesium deficiency prevents high-fat-diet-induced obesity in mice

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Abstract

Hypomagnesemia (blood Mg^{2+} <0.7 mmol/L) is a common phenomenon in individuals with type 2 diabetes (T2D). However, it remains unknown how a low blood Mg^{2+} concentration affects lipid and energy metabolism. Therefore, the importance of Mg^{2+} in obesity and T2D has been largely neglected to date. This study aims to determine the effects of hypomagnesemia on energy homeostasis and lipid metabolism. To this aim, mice were fed either a low-fat diet (LFD) or a high-fat diet (HFD) in combination with a normal- or low-Mg^{2+} content for 17 weeks.

In this study, we show that low dietary Mg^{2+} intake ameliorates HFD-induced obesity in mice. Consequently, fasting serum glucose levels decreased and insulin sensitivity improved in low Mg^{2+}-HFD-fed mice. Moreover, HFD-induced liver steatosis was absent in the low Mg^{2+} group. In hypomagnesaemic HFD-fed mice, mRNA expression of key lipolysis genes was increased in epididymal white adipose tissue (eWAT), corresponding to reduced lipid storage and high blood lipid levels. Low Mg^{2+}-HFD-fed mice had increased brown adipose tissue (BAT) *Ucp*1 mRNA expression and a higher body temperature. No difference was observed in energy expenditure between the two HFD groups.

In conclusion, Mg^{2+}-deficiency abrogates HFD-induced obesity in mice through enhanced eWAT lipolysis and BAT activity.

Keywords: β-Adrenergic receptor; brown adipose tissue; energy homeostasis; hypomagnesaemia; lipid metabolism; lipolysis; magnesium; obesity; white adipose tissue.

Introduction

Hypomagnesemia (blood Mg^{2+} concentration <0.7 mmol/L) affects approximately 30% of individuals with T2D (1, 2). Hypomagnesemia is an important risk factor for the development and progression of T2D (3-5). Low dietary Mg^{2+} intake and reduced serum Mg^{2+} have also been associated with obesity, although with conflicting results (1, 6-8). Moreover, reduced blood Mg^{2+} levels have been correlated with elevated glucose and triglyceride concentrations in individuals with T2D, suggesting that hypomagnesemia is associated with insulin resistance and dyslipidaemia (1).

Mg^{2+} fulfils many roles including cell growth, membrane stability, enzyme activity and energy metabolism (9). It is a cofactor for numerous enzymes, primarily because it stabilises ATP and facilitates phosphate transfer reactions (10, 11). Mg^{2+} is essential for glycolysis and the citric acid cycle (12, 13). Because Mg^{2+} is critical for insulin receptor tyrosine kinase activity, hypomagnesemia has also been implicated in insulin resistance (14-16). Recently, hypomagnesemia in mice was shown to contribute to enhanced catabolism, but no in-depth metabolic phenotype analysis was performed (17).

In T2D, restoring serum Mg^{2+} values by oral Mg^{2+} supplementation improves insulin sensitivity, decreases fasting glucose levels (18) and corrects the lipid profile (19-21). Although Mg^{2+} is essential for key enzymes in lipid metabolism, including hepatic lipase and lecithin-cholesterol acyltransferase (22, 23), the effects of chronic Mg^{2+} deficiency on adipocyte function and lipid metabolism remain largely unknown. In this study, we explored the role of Mg^{2+} in energy homeostasis, insulin sensitivity and lipid metabolism, by feeding mice a low-fat diet (LFD) or a high-fat diet (HFD) combined with low or normal Mg^{2+} for 17 weeks. The resulting metabolic effects were extensively characterised. Data were confirmed by an independent replication experiment.

Results

Low dietary Mg^{2+} intake reduces diet-induced obesity in mice

The mice were fed a LFD or HFD containing either a low (0.03% wt/wt) or normal (0.21% wt/wt) Mg^{2+} concentration for 17 weeks (Fig. 1A). There was no difference in body weight between low and normal Mg^{2+} groups on the LFD, but mice on the LowMg-HFD gained significantly less weight (58% less) than those on the normal Mg^{2+} (NormMg)-HFD (47.00 ± 1.53 g vs 38.62 ± 1.51 g in mice given a NormMg-HFD and LowMg-HFD, respectively, $p<0.05$, Fig. 1A,B). The lower body weight of the LowMg-HFD group could not be explained by differences in dietary intake, as shown by similar food intake and faeces production between the two HFD groups (Fig. 1C,D).

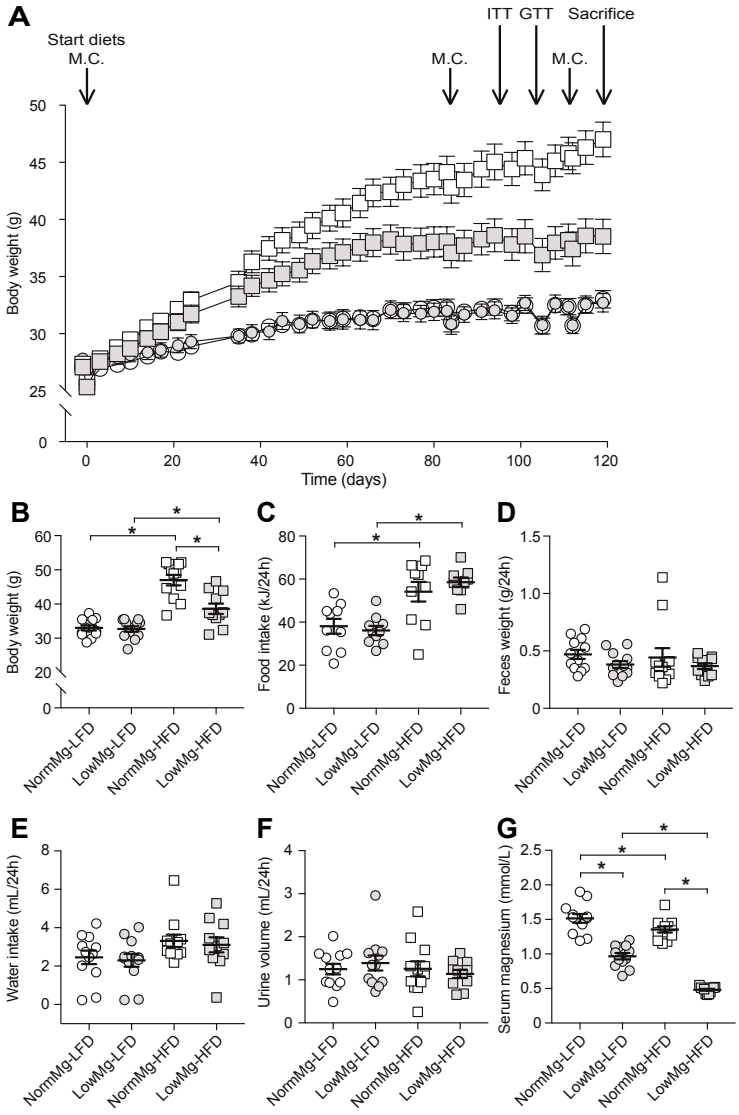

Figure 1 | Low dietary Mg^{2+} intake reduces diet-induced obesity in mice

(**A**) Body weight of mice during the experiment and (**B**) at week 17 (end of experiment). Arrows indicate experimental interventions: metabolic cage (MC), ITT, GTT. (**C**) Food intake, (**D**) total faeces weight, (**E**) water intake (two-way ANOVA for dietary fat effect $p<0.05$) and (**F**) urinary production determined over a period of 24 h, using metabolic cages, at week 16. (**G**) Serum Mg^{2+} levels at death. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.

There was also no difference in water intake or urinary volume between the HFD groups (Fig. 1E,F). Hypomagnesemia was detected in both the LowMg groups, but was significantly more pronounced in mice that were concomitantly fed a HFD (Fig. 1G).

Reduced diet-induced obesity in Mg^{2+}-deficient mice is accompanied by improved insulin sensitivity

To explore glucose metabolism in more detail, beta cell function and insulin sensitivity were determined by an intraperitoneal (IP) glucose tolerance test (GTT) and IP insulin tolerance test (ITT). In the IPGTT (a measure for beta cell dysfunction and insulin resistance), glucose clearance was reduced in both HFD groups (Fig. 2A). Glucose clearance was not significantly different between NormMg-HFD-fed mice and LowMg-HFD-fed mice (2.58 ± 0.08 vs 2.26 ± 0.13 mol/L× min in NormMg-HFD- and LowMg-HFD-fed mice, respectively, $p=0.07$, Fig. 2C). LowMg-HFD-fed mice required significantly less insulin than NormMg-HFD-fed mice to clear the glucose, consistent with LowMg-HFD-fed mice being more insulin sensitive (Fig. 2B). Fasting blood glucose and insulin concentrations were significantly increased in the HFD-fed mice, in accordance with the increased body weight (Fig. 2D,E). Compared with the NormMg-HFD-fed mice, fasting blood glucose was lower in the LowMg-HFD-fed mice (Fig. 2D). The effect of dietary Mg^{2+} on fasting insulin was not statistically significant (Fig. 2E, two-way ANOVA for dietary Mg^{2+} effect, $p=0.07$).

Both HFD-fed groups demonstrated increased insulin resistance in the ITT compared with their respective controls (Fig. 2F,G). Low dietary Mg^{2+} content resulted in a significantly lower AUC of the ITT (Fig. 2G, two-way ANOVA for dietary Mg^{2+} effect $p<0.05$). In the LowMg-HFD group compared to the NormMg-HFD-fed mice, the AUC of the ITT was not significantly different (0.91 ± 0.05 vs 0.72 ± 0.05 mol/L × min in NormMg-HFD-fed and LowMg-HFD-fed mice, respectively, Tukey's test $p=0.07$, Fig. 2F,G).

Insulin resistance is often accompanied by hyperlipidemia, in particular, high triglyceride and FFA levels. As expected, the HFD-fed mice had higher serum triglyceride and FFA levels than LFD-fed mice (Fig. 2H,I). Interestingly, despite their lower body weight and increased insulin sensitivity, LowMg-HFD-fed mice also had high serum triglyceride and FFA (Fig. 2H,I). In contrast, serum leptin levels correlated with body weight; hence, reduced leptin levels were observed in the LowMg-HFD-fed mice compared with NormMg-HFD-fed mice (Fig. 2J). No difference between the two HFD groups was observed in serum adiponectin and cholesterol (Supplementary Fig. 1 A,B).

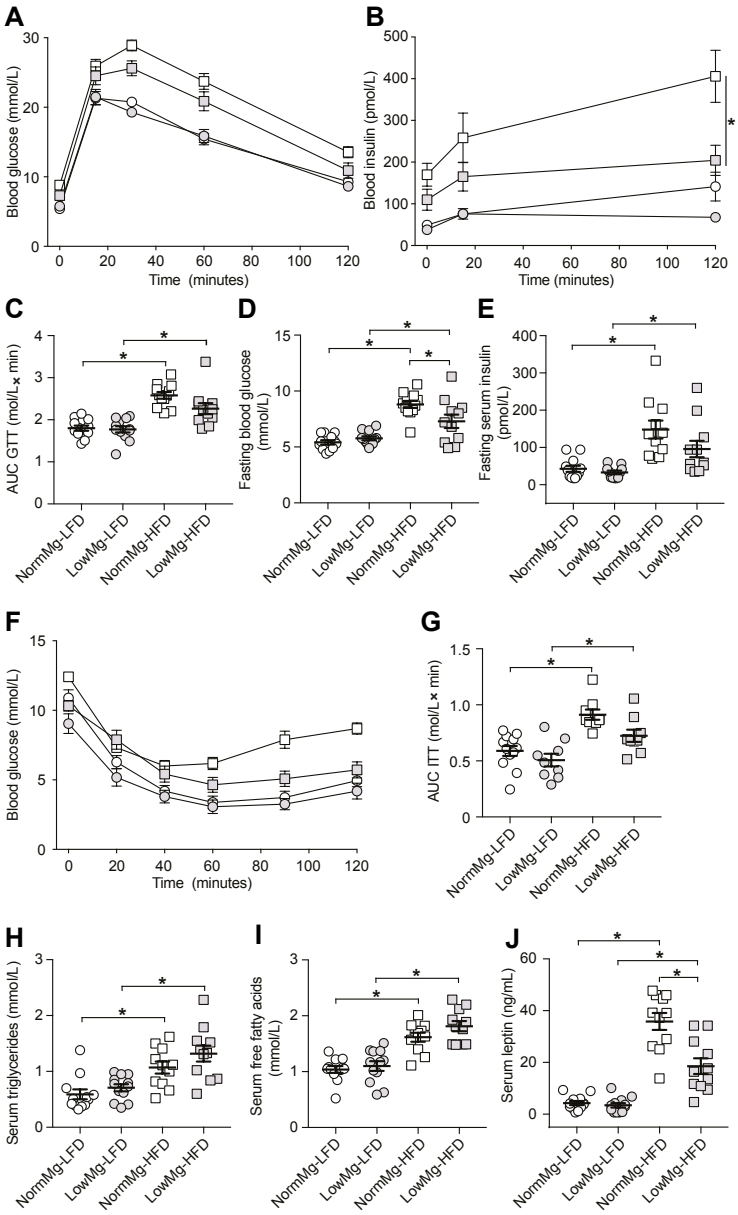

Figure 2 | Reduced diet-induced obesity in Mg^{2+}-deficient mice is accompanied by high triglyceride levels and improved insulin sensitivity

Figure 2 | Continued.

(**A**) Glucose clearance determined by IPGTT at week 15. (**B**) Serum insulin measured at 0, 15 and 120 mins of the GTT. (**C**) AUC determined between 0 and 120 min. (**D**) Fasting blood glucose and (**E**) serum insulin measured during the GTT. (**F**) Blood glucose measured during an intraperitoneal ITT at week 14. (**G**) AUC determined between 0 and 120 min (two-way ANOVA for dietary Mg^{2+} effect, $p<0.05$). Non-fasted serum (**H**) triglyceride, (**I**) FFA and (**J**) leptin concentrations at killing. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.

Mg^{2+} deficiency prevents diet-induced hepatic lipid storage

Liver function is often impaired in T2D as a consequence of insulin resistance and hepatic steatosis (28). Feeding mice a NormMg-HFD resulted in a significantly heavier liver. However, this effect was abrogated in mice fed a LowMg-HFD (Fig. 3A). In Mg^{2+}-deficient mice, the HFD did not increase liver triglyceride content (Fig. 3B). In line with the triglyceride measurements, H&E and Oil Red O staining showed reduced hepatic lipid accumulation in the Mg^{2+}-deficient HFD-fed mice (Fig. 3C,D, respectively). Hepatic mRNA expression of *Cd36*, a long-chain fatty acid transporter, was reduced in the LowMg-HFD-fed mice compared with the NormMg-HFD-fed mice (Supplementary Fig. 2A).

Low dietary Mg^{2+} increased hepatic mRNA expression of sterol regulatory element-binding protein 1c (*Srebp1c*), which is involved in cholesterol and fatty acid metabolism, and phosphoenolpyruvate carboxykinase 1 (*Pepck1*), essential for gluconeogenesis, glyceroneogenesis and fatty acid re-esterification (Suppelementary Fig. 2B,C two-way ANOVA for dietary Mg^{2+} effect $p<0.05$). No differences were observed between the two HFD-fed groups in the expression of key genes involved in hepatic glycolysis, ketogenesis and beta-oxidation (Supplementary Fig. 2D-L).

Reduced adipose tissue mass in Mg^{2+}-deficient HFD-fed mice is associated with increased mRNA expression of lipolysis genes

Our results show that mice fed a LowMg-HFD diet exhibit reduced body weight and high triglyceride levels compared with their NormMg-HFD-fed littermates. Interestingly, the LowMg-HFD group had decreased mass of epididymal and inguinal white adipose tissue (eWAT and iWAT, respectively) (Fig. 4A,B), which may point towards defective lipid handling in WAT. The HFD increased adipocyte size (Fig. 4C,D), but no significant difference was observed between the two HFD groups (Fig. 4C,D). Nevertheless, quantitative PCR showed that mRNA expression of*Srebp1c, Pepck1* and genes involved in beta-oxidation was increased in the eWAT of the LowMg-HFD group compared with the NormMg-HFD group (Supplementary Fig. 3A-F).

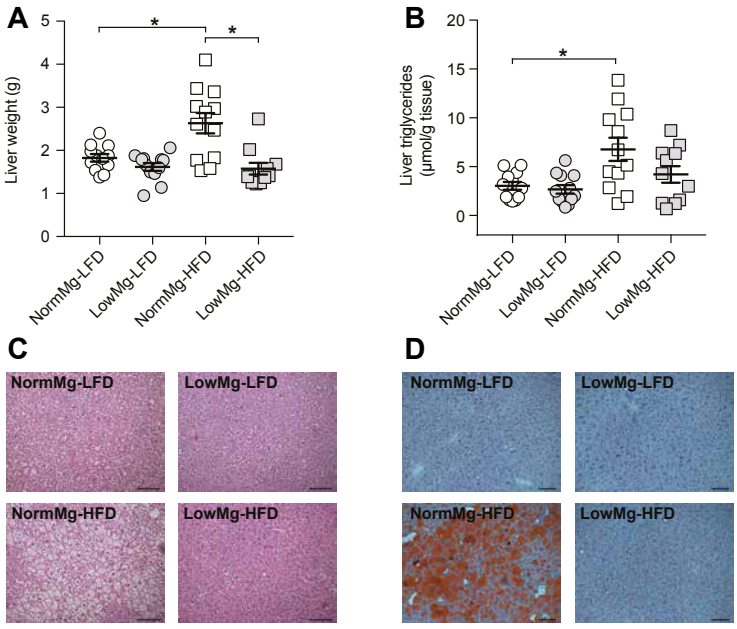

Figure 3 | Mg^{2+} deficiency prevents diet-induced hepatic lipid storage

Liver (**A**) weight and (**B**) triglyceride content at death. Representative images of livers stained with (**C**) H&E or (**D**) Oil Red O. Scale bars, 100 μm. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.

To determine the consequences of low Mg^{2+} on lipid metabolism, we performed RNA sequencing on eWAT. A principal component analysis using the $\log_2$ transformed expression values shows that the samples from both LFD groups cluster closely together, indicating the absence of a strong Mg^{2+} effect, whereas there is a clear separation of NormMg-HFD vs LowMg-HFD gene expression profiles (Fig. 4E). To investigate the effect of Mg^{2+} on specific biological pathways, the fold changes for groups of genes belonging to the same gene ontology were analysed. Gene ontology term analysis indicated that processes associated with adiposity (e.g. inflammation) were downregulated in LowMg-HFD-fed vs NormMg-HFD-fed mice, in accordance with decreased adipose tissue mass (Supplementary Table 1). Interestingly, despite the increased insulin sensitivity of the LowMg-HFD-fed mice, several key genes involved in the triglyceride catabolism pathway (lipolysis) were upregulated in the LowMg-HFD vs the NormMg-HFD group, which may explain the reduced lipid

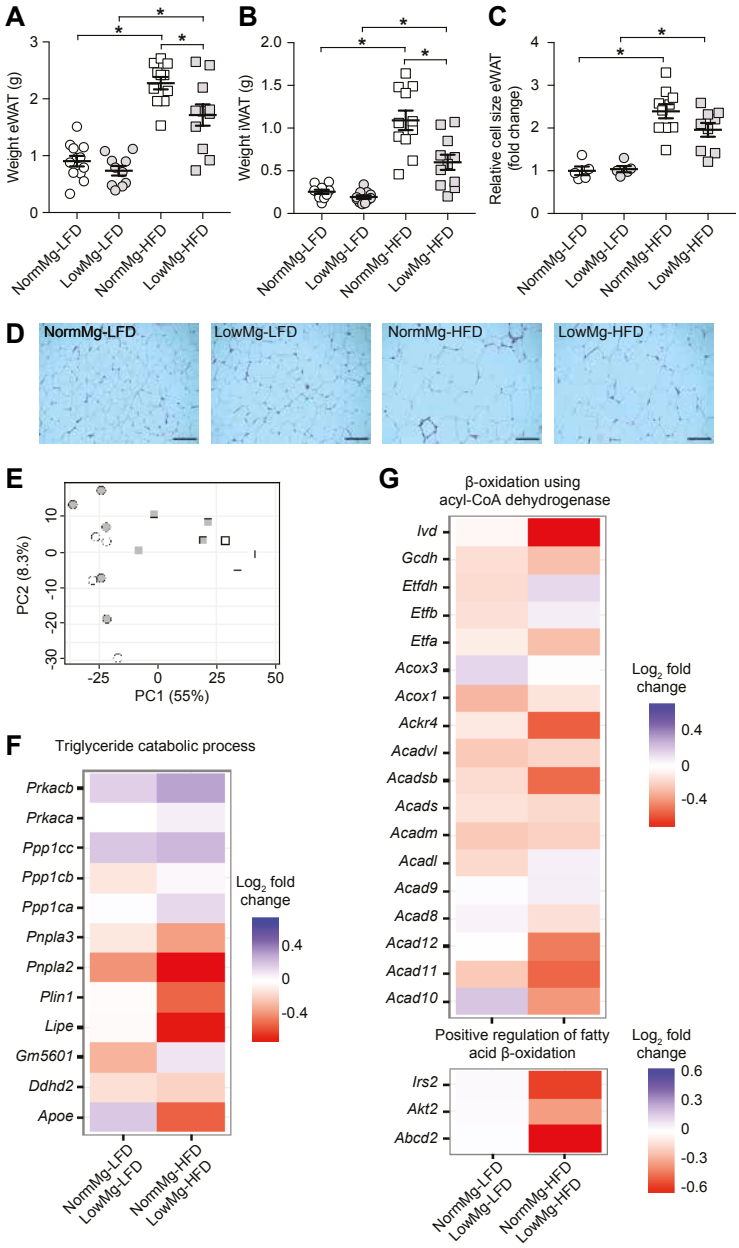

Figure 4 | Reduced adipose tissue mass in Mg^{2+}-deficient HFD-fed mice is associated with increased mRNA expression of lipolysis genes in eWAT

Figure 4 | Continued.

Weight of (**A**) eWAT and (**B**) iWAT, and (**C**) eWAT cell size at killing. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. (**D**) Representative images of H&E stained eWAT. Scale bars, 100 μm. (**E**) Principal component (PC) analysis of RNA sequencing on eWAT. The percentages on the *x*-axis and *y*-axis indicate the total percentage of variance explained by the first two principal components respectively. GO-term analyses of the pathways (**F**) 'triglyceride catabolic process' and (**G**) 'β-oxidation using acyl-CoA dehydrogenase'; and 'positive regulation of fatty acid β-oxidation'. Gene expression changes are presented as $\log_2$ fold changes with the NormMg^{2+} diet as reference, so that a negative value (in red) indicates a decrease in expression in the NormMg^{2+} vs LowMg^{2+} groups. *$p<0.05$ for the comparisons shown.

storage as well as the high serum FFA levels (Fig. 4F). A modest increase in acyl-CoA dehydrogenase dependent beta-oxidation was observed in the LowMg-HFD-fed mice vs the NormMg-HFD-fed mice (Fig. 4G). The metabolic effects of Mg^{2+} in eWAT appear to be specific to lipid homeostasis, as there was no clear effect on glycolysis (Supplementary Fig. 3G). Although serum cholesterol levels were not different between the experimental groups, cholesterol biosynthesis was greatly reduced in the LowMg-HFD-fed vs the NormMg-HFD-fed mice (Supplementary Fig. 3H).

To investigate whether hypomagnesemia has a direct effect on lipolysis in eWAT, we examined the effect of Mg^{2+} on lipolysis in differentiated 3T3-L1 cells *in vitro*. Unstimulated lipolysis was not different at 0 or 1 mmol/L Mg^{2+}, indicating that Mg^{2+} deficiency does not directly induce lipolysis in adipocytes (Supplementary Fig. 4).

mRNA expression of the β_3-adrenergic receptor is increased in LowMg-HFD mice

β_3-Adrenergic receptors (ADRB3) are essential regulators of lipid metabolism, increasing brown adipose tissue (BAT) activity and reducing WAT lipid storage *via* activation of lipolysis (29-32). We therefore explored whether enhanced β-adrenergic signalling could explain the high triglyceride levels, increased lipolysis and reduced body weight of Mg^{2+}-deficient HFD-fed mice. Expression of *Adrb3* was significantly increased by 2.5-fold in the eWAT of LowMg-HFD-fed compared with NormMg-HFD-fed mice (Fig. 5A). Additionally, both HFD-fed groups showed elevated mRNA expression of *Adrb3* in BAT, but this upregulation was more pronounced in the LowMg-HFD group (Fig. 5B). No significant difference was observed in the serum level of the dopamine metabolite 3-methoxytyramine (Fig. 5C).

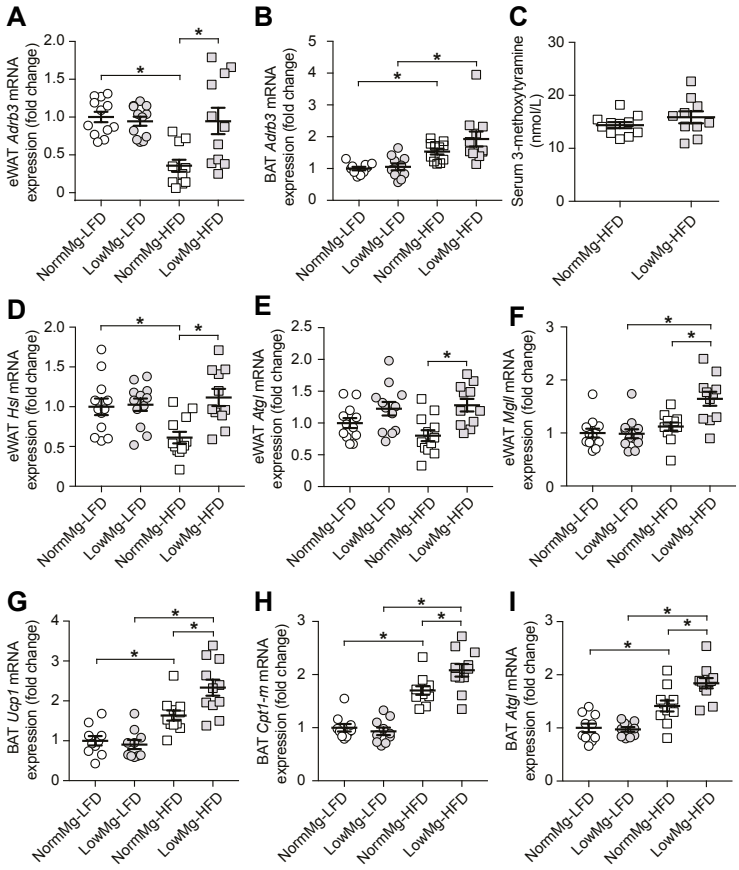

Figure 5 | mRNA expression of the β_3-adrenergic receptor is increased in eWAT of LowMg-HFD-fed mice

Relative mRNA expression of *Adrb3* in (**A**) eWAT and (**B**) BAT, normalised to *Gapdh* expression, relative to NormMg-LFD. (**C**) Serum 3-methoxytyramine concentration at death. eWAT mRNA expression of genes essential for lipolysis, (**D**) *Hsl*, (**E**) *Atgl* and (**F**) *Mgll*, and BAT mRNA expression of (**G**) *Ucp1*, (**H**) *Cpt1-m* and (**I**) *Atgl*. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.

Gene expression of the lipolysis genes adipose triglyceride lipase (*Atgl*), hormone-sensitive lipase (*Hsl*) and monoglyceride lipase (*Mgll*) was significantly increased in eWAT of Mg^{2+}-deficient HFD-fed mice compared with the NormMg-HFD group (Fig. 5D-F).

Expression of *Ucp1* in BAT, which is essential for non-shivering thermogenesis, was upregulated in NormMg-HFD-fed mice compared with NormMg-LFD-fed mice (Fig. 5G). In line with increased β_3-adrenergic signalling, *Ucp1* expression was further increased in BAT of LowMg-HFD-fed mice. BAT thermogenesis is strongly regulated by fatty acid availability (33). Indeed, essential genes involved in FFA metabolism of BAT are upregulated (Fig. 5H,I).

LowMg-HFD-fed mice have increased body temperature but equal energy expenditure

To investigate the energy metabolism in Mg^{2+}-deficient HFD-fed mice, the dietary intervention study was repeated with respiratory cages. Respiration, body temperature and activity were measured at week 8, which was when the weight differences developed in our first experiment. In line with the previous experiment, the Mg^{2+}-deficient HFD-fed mice had reduced body weight compared with the NormMg-HFD-fed mice (Fig. 6A). Lean body mass was not different between the two HFD groups, indicating that the weight difference depends on adipose tissue mass (Fig. 6B). As with our previous experiment, the HFD caused a reduction in serum Mg^{2+} levels (Fig. 6C). A significant increase was observed in serum triglyceride when animals were killed (after 9 weeks) in the LowMg-HFD group compared with the NormMg-HFD group (Fig. 6D). Low dietary Mg^{2+}-fed mice had decreased non-fasted serum glucose (Fig. 6E; two-way ANOVA for dietary Mg^{2+} effect $p<0.05$), while the difference between the two HFD-fed groups was not significant (NormMg-HFD vs LowMg-HFD, Tukey's test $p=0.07$). Hypomagnesemia and HFD decreased core body temperature (Fig. 6F). In contrast, the body temperature of LowMg-HFD-fed mice was higher than NormMg-HFD-fed mice (Fig. 6F; 35.8 ± 0.1 vs 36.4 ± 0.2 °C in NormMg-HFD and LowMg-HFD, $p<0.05$), in line with increased BAT activity. Moreover, mice fed a Mg^{2+}-deficient diet showed increased locomotor activity (Supplementary Fig. 6A,B; two-way ANOVA for dietary Mg^{2+} effect $p<0.05$). However, energy expenditure was not different between the two HFD-fed groups (Fig. 6G,H). In both HFD groups, the respiratory exchange ratio (RER) was approximately 0.7, indicating that fatty acids are the main energy source (Fig. 6I,J). Interestingly, in the LFD groups, hypomagnesemia resulted in a reduction of the RER (Fig. 6I,J), suggesting an increased use of fatty acids as an energy substrate over glucose. However, this reduction was not statistically significant (Fig. 6J; 0.82 ± 0.01 vs 0.79 ± 0.01 average RER in NormMg-LFD and LowMg-LFD, respectively; $p=0.10$).

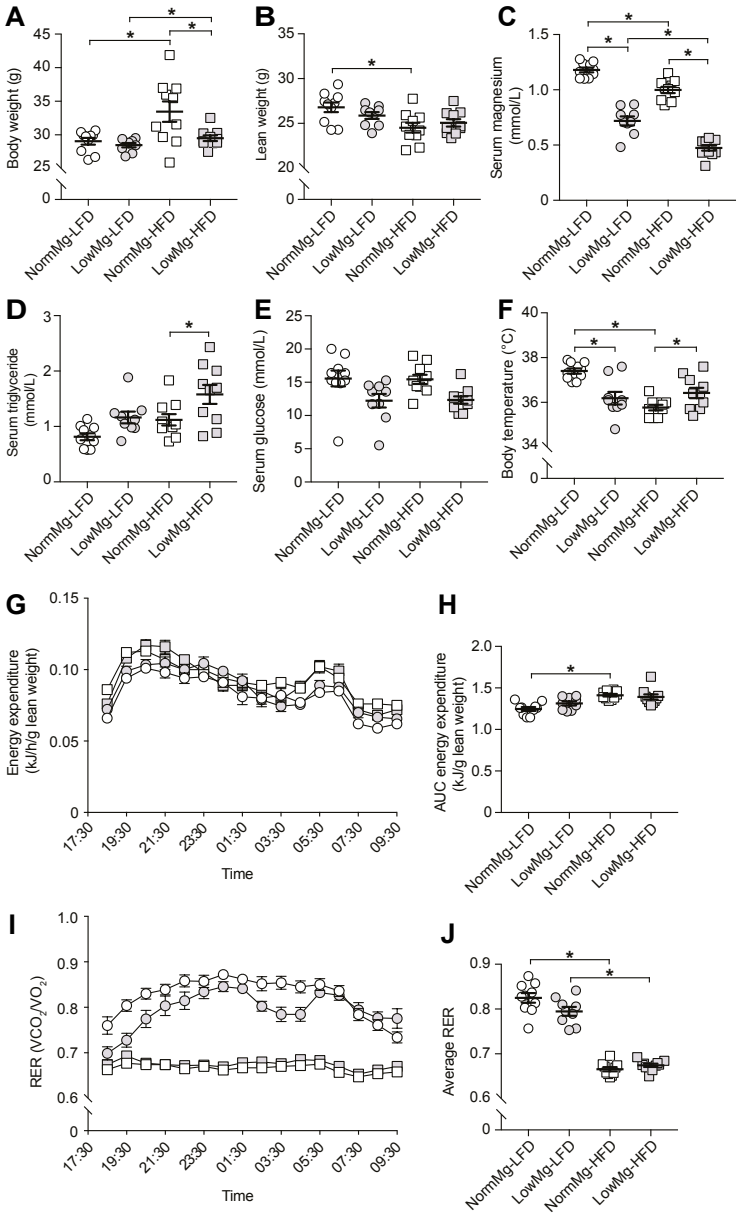

Figure 6 | LowMg-HFD-fed mice have increased body temperature but equal energy expenditure

Figure 6 | Continued.

A replication animal study was performed for a duration of 9 weeks. (**A**) Body weight and (**B**) lean body weight of the animals at death. Non-fasted serum (**C**) Mg^{2+}, (**D**) triglyceride and (**E**) glucose concentrations at killing (serum glucose two-way ANOVA for dietary Mg^{2+} effect $p<0.05$; NormMg-HFD vs LowMg-HFD Tukey's test $p=0.07$). (**F**) Body temperature, (**G**) energy expenditure averaged per hour and corrected for lean weight, (**H**) from which the AUC is calculated, measured after 8 weeks of dietary intervention. (**I**) RER averaged per hour, measured after 8 weeks of dietary intervention. RER is determined by dividing the CO_2 production by the O_2 intake. (**J**) Average RER over the entire duration of the measurement (from 18:30 to 09:30 hours). NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.

Discussion

Hypomagnesemia has been repeatedly reported in T2D or the metabolic syndrome (1, 2, 14), but the role of Mg^{2+} in lipid metabolism has been largely overlooked. Here, we demonstrate that low dietary Mg^{2+} intake ameliorates HFD-induced obesity. The lower body weight results in beneficial metabolic effects including improved insulin sensitivity, reduced hepatic steatosis and lower WAT inflammation. Nevertheless, serum triglyceride and FFA concentrations were increased in the low Mg^{2+} HFD group, corresponding to increased eWAT mRNA expression of lipolysis genes. These findings establish Mg^{2+} as an important regulator of body weight and lipid metabolism.

In this study, a Mg^{2+}-deficient diet ameliorated HFD-induced weight gain in mice. This was the result of reduced adiposity, because lean body mass was similar between the two HFD groups and both eWAT and iWAT weight were lower in mice fed a LowMg-HFD compared with a NormMg-HFD. The reduced body weight was associated with favourable metabolic effects. IPGTT, IPITT and fasting glucose levels indicated enhanced insulin sensitivity. Moreover, the reduced body weight of the LowMg-HFD mice led to a complete absence of hepatic steatosis and RNA sequencing of the eWAT demonstrated downregulation of pro-inflammatory pathways. Despite these beneficial effects, blood lipid levels remained high. In line with our data, others have demonstrated that low dietary Mg^{2+} intake reduced body weight in several rat models of Mg^{2+} deficiency (34-37). However, these studies did not address the underlying cause or investigate the effects on lipid metabolism.

Our animal data is strengthened by the results of Chubanov *et al* (17) where severe hypomagnesemia *via Trpm6* knockout also resulted in a catabolic phenotype and improved insulin sensitivity (17). The catabolic phenotype of Mg^{2+}-deficient mice leads to hyperlipidemia, which has considerable adverse effects in individuals with

T2D (38, 39). Nevertheless, the low Mg^{2+} HFD does not completely mimic the human situation because the hypomagnesemia induced in mice is more severe (1). Moreover, an unhealthy human diet consists of both high fat and sugar, whereas the HFD in mice purely depends on palm oil. Indeed, Mg^{2+}-deficiency in high-fructose diets has adversely affected insulin sensitivity and lipid homeostasis in rats. This shows the considerable differences in the role of Mg^{2+} in the metabolism of lipids vs carbohydrates (40, 41). Future studies should investigate the role of Mg^{2+} in combined fat and sugar diets. These differences may explain why, in humans, higher oral Mg^{2+} intake and Mg^{2+} supplementation have beneficial effects on metabolic variables, which apparently contrasts with our animal data (18-20).

In our study, the reduced WAT mass of LowMg-HFD-fed mice was associated with enhanced lipolysis gene expression, causing high serum FFA and triglyceride levels. These findings suggest that LowMg-HFD-fed mice depend more on mitochondrial beta-oxidation, rather than glycolysis, for energy production. However, our energy metabolism experiments demonstrated neither differences in energy expenditure nor in RER between the NormMg-HFD and LowMg-HFD groups. It should be noted, however, that both HFD groups mainly depend on lipids for energy metabolism, masking potential RER differences between these groups. Moreover, despite equal energy expenditure, the NormMg-HFD-fed mice are considerably heavier than LowMg-HFD-fed mice and therefore have a higher energy demand. Several studies have discussed the considerable difficulties associated with the interpretation of energy expenditure data and emphasised that body weight differences complicate interpretation (42, 43). Increased thermogenesis may explain why energy expenditure does not differ between LowMg-HFD-fed and the heavier NormMg-HFD-fed mice. Although the effects are modest, the LowMg-HFD-fed mice had a significantly higher body temperature and increased *Ucp1* expression in BAT, indicative of higher thermogenesis. Cold-exposure studies are necessary to further investigate the role of Mg^{2+} status in BAT activation, WAT browning and thermogenesis.

The increased lipolysis and brown adipose tissue activity were associated with higher β_3-adrenergic receptor expression in eWAT and BAT of LowMg-HFD-fed mice. β_3-receptor knockout mice have increased lipid stores and impaired WAT browning (44, 45). Activation of the β_3-adrenergic receptors in mice using agonist CL316243 decreases adipose tissue mass, improves insulin sensitivity, increases uncoupling protein-1 (UCP1)-dependent thermogenesis and activates a cycle of concomitant lipolysis and *de novo* lipogenesis (46, 47). Interestingly, this is exactly the phenotype that was observed in the LowMg-HFD-fed mice, although to a lesser extent. A link between Mg^{2+}, β-adrenergic signalling and lipolysis is not without precedent. Use of β-adrenergic agonists, which stimulate lipolysis, have been associated with decreased blood Mg^{2+} levels (1, 48, 49). Mg^{2+} has also been shown to reduce catecholamine release from the adrenal medulla (50) and Mg^{2+} deficiency is associated with higher

urinary levels of adrenaline and noradrenaline (norepinephrine) (37). Moreover, Mg^{2+} supplementation has been suggested to regulate lipolysis, as it prevents hyperlipidemia in diabetic rats and reduces serum triglyceride levels in individuals with T2D (20, 51). Further research is required to determine exactly how hypomagnesemia increases β-adrenergic signalling and how β-adrenergic signalling can induce hypomagnesemia.

A strength of this study is that the model used to induce T2D and low dietary Mg^{2+} intake closely resembles the human situation. The Western diet contains high amounts of processed foods consisting of high energy and low Mg^{2+}. Moreover, the extensive phenotyping of the animals in this study provides new avenues for research into the pivotal role of Mg^{2+} in metabolism. The data obtained in this study are robust, as a replicate animal experiment was performed in a separate institution, confirming our results.

Our study has limitations. First, because of the large weight differences induced by the Mg^{2+} deficient diet, it is difficult to specifically attribute the metabolic changes of the mice to their lower body weight or their Mg^{2+} deficiency. In addition, our study design did not allow us to study in more depth the contribution of disturbed β-adrenergic signalling to the differences in body weight, eWAT lipolysis, BAT activity and hyperlipidemia. Although our data and previous studies support a role for Mg^{2+} in β-adrenergic signalling (37, 50), further studies are required to establish the exact role of Mg^{2+} in catecholamine secretion and signalling.

In conclusion, our results demonstrate that hypomagnesemia in mice prevents HFD-induced weight gain by enhanced BAT activity and increased eWAT lipolysis gene expression. Consequently, this led to improved insulin sensitivity and absent hepatic steatosis. These results underline the pivotal function of Mg^{2+} in maintaining a healthy energy metabolism.

Materials and methods

17-week mouse study – Radboud university medical center

This study was approved by the animal ethics board of the Radboud University Nijmegen (RU DEC 2015-0073) and the Dutch Central Commission for Animal Experiments (CCD, AVD103002015239). To study the effect of dietary Mg^{2+} on HFD-induced obesity, 48 male C57BL6/J mice (Charles River, Germany), age 9-10 weeks, were randomly allocated into four experimental groups of n=12 mice. Mice were acclimatized for two weeks in a temperature- and light-controlled room, six per cage (Eurostandard Type III), and had *ad libitum* access to acidified tap water and standard pellet chow (Ssniff Spezialdiäten, GmbH, Germany). Experimental diets consisted of both 10 or 60 kcal% palm oil and 0.03 or 0.21 w/w% magnesiumoxide (Ssniff Spezialdiäten, NormalMg-LFD #S9074-E0277, LowMg-LFD #S9074-E0287,

NormalMg-HFD #S9074-E0297, LowMg-HFD #S9074-E0317). Researchers and animal caretakers were blinded for the Mg^{2+} content of the experimental diets throughout the experiment. At day -1, 84 and 112, mice were housed individually in metabolic cages for 24 hours for the collection of urine and faeces and determining food and water intake. Mice were weighed twice weekly and blood was collected *via* cheek puncture at day -1, 28, 56 and 84. At week 14 and 15 insulin and glucose tolerance tests, respectively, were performed. One mouse died unrelated to the dietary intervention and was excluded from future analyses. After 17 weeks on the diets, mice were anaesthetized by 4 v/v% isoflurane and exsanguinated *via* orbital sinus bleeding. Death was confirmed by cervical dislocation. Tissues were stored in 10 v/v% formalin or snap frozen in liquid nitrogen.

Intraperitoneal insulin and glucose tolerance tests

After 14 weeks on the diets, three mice per group per day underwent an intraperitoneal insulin tolerance test (IPITT), over a period of four days (n=9 mice/group). After 6 hours of fasting from 08:00 AM, mice were injected with 0.75 U/kg bodyweight human insulin (Novorapid, Novo Nordisk, Denmark) dissolved in phosphate buffered saline (PBS). Blood glucose levels were measured at 0, 20, 40, 60, 90 and 120 minutes after the insulin injection. Blood was taken *via* a tail cut and glucose levels were measured using a StatStrip® Xpress glucose meter (Nova Biomedical, USA). Three mice developed severe hypoglycaemia and were injected with D-glucose (Invitrogen, The Netherlands) between 15 and 30 minutes post insulin-injection. For the subsequent time points, these mice were given the same glucose values as the lowest values within that group. After 15 weeks on the diets, three mice per group per day underwent an intraperitoneal glucose tolerance test (IPGTT), over a period of four days (n=12 mice/group). After an overnight fast (from 18:00 to 09:00), mice were injected with 2 g/kg body weight D-glucose (Invitrogen, The Netherlands) dissolved in PBS, with an injection volume of 50ul/10g body weight. Blood glucose levels were measured at 0, 15, 30, 60 and 120 minutes after the glucose injection.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK), subjected to DNase (Promega, Fitchburg, WI, USA) treatment and measured using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, Bleiswijk, the Netherlands). Gene expression levels were quantified by SYBR-Green (Bio-Rad, Veenendaal, the Netherlands) on a CFX96 real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands) and normalised for *Gapdh*. Primer sequences are provided in Table 2.

Table 1 | RT-qPCR Primer sequences

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Adrb3	TCCGTCGTCTTCTGTGTAGC	GCCATCAAACCTGTTGAGCG
Atgl	GAGGAATGGCCTACTGAACCAAC	AGGCTGCAATTGATCCTCCTC
Cact	CGATTCCAGACTGCACCTCC	CGCGGATCATGACTGCATTG
Cd36	TGCATGAATTAGAACCGGGC	TCTCCTCGTGCAGCAGAATC
Cpt1-l	GTGAGCCTGGCCTCGCC	TGAGTGGTGACCGAGTCTGC
Cpt1-m	CTGGGCTATCTGTGTCCGTC	GGGACAGGAAGCTTAGGCAG
Cpt2	GTATCTGCAGCACAGCATCG	GTTTAGGGATAGGCAGCCTGG
Fbp1	CCCAGCTGCTGAATTCGCTC	AGCGATACCATAGAGCTGTGC
G6pase	TTGGACAACGCCCGTATTGG	GGACTTCCTGGTCCGGTCTC
Gapdh	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC
Glut1	GGGTCTTAAGTGCGTCAGGG	TCACCTTCTTGCTGCTGGG
Glut2	AGAAGACAAGATCACCGGAACC	TCACACCGATGTCATAGCCG
Glut4	CTTATTGCAGCGCCTGAGTC	GTTCCCCATCGTCAGAGCC
Gs	TGACTGAGCTCAAACGAAATGATTC	TGCATCAGGGTGTGGATCTG
Gyk	GTTGTCCCCTCTGGCTCTTC	AGGCCTGTCTTGGACTTGAC
Hmgcs1	TCCCCTTTGGCTCTTTCACC	TCCCACATCTTTTGGCCAGC
Hsl	AGGGAGGGCCTCAGCG	TGTCTTCTGCGAGTGTCACC
Mgll	CGGAACAAGTCGGAGGGTTC	TGTTTTGTCCTGACTCCGGG
Pepck1	CCTAGTGCCTGTGGGAAGAC	AGCCCTTAAGTTGCCTTGGG
Pk-lr	CAGTATGGAAGGGCCAGCAG	CAGGAAGGTGTCCGCCATAG
Ppar-α	GTGGTGCATTTGGGCGTATC	TGAACTTCAACTTGGCTCTCC
Ppar-γ	CTGACGGGGTCTCGGTTG	CAACCATGGTAATTTCAGTAAAGGGCC
Srebp1	CAGCCACACTTCATCAAGGC	ACTCACCAGGGTCTGC
Ucp1	TGGCCTCTTTAACCCTGCTG	GATTAGGGGTCGTCCCTTTCC

Histology

Epididymal fat and liver tissues were fixed in 10% vol./vol. neutral-buffered formalin (KLINIPATH, Duiven, the Netherlands) in PBS. Samples were dehydrated through alcohol, embedded in paraffin and cut into 4 μm sections. Sections were stained with H&E using standard procedures. The average cell size of 100-300 cells per mouse was determined manually using ImageJ software (v1.48, NIH, USA, RRID:SCR_003070). Liver samples were snap frozen in liquid nitrogen, cut into 10 μm sections, stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with haematoxylin.

RNA-Sequencing

Total RNA was extracted from epididymal white adipose tissue using TRIzol reagent (Invitrogen, UK) according to manufacturer's protocol. RNA integrity was validated by investigating the 18S/28S bands on a 2 w/v% agarose gel. Five randomly selected

samples of each group were RNA-sequenced (RNA-Seq). Quality control and RNA-Seq were performed by Beijing Genomics Institute (BGI), Hong Kong). Quality control was performed by using Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System to qualify and quantify the sample library. One sample from the NormalMg-LFD group failed the quality control, and was excluded from subsequent sequencing and analyses. One sample from the LowMg-HFD group showed a small contamination with pancreatic tissue and was excluded from subsequent analyses. 13 million reads were sequenced using the Hiseq 4000 platform (Illumina, USA) using a 50 bp single-end module. Clean reads were mapped to Mus Musculus transcriptome (GRCm38/mm10) using HISAT/Bowtie2 tool (24, 25). RSEM software v1.2.31 was used to quantify gene expression levels (FPKM values)(26). FPKM values were $\log_2$ transformed and further analysed in R (www.r-project.org, RRID:SCR_001905). In order to filter non-expressed transcripts from the data, only transcripts that showed an average expression level of 8 within a group and for which the transcript levels were above 8 in at least four replicates from an experimental group were retained, yielding a total of 8808 transcripts. To calculate the differences between expression levels for genes belonging to the same Gene Ontology group, the fold change between the LowMg and NormalMg condition for both the HFD and LFD groups were collected for each gene in the group. Subsequently a *t*-test was used to test for the hypothesis of equal means. The procedure was repeated for all GO terms and the *p*-values for the tests were corrected for multiple testing using the Benjamini-Hochberg method as implemented in the p.adjust method in R. Heatmaps for individual GO terms were created using the ggplot2 library (RRID:SCR_014601) (27).

Analytical procedures

Serum Mg^{2+} was determined using a spectrophotometric assay at 600 nm (Roche/Hitachi, Tokyo, Japan) according to the manufacturer's protocol. Liver samples were weighed and lysed in lysis buffer (10% wt/vol.) containing 50 mmol/L Tris-HCl pH 7.5, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% vol./vol. Triton X-100, 10 mmol/L glycerophosphate, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate and 150 mmol/L sodium chloride. Triglyceride concentrations in serum and liver lysate were assayed using an enzymatic kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to the manufacturer's protocol. Serum FFA (NEFA-C kit, WAKO Diagnostics, Delfzijl, the Netherlands), cholesterol (Human Diagnostics, Wiesbaden, Germany), glucose (Instruchemie, Delfzijl, the Netherlands), leptin (R&D Systems, Minneapolis, MN, USA) and adiponectin (R&D Systems, Minneapolis, MN, USA) concentrations were determined according to manufacturers' protocols. 3-Methoxytyramine and normetadrenaline (normetanephrine) were analysed by a 6490 LC-MS/MS (Agilent Technologies, Amstelveen, the Netherlands) after solid phase extraction (SPE) Oasis WCX μElution sample cleanup (Waters,

Etten-Leur, the Netherlands). A calibration curve was used with 3-methoxytyramine-HCl (Sigma-Aldrich, St. Louis, MO, USA) as calibrator. 3-Methoxytyramine-d_4-HCl (Medical Isotopes, Pelham, NH, USA) was used as internal standard. An ethylene bridged hybrid (BEH) Amide 1.7 μm 100A, 2.1 x 100 mm column (Waters, Etten-Leur, the Netherlands) was used as an analytical column.

9-Week replication mouse study – MRC Harwell Institute

All experimental procedures were conducted in compliance with the UK Animals Scientific Procedures Act (1986) and University of Oxford ethical guidelines. 39 male C57BL6/J mice (MRC Harwell, UK) were randomly allocated into 4 groups of n=10 mice (n=9 in the LowMg-LFD group) housed with five per cage (1284L and 1285L IVC, Tecniplast, Italy). Mice had *ad libitum* access to demineralized chlorinated tap water and standard pellet chow. At 8 weeks old, mice were put on experimental diets identical to the first animal experiment at the Radboudumc, for a period of 9 weeks. At day 14, mice were housed individually in metabolic cages (Tecniplast, Italy) for 24 hours for the collection of urine and faeces and determining food and water intake. Mice were weighed twice weekly and blood was collected *via* tail bleed at day -1 and 14. Respiration metabolic cages (TSE Phenomaster Cages, Germany) were used at day 28 and 56 of the experiment and body temperatures were measured by rectal probe (ATP-instrumentation, UK). Data were averaged per hour and plotted from 6:30 PM to 9:30 AM. After 9 weeks on the diets, mice were anaesthetized by 4 vol./vol.% isoflurane and exsanguinated *via* orbital sinus bleeding. Death was confirmed by cervical dislocation. Tissues were stored in 10 v/v% formalin or snap frozen in liquid nitrogen.

Lipolysis in 3T3-L1 adipocytes

3T3-L1 fibroblasts (ATCC, mycoplasma-free) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza Westburg, Leusden, the Netherlands) containing 2 mg/ml ciproxin (Fresenius Kabi, Zeist, The Netherlands), 200 mmol/L L-glutamine (GE healthcare Life Sciences, Logan, UT, USA) and 10 vol./vol.% fetal bovine serum (FBS, Greiner Bio One), at 37 °C, in 5 vol./vol.% CO_2. 3T3-L1 cells between passages 10 and 20 were differentiated according to ATCC's protocol. In short, cells were seeded in PLL (Sigma-Aldrich) coated 6-well plates in DMEM and upon confluence medium was refreshed. Two days post-confluence the induction process was initiated by changing the medium to induction medium containing 1 μg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mmol/L IBMX (Sigma-Aldrich), and 1 μmol/L dexamethasone (Sigma-Aldrich) for 48 hours. The cells were then washed (PBS) and DMEM containing 1 μg/mL insulin was added. Hereafter, the medium was refreshed every two days until >90% of the cells were completely differentiated into adipocytes. To determine the effect of Mg^{2+} on lipolysis, differentiated 3T3-L1 cells were incubated

for 20 hours in DMEM without added insulin, containing 1.0 or 0 mmol/L $MgCl_2$, followed by 2 hours serum-starvation. Hereafter, cells were incubated for 4 hours in 700 μl KRPH buffer (20 mmol/L HEPES pH 7.4, 5 mmol/L KH_2PO_4, 1 mmol/L $CaCl_2$, 136 mmol/L NaCl, 4.7 mmol/L KCl at 37 °C) containing 1.0 or 0 mmol/L $MgCl_2$, 0.1 wt/vol.% glucose (Merck Millipore, Amsterdam, the Netherlands) and 3.5 wt/vol.% fatty acid free BSA (Sigma-Aldrich). 50 μL aliquots of the medium were taken every hour and heated for 8 minutes at 65 °C. The concentration of FFAs was assessed using the WAKO NEFA-C kit (Instruchemie, Delfzijl, the Netherlands) according to the manufacturer's protocol.

Statistics

For the animal experiments, a two-way ANOVA was used to look for a significant interaction effect between the two main variables (dietary fat and Mg^{2+} content). If there was none, significant differences between the groups were assessed using a two-way ANOVA approach with a Tukey's multiple comparisons test. If the two-way ANOVA demonstrated a significant interaction effect between the two main variables, an unpaired multiple *t* test approach using the Holm–Sidak method for multiple comparisons was used. Statistical significance was determined using Graphpad Prism v7 (California, USA, RRID: SCR_002798). For the lipolysis assays, an unpaired Student's *t* test was used. Differences with a *p*-value of <0.05 were considered statistically significant. Results are presented as mean ± SEM.

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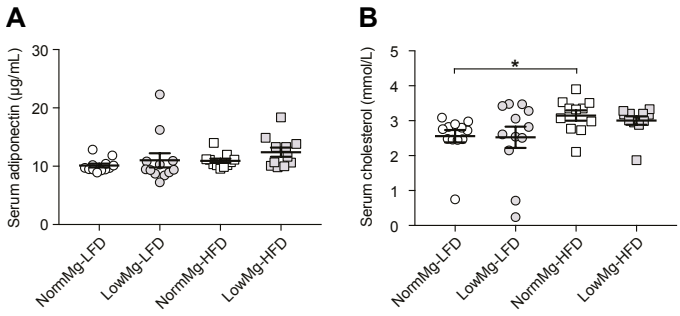
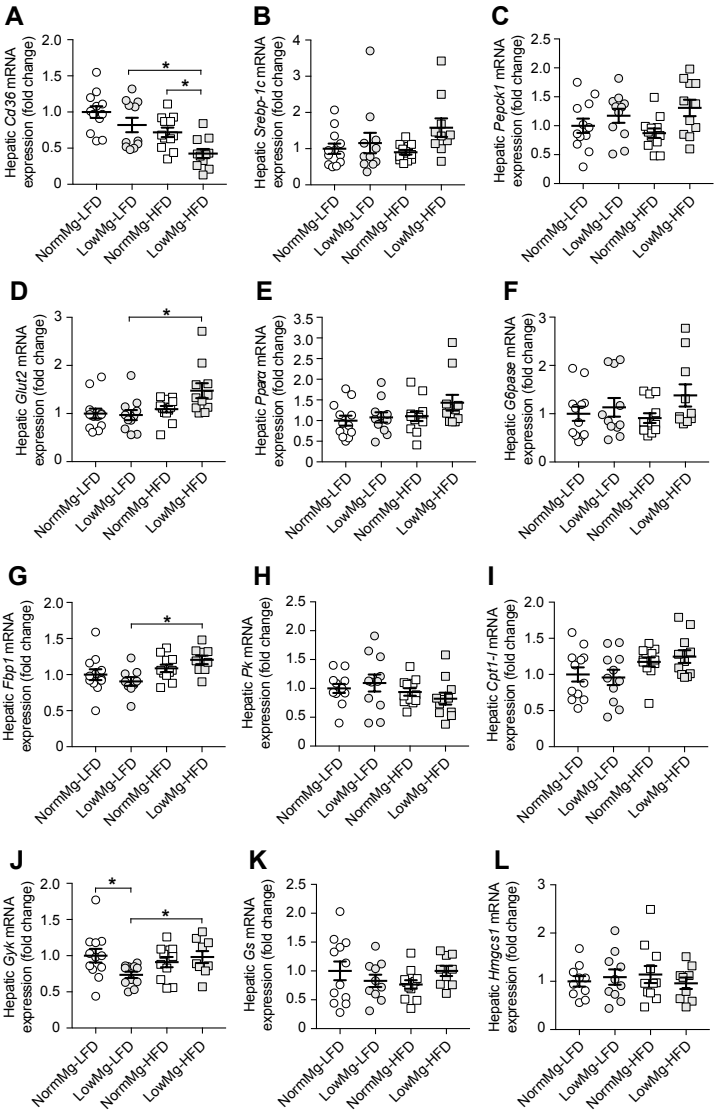
Supplementary data



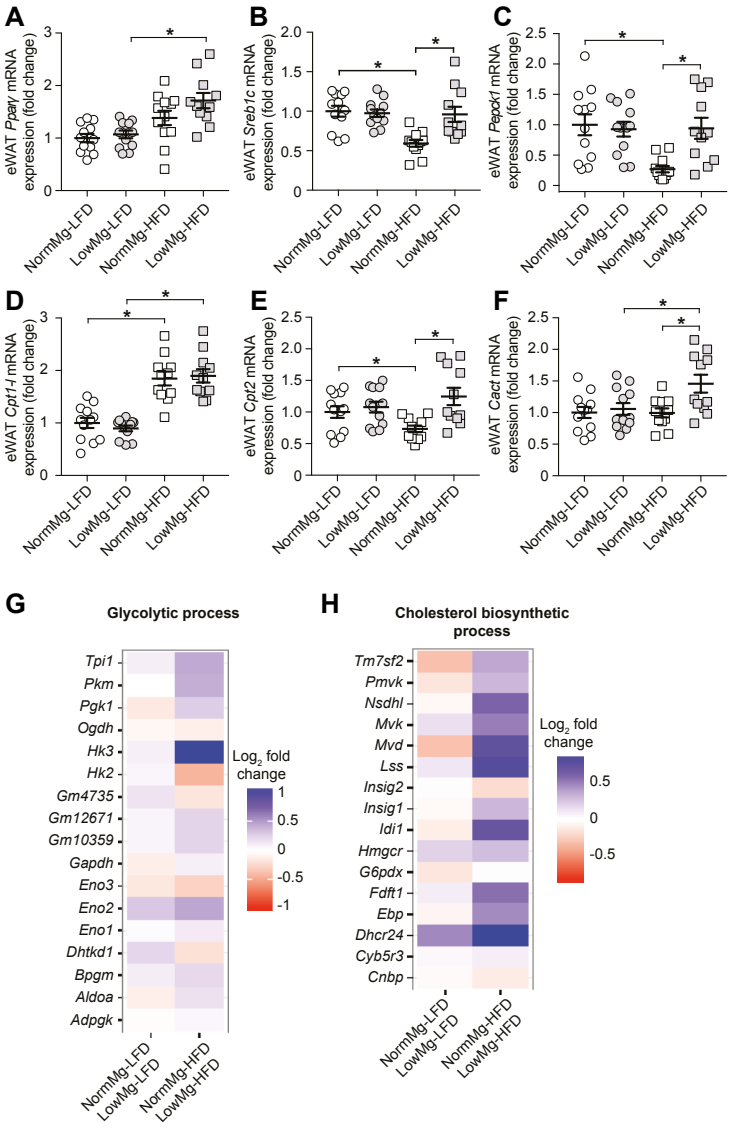
Supplementary figure 1 | Changes in dietary Mg^{2+} do not affect serum adiponectin and cholesterol concentrations

Non-fasted serum concentrations of (**A**) adiponectin and (**B**) cholesterol at killing. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. *$p<0.05$ for the comparisons shown.



Supplementary figure 2 | Hepatic mRNA expression of key genes involved in energy metabolism

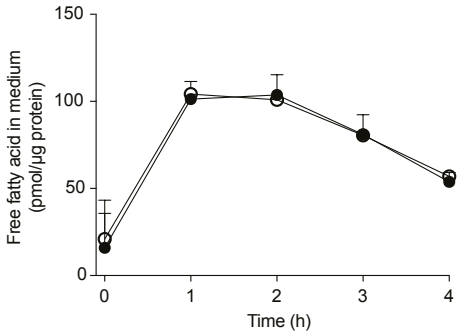
Hepatic mRNA expression of (**A**) *Cd36,* (**B**) *Srebp1c* (two-way ANOVA for dietary Mg^{2+} effect $p<0.05$), (**C**) *Pepck1* (two-way ANOVA for dietary Mg^{2+} effect $p<0.05$; NormMg-HFD vs. LowMgHFD Tukey's test $p=0.06$), (**D**) *Glut2,* (**E**) *Ppara,* (**F**) *G6pase*, (**G**) *Fbp1*, (**H**) *Pk-lr*, (**I**) *Cpt1-l* (two-way ANOVA dietary fat effect $p<0.05$), (**J**) *Gyk*, (**K**) *Gs* and (**L**) *Hmgcs1* normalized to *Gapdh* expression, relative to NormMg-LFD. NormMg-LFD (white circles), LowMg-LFD (grey circles), NormMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. * Indicates $p<0.05$.



Supplementary figure 3 | Increased mRNA expression of key genes involved in gluconeogenesis and beta-oxidation in the eWAT of LowMg-HFD mice and heatmaps of eWAT RNA-Seq GO-Term glycolysis and cholesterol biosynthesis pathways

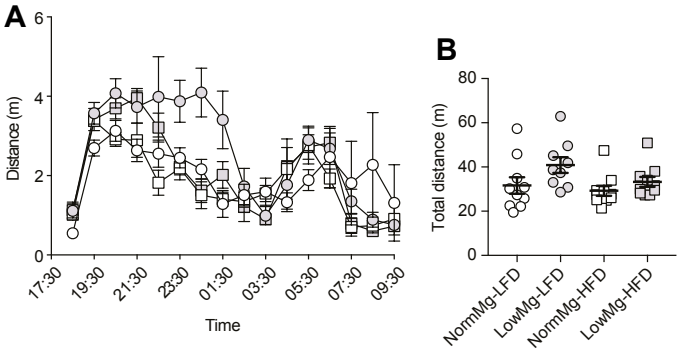
Supplementary figure 3 | Continued.

Epididymal white adipose tissue (eWAT) mRNA expression of (**A**) *Pparγ*, (**B**) *Srebp1c*, (**C**) *Pepck1*, (**D**) *Cpt1-l*, (**E**) *Cpt2* and (**F**) *Cact*, normalized to *Gapdh* expression, relative to NormalMg-LFD. GO-Term Analyses of the Pathways (**G**) 'Glycolytic Process' and (**H**) 'Cholesterol Biosynthesis'. Gene expression changes are presented as $\log_2$ fold changes with the NormalMg^{2+} diet as reference, so that a negative value (in red) indicates a decrease in expression in the NormalMg^{2+} versus LowMg^{2+} groups. NormalMg-LFD (white circles), LowMg-LFD (grey circles), NormalMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. * Indicates $p<0.05$.



Supplementary figure 4 | Extracellular Mg^{2+} deficiency does not directly induce lipolysis in 3T3-1L adipocytes

3T3-1L cells were differentiated into adipocytes and cultured in 0 (open circles) or 1 (closed circles) mmol/L extracellular $MgCl_2$ for 22 hours. As a measure of the rate of lipolysis, the free fatty acid concentration in the medium was measured over a period of 4 hours (n=3 wells per time point per condition). A figure of a representative experiment is shown. The experiment was repeated with similar results. Data are mean ± SEM.



Supplementary figure 5 | Mice on a low dietary Mg^{2+} content have increased locomotor activity

(**A**) Distance walked by mice, averaged per hour from 18:30 to 09:30, measured after 8 weeks of dietary intervention, (**B**) from which the total distance walked during this period is calculated (two-way ANOVA for dietary Mg^{2+} effect $p<0.05$). NormalMg-LFD (white circles), LowMg-LFD (grey circles), NormalMg-HFD (white squares), LowMg-HFD (grey squares). Data are mean ± SEM. * Indicates $p<0.05$.

Supplementary table 1 | List of differentially regulated GO-terms from the RNA-Seq on white adipose tissue between NormalMg-HFD and LowMg-HFD. A negative log_2 fold change indicates a higher expression in the NormalMg-HFD group compared to the LowMg-HFD.

GO-Term; 10 lowest *p*-values	Corrected *p*-value	Log_2 Fold change
Translational initiation	3.5E-06	-0.26
Protein transport	6.5E-06	-0.12
SRP-dependent cotranslational protein targeting to membrane	9.2E-06	-0.30
Intracellular protein transport	1.1E-05	-0.15
RRNA processing	1.4E-05	-0.21
Viral transcription	7.1E-05	-0.31
Cell division	7.7E-05	-0.15
Cytoplasmic translation	7.7E-05	-0.21
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	7.7E-05	-0.24
Cell proliferation	8.2E-05	-0.17
GO-Term; 10 largest positive fold change (decreased in NormalMg-HFD compared to LowMg-HFD)	**Corrected *p*-value**	**Log_2 Fold change**
Positive regulation of cholesterol esterification	0.57	1.00
Positive regulation of heat generation	0.55	0.68
Negative regulation of neurotrophin TRK receptor signalling pathway	0.74	0.67
Positive regulation of double-strand break repair	0.74	0.67
L-glutamate import	0.62	0.65
Positive regulation of renal sodium excretion	0.65	0.63
Drug metabolic process	0.40	0.62
DNA dealkylation involved in DNA repair	0.76	0.61
Angiotensin mediated vasoconstriction involved in regulation of systemic arterial blood pressure	0.76	0.61
Desmosome assembly	0.36	0.61
GO-Term; 10 largest negative fold change (increased in NormalMg-HFD compared to LowMg-HFD)	**Corrected *p*-value**	**Log_2 Fold change**
Positive regulation of T cell differentiation in thymus	0.51	-1.11
Negative regulation of B cell receptor signalling pathway	0.21	-1.09
Positive regulation of type III hypersensitivity	0.05	-1.07
Cell activation	0.70	-1.06
Mast cell degranulation	0.49	-1.06
Antigen processing and presentation of exogenous peptide antigen via MHC class I	0.11	-1.02
Positive regulation of type I hypersensitivity	0.16	-1.02
Sphingosine metabolic process	0.67	-1.02
Positive regulation of CD4-positive, alpha-beta T cell differentiation	0.24	-0.99
Positive regulation of type IIa hypersensitivity	0.26	-0.95

"There is a god in man
And in nature
He who sits in the dark
The bringer of light"
– Gorgoroth | The Sign of an Open Eye

4

Renal phospholipidosis and impaired magnesium handling in diabetic mice

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Abstract

In T2D patients, reduced blood Mg^{2+} levels are associated with an increased decline in renal function, independent of glycemic control and hypertension. To study the underlying mechanism of this phenomenon, we investigated the renal effects of hypomagnesemia in a diet-induced mouse model of T2D.

In mice fed a low dietary Mg^{2+}, the HFD resulted in severe hypomagnesemia within 4 weeks. Renal or intestinal Mg^{2+} wasting was not observed after 16 weeks on the diets. Despite the absence of urinary or fecal Mg^{2+} loss, the HFD induced a reduction in the mRNA expression *Trpm6* in both the kidney and colon. mRNA expression of distal convoluted tubule (DCT)-specific genes was down-regulated by the LowMg-HFD, indicating atrophy of the DCT. The low dietary Mg^{2+} resulted in severe HFD-induced proximal tubule phospholipidosis, which was absent in mice on a NormalMg-HFD. This was accompanied by alterations in renal energy metabolism including enhanced gluconeogenesis and cholesterol synthesis.

In conclusion, this study shows that hypomagnesemia is a consequence of T2D. Moreover, hypomagnesemia induces major structural changes in the diabetic kidney, including proximal tubular phospholipidosis, providing a novel mechanism for the increased renal decline in hypomagnesemic T2D patients.

Keywords: Hypomagnesemia; type 2 diabetes; proximal tubule; phospholipidosis; distal convoluted tubule.

Introduction

Hypomagnesemia (blood magnesium (Mg^{2+}) concentration <0.7 mmol/L) is present in approximately 30% of patients with type 2 diabetes mellitus (T2D), compared to 2.5% in the general population (1, 2). Hypomagnesemia in T2D patients has a number of clinical implications. Besides general complaints such as fatigue and headache, hypomagnesemia increases T2D disease progression and leads to more diabetes-related complications, such as renal failure, hypertension and cardiovascular events (3-6). Mg^{2+} deficiency has been shown to inversely correlate with blood glucose and lipid levels, which could underlie the increased risk of cardiovascular events and the more rapid renal function decline in hypomagnesemic T2D patients (1, 4, 7, 8). In the developed world, diabetic nephropathy accounts for approximately 50% of end-stage renal disease cases, making it the main cause of end-stage renal disease (9). How Mg^{2+} deficiency contributes to a more rapid renal function decline in T2D patients remains unclear.

In the physiological situation, blood Mg^{2+} levels are tightly regulated between 0.7-1.05 mmol/L by the interplay of intestine, bone and kidney (10). In the kidney, paracellular reabsorption of Mg^{2+} takes place in the proximal tubule (10-25%) and in the thick ascending limb (TAL, 50-70%), where Mg^{2+} reabsorption is dependent on tight junction permeability (11, 12). Despite the bulk of Mg^{2+} uptake occurring in the TAL, inhibiting Mg^{2+} reabsorption in this segment by furosemide does not lead to severe hypomagnesemia, indicating the immense compensatory capacity of the distal convoluted tubule (DCT) (13). Therefore, fine-tuning of urinary Mg^{2+} excretion is determined by transcellular reabsorption in the DCT (10%, but fluctuant) (14). Mg^{2+} reabsorption in the DCT is regulated by hormones such as epidermal growth factor (EGF) and insulin (15, 16). When blood Mg^{2+} levels decrease, renal Mg^{2+} reabsorption in the DCT is enhanced by upregulating the gene expression and activity of the epithelial Mg^{2+} channel, transient receptor potential melastatin type 6 (*TRPM6*), and of Mg^{2+}-transport regulators, such as Cyclin M2 (*CNNM2*) (17, 18). Over 40% of T2D patients have excessive urinary Mg^{2+} wasting; however, the exact origin of this renal Mg^{2+} leak remains to be elucidated (1).

This study investigates the effects on mineral and lipid metabolism that are induced in the kidney by feeding mice a Mg^{2+}-deficient high fat diet (HFD). Repeated blood samples were taken to monitor Mg^{2+} levels over time and metabolic cages were used to determine urinary and fecal Mg^{2+} excretion. After 17 weeks, kidney tissue was analyzed by immunohistochemistry, electron microscopy and real-time qPCR (RT-qPCR).

Results

High fat diet feeding induces severe hypomagnesemia

To investigate the effect of T2D on Mg^{2+} homeostasis, mice were fed a low-fat diet (LFD) or a HFD combined with a normal or low dietary Mg^{2+} content. Both low Mg^{2+} diet groups showed decreased serum Mg^{2+} levels after 17 weeks on the diet (Fig. 1A,B). Interestingly, the LowMg-HFD mice developed an almost two-fold larger decrease in serum Mg^{2+} compared to LowMg-LFD mice (Fig. 1A,B, 0.39 ± 0.21 vs. 0.74 ± 0.12 mmol/L decrease in NormMg-HFD vs. LowMg-HFD, respectively, $p \leq 0.05$). After 16 weeks on the diet, urinary Mg^{2+} excretion was minimal in both LowMg groups (Fig. 1C). Renal loss of Mg^{2+} was lower in NormalMg-HFD mice compared to NormalMg-LFD mice (Fig. 1C). The fecal Mg^{2+} excretion matched the results observed in the urine (Fig. 1D). Moreover, serum calcium (Ca^{2+}) levels were reduced in mice fed a LowMg-HFD compared to a NormalMg-HFD (Fig. 1E). Serum sodium (Na^{+}) and potassium (K^{+}) concentrations were not different among all groups (Fig. 1F,G). Both LowMg-fed groups showed reduced renal Ca^{2+} excretion, while the HFD did not affect urinary Ca^{2+} excretion (Fig. 1H). In line with the serum levels, no changes were observed on urinary K^{+} and Na^{+} excretion between the two HFD-fed groups (Fig. 1I,J).

A Mg^{2+} deficient HFD reduces mRNA expression of DCT-specific genes

Mice on the LowMg-LFD had increased mRNA expression of *Trpm6* compared to NormalMg-LFD mice (Fig. 2A). Despite the severe hypomagnesemia of the LowMg-HFD mice, *Trpm6* mRNA levels were not upregulated compared to NormalMg-HFD mice (Fig. 2A). The mRNA expression of *Cnnm2*, a DCT and connecting tubule (CNT) specific Mg^{2+}-regulating gene, was also reduced in the LowMg-HFD fed mice compared to NormalMg-HFD (Fig. 2B). To investigate if a similar expression pattern was observed for other DCT-specific genes involved in electrolyte transport, the mRNA levels of parvalbumin (*Pvalb*) and Na^{+}-Cl^{-} cotransporter (*Slc12a3*) were examined. Indeed, the mRNA expression of *Pvalb* and *Slc12a3* was decreased in the LowMg-HFD versus the NormalMg-HFD mice (Fig. 2C,D). Interestingly, the mRNA transcript of Kallikrein 1 (*Klk1*), a DCT/CNT-specific gene that is not directly involved in electrolyte transport, was also reduced in the LowMg-HFD mice (Fig. 2E). The renal mRNA expression of the ubiquitous Mg^{2+}-channel *Trpm7* remained unchanged between all mice groups (Fig. 2F). Dedifferentiation of DCT cells as a cause of lower mRNA expression of DCT-specific genes was ruled out based on distinct apical localization of NCC (Fig. 3A). To study whether there was a reduction in DCT-length, immunohistological stainings were performed (Fig. 3B). Based on an NCC-staining, the total DCT-area was 26% lower in LowMg-HFD-fed mice than NormMg-HFD mice, however, this did not attain statistical significance (Fig. 3B,C 2.3 ± 0.3 vs. 1.7 ± 0.3 % DCT-area in NormalMg-HFD vs. LowMg-HFD, respectively, $p > 0.05$).

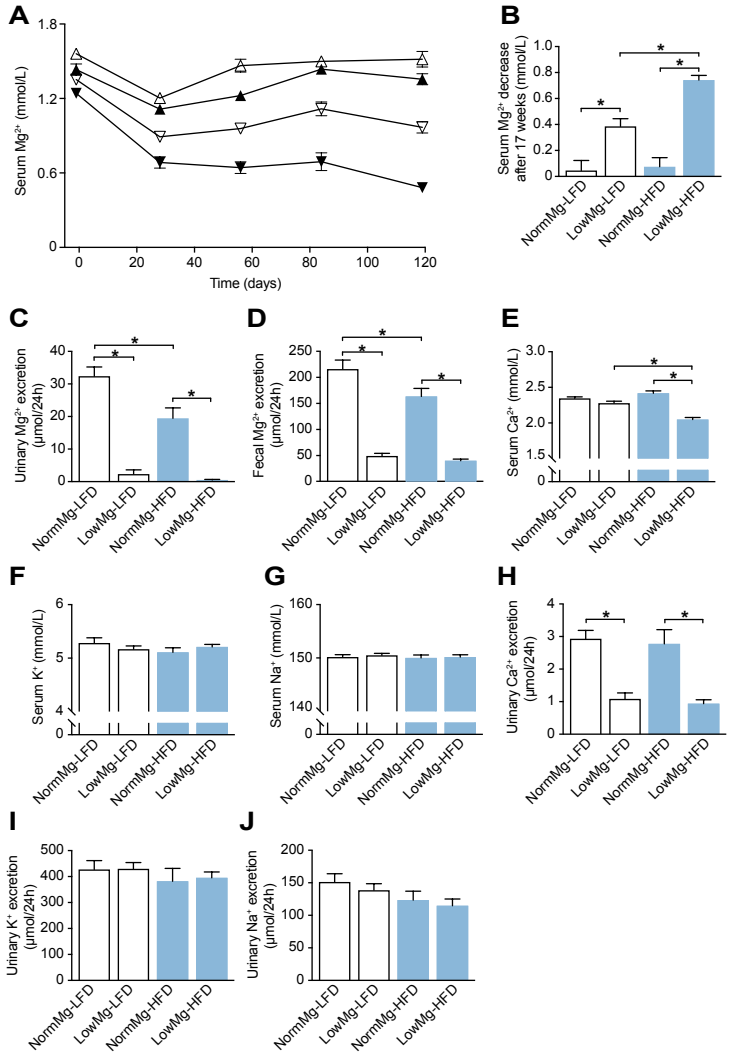

Figure 1 | Feeding mice a HFD induces hypomagnesemia within 4 weeks

(**A**) Serum Mg^{2+} levels over time. NormalMg-LFD (open triangle), LowMg-HFD (inverted open triangle), NormalMg-HFD (closed triangle), LowMg-HFD (inverted closed triangle). (**B**) Decrease in the serum Mg^{2+} concentration at 17 weeks compared to 0 weeks of dietary intervention. (**C-D**) 24-Hour excretion of (**C**) urinary and (**D**) fecal Mg^{2+} determined using metabolic cages at 16 weeks. (**E-G**) Serum (**E**) Ca^{2+}, (**F**) K^+ and (**G**) Na^+ concentrations at sacrifice. (**H-J**) 24-Hour urinary excretion of (**H**) Ca^{2+}, (**I**) K^+ and (**J**) Na^+ (urinary Na^+ at 16 weeks, two-way ANOVA for dietary calorie effect, $p \leq 0.05$) determined using metabolic cages at 16 weeks. Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates $p \leq 0.05$.

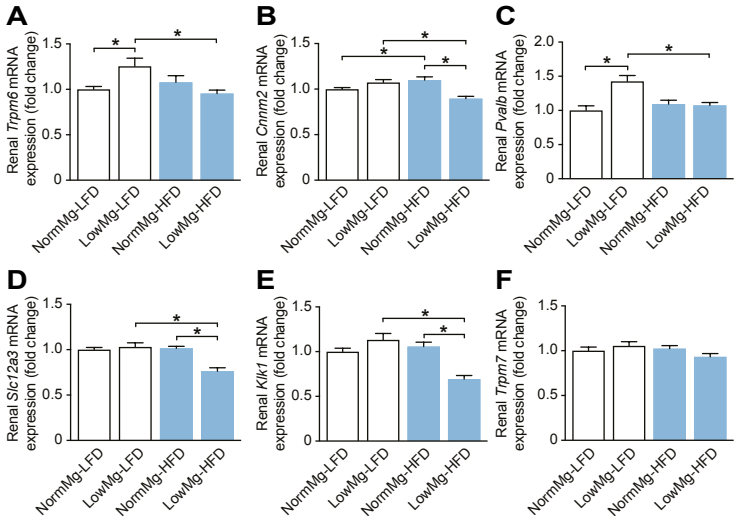

Figure 2 | A Mg^{2+} deficient HFD reduces mRNA expression of DCT-specific genes

(**A-F**) Renal mRNA expression of (**A**) *Trpm6*, (**B**) *Cnnm2*, (**C**) *Pvalb*, (**D**) *Slc12a3*, (**E**) *Klk1* and (**F**) *Trpm7* normalized to *Gapdh* expression, relative to NormalMg-LFD. Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates $p \leq 0.05$.

No significant changes were observed between the two HFD groups in other genes involved in renal Mg^{2+} handling, including solute carrier family 41 member 1 (*Slc41a1)*, claudin 10 (*Cldn10*), *Cldn14*, *Cldn16*, *Cldn19*, hepatocyte nuclear factor 1 homeobox B (*Hnf1b*), *Egf* and Mg^{2+} transporter MRS2 homolog (*Mrs2)* (Supplementary Fig. 1A-H).

A HFD reduces mRNA expression of *Trpm6* in distal colon

When blood Mg^{2+} levels decrease, the colon compensates by increasing the uptake of Mg^{2+} from the diet (19). Indeed, in mice fed a low dietary Mg^{2+} content colonic *Trpm6* mRNA expression was upregulated (Fig. 4A). Interestingly, despite a lower serum Mg^{2+} concentration in the LowMg-HFD fed mice, colonic *Trpm6* mRNA expression was reduced compared to LowMg-LFD mice (Fig. 4A). The colonic mRNA expression of *Trpm7*, *Cnnm2* and *Cnnm4* remained unchanged (Fig. 4B-D). In the HFD-fed mice, low dietary Mg^{2+} content reduced mRNA expression of *Slc41a1*, however did this not attain statistical significance (Fig. 4E, 1.23 ± 0.07 vs. 0.87 ± 0.09 relative fold change in NormalMg-HFD vs. LowMg-HFD, respectively, $p=0.06$).

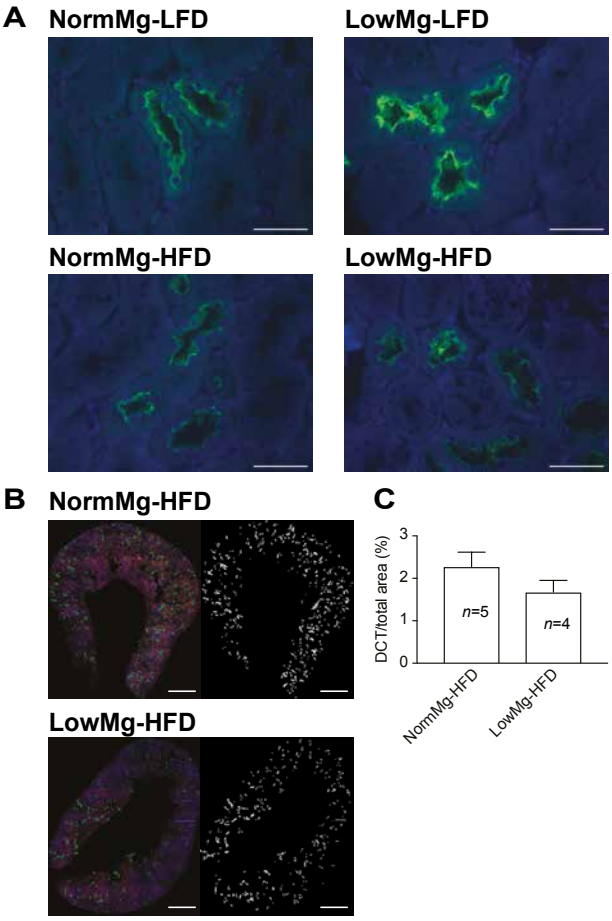

Figure 3 | Characterizing the effects of the dietary interventions on DCT differentiation and length

(**A**) Representative images of NCC-stained kidney slices indicating apical expression of NCC (scale bars, 50 μm). NCC: green, DAPI: blue. (**B**) Representative images of NCC- and BCRP-stained whole-kidney slices (left) and the image after automatic segmentation of the NCC-channel in the cortex area (right, scale bars, 1 mm). NCC: green, BCRP: red, DAPI: blue. (**C**) Total percentage of NCC-stained area corrected for total kidney cortex area. Data are mean ± SEM.

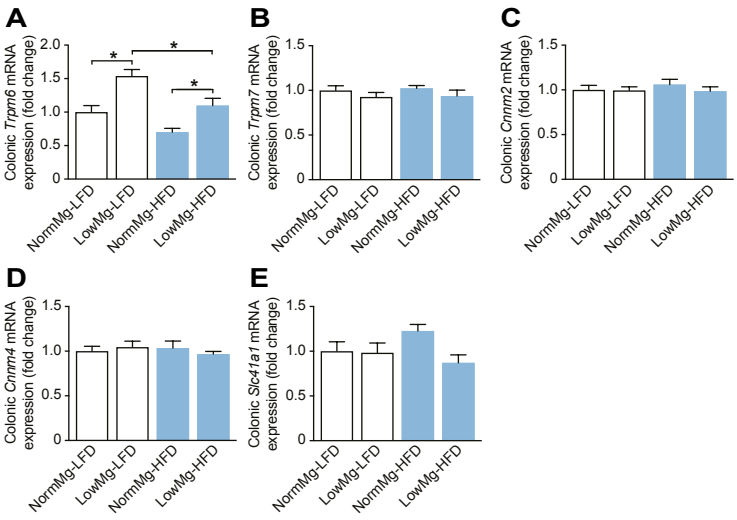

Figure 4 | A HFD reduces mRNA expression of *Trpm6* in distal colon

(**A-E**) Colonic mRNA expression of (**A**) *Trpm6*, (**B**) *Trpm7*, (**C**) *Cnnm2*, (**D**) *Cnnm4* and (**E**) *Slc41a1* normalized to *Gapdh* expression, relative to NormalMg-LFD. Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates $p \leq 0.05$.

Magnesium deficient high-fat-diet-fed mice develop proximal tubule phospholipidosis

To further assess the impact of the dietary interventions on the kidney, several histological stainings were performed. H&E staining of the kidneys revealed massive vesicle-like structures in the proximal tubules of the LowMg-HFD mice (Fig. 5A). This phenomenon was not observed in the other animal groups. To further characterize these vesicle accumulations, kidneys were stained with Oil Red O, staining neutral lipids and with periodic acid-Schiff, which stains polysaccharides (Supplementary Fig. 2A,B). However, both detections were negative. Toluidine blue staining revealed the presence of acidic components in the proximal tubule of the LowMg-HFD fed mice (Fig. 5B). Electron microscopy (EM) demonstrated multi-lamellar onion-like structures, located specifically in the proximal tubule cells, which exceeded the size of mitochondria, indicative of advanced proximal tubule phospholipidosis (Fig. 5C). In Mg^{2+}-deficient HFD mice kidney injury molecule 1 (*Kim1*) mRNA expression was upregulated compared to LowMg-LFD mice (Supplementary Fig. 3A).

We hypothesized that the accumulation of phospholipids could be due to a disturbed breakdown of phospholipids or sphingolipids. LowMg-HFD mice had lower mRNA expression of phospholipase C isoform beta 1 (*Plcb1,* Fig. 6A), essential

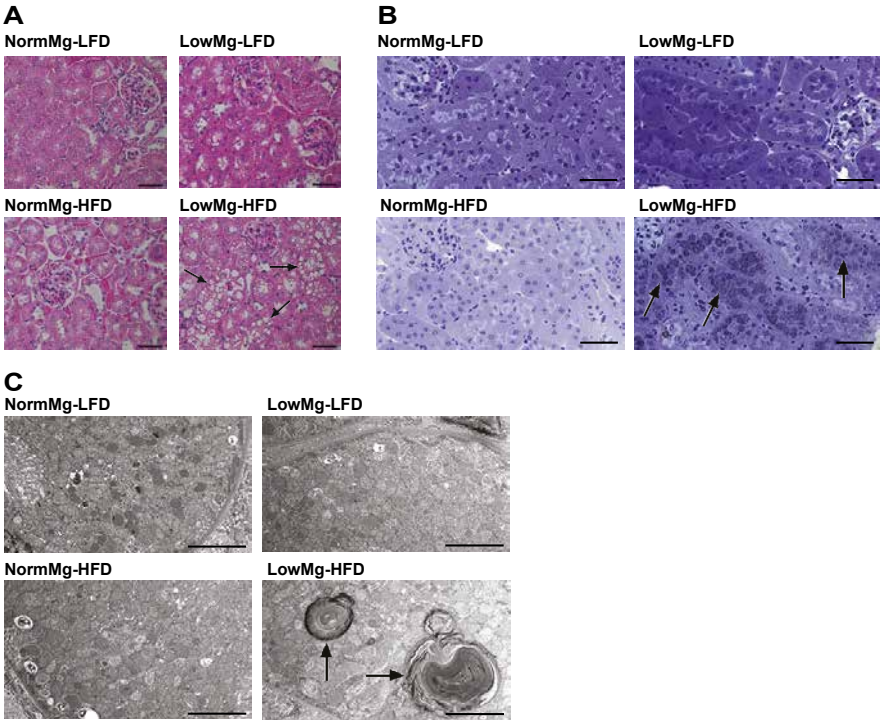

Figure 5 | LowMg-HFD fed mice develop proximal tubule phospholipidosis

(**A**) Representative images of H&E stained kidney slides (scale bars, 50 μm). (**B**) Representative images of Toluidine-blue stained kidney slides (scale bars, 50 μm). (**C**) Representative images of the proximal tubule cells by electron microscopy (scale bars, 5 μm). Black arrows indicate the vesicles.

for phospholipid breakdown, compared to NormalMg-HFD mice. There was no significant reduction in phospholipase C isoforms gamma 1 and 2 (*Plcg1* and *Plcg2*, Supplementary Fig. 3B-C). Moreover, there were no differences in expression of enzymes that catabolize sphingomyelin, sphingomyelin phosphodiesterase 1 (*Smpd1*), *Smpd2* and *Smpdl3a,* nor in the expression of the enzyme that catabolizes ceramides, alpha galactosidase (*Gla*, Supplementary Fig. 3D-G).

Magnesium deficiency alters renal energy metabolism

As LowMg-HFD mice developed renal phospholipidosis, we examined whether this was associated with changes in renal energy metabolism. In both low Mg^{2+} fed groups a striking increase was observed in mRNA expression of phosphoenolpyruvate

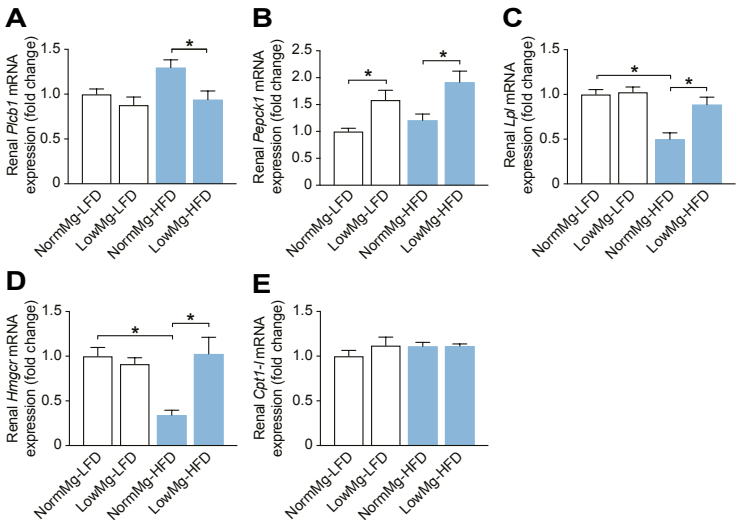

Figure 6 | Mg^{2+} deficiency induces renal metabolic changes on mRNA level

(**A-E**) Renal mRNA expression of (**A**) *Plcb1,* (**B**) *Pepck1*, (**C**) *Lpl*, (**D**) *Hmgcr* and (**E**) *Cpt1-l.* Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates $p \leq 0.05$.

carboxykinase 1 (*Pepck1*), the rate-limiting enzyme in gluconeogenesis, which is predominantly expressed in the proximal tubule (Fig. 6B) (20). mRNA levels of lipoprotein lipase (*Lpl*), which hydrolyses triglycerides in lipoprotein particles, was elevated in LowMg-HFD compared to NormalMg-HFD fed mice (Fig. 6C). Moreover, mRNA expression of HMG-CoA reductase (*Hmgcr*), the rate-limiting enzyme in cholesterol synthesis and the target of statin drugs, was also enhanced (Fig. 6D). Mitochondrial long chain fatty acid oxidation was equal between all groups, indicated by similar mRNA expression of carnitine palmitoyltransferase 1 (*Cpt1-l*), the rate-controlling enzyme in this process (Fig. 6E).

Discussion

Hypomagnesemia is a commonly observed electrolyte disorder in T2D patients. In this study we demonstrate that HFD-feeding causes severe hypomagnesemia when dietary Mg^{2+} intake is low. The LowMg-HFD reduced colonic and renal *Trpm6* mRNA expression and resulted in a diminished mRNA expression of DCT-specific genes. Moreover, the Mg^{2+}-deficient HFD caused severe proximal tubule phospholipidosis.

This was accompanied by alterations in renal energy metabolism, including enhanced gluconeogenesis and cholesterol synthesis.

In mice on a low Mg^{2+} diet, HFD feeding induced hypomagnesemia, which was observed within 4 weeks on the diet, indicating that hypomagnesemia is a consequence of T2D-related factors. Importantly, the HFD did not affect serum or urinary Na^{+} and K^{+} levels, which corresponds with the normal plasma distribution of these cations in T2D patients (1). The HFD induced hypomagnesemia in the mice on a Mg^{2+}-deficient diet, but not in a normomagnesemic diet. The Mg^{2+} content of the normal Mg^{2+} diet (0.21% w/w) is much higher than the minimal dietary Mg^{2+} requirement for mice (0.05% w/w) (21). Therefore, it is likely that high dietary Mg^{2+} intake in these animals prevents the HFD-induced hypomagnesemia.

HFD-induced hypomagnesemia has been previously reported, but the molecular basis for this Mg^{2+} deficiency was not further explored (22). In our study, despite the absence of fecal or urinary Mg^{2+} wasting, colonic and renal *Trpm6* mRNA expression were reduced in LowMg-HFD-fed mice. Moreover, in kidneys of the LowMg-HFD mice a lower mRNA expression of other DCT-specific genes such as *Slc12a3*, *Pvalb* and *Klk1,* was observed (20). *Klk1* is a serine protease that is not directly involved in Mg^{2+} or Ca^{2+} handling, thus the reduced expression of *Klk1* suggests dedifferentiation or shortening of the DCT segment. As NCC was normally present on the apical membrane, dedifferentiation of DCT cell was ruled out. Immunohistochemistry on whole-kidney slides indicated a non-significant reduction in DCT-area. Atrophy of the DCT segment has been previously described after thiazide use, which blocks NCC activity and also results in hypomagnesemia (23). However, we are the first to show effects on the DCT in response to LowMg-HFD feeding.

The hypomagnesemia observed in the HFD-fed mice is irrespective of urinary Mg^{2+} wasting, which differs from the excessive urinary Mg^{2+} wasting in T2D patients (1, 24, 25). In our study the urinary measurements were performed after 16 weeks on the diets. Possibly, the urinary Mg^{2+} wasting occurred during the initial phase. Moreover, the reduced 24h urinary Mg^{2+} loss after is also partially explained by the lower serum Mg^{2+} levels of the HFD-fed animals.

Feeding mice a Mg^{2+}-deficient HFD resulted in proximal tubule phospholipidosis, a lysosomal storage disorder. In humans, phospholipidosis can be caused by the use of cationic amphiphilic drugs or it can be inherited, such as in Niemann-Pick or Fabry's disease (26-28). In these diseases the breakdown of sphingolipids is disturbed, leading to an excessive accumulation of lipids in the lysosomes. EM pictures of renal cells in models of these diseases appear identical to the EM pictures of our LowMg-HFD fed mice (Fig. 5C) (28, 29). On mRNA expression level, no differences were observed in the key genes underlying these diseases. Possibly, reduced *Plcb1* expression in the kidneys of LowMg-HFD mice could underlie the phospholipid accumulation. Inhibition of phospholipase C by cationic amphiphilic drugs, has been

shown to induce renal cortical phospholipidosis (30). Interestingly, phospholipase C requires Mg^{2+} to function (31, 32). Moreover, hypomagnesemic *Trpm6* knockout mice have elevated serum and liver sphingolipid levels (19). How hypomagnesemia affects phospholipid and sphingolipid metabolism requires further investigation.

Numerous studies have investigated HFD-induced histological changes in the kidney. The reported histological findings include, but are not limited to, glomerulosclerosis, thickened basal membrane, neutral lipid accumulation and macrophage infiltration (33-35). Interestingly, only one study observes HFD-induced proximal tubule phospholipidosis (35). In our study, renal phospholipidosis by HFD-feeding exclusively occurs in the Mg^{2+}-deficient mice and not in the normomagnesemic mice, indicating a key role for Mg^{2+} in preventing the onset of renal phospholipidosis. Possibly, differences in dietary Mg^{2+} content explain the discrepancy between different studies in the observation of HFD-induced renal phospholipidosis.

Lower blood Mg^{2+} levels in T2D patients leads to a more rapid renal decline, but the underlying mechanism remains unclear (4). The increased renal decline was shown to be independent of glycemic control and blood pressure, which are currently the main targets in the treatment of diabetic nephropathy (4, 36, 37). Renal lipid accumulation could be a new possible mechanism explaining the more rapid decline in renal function caused by lower blood Mg^{2+} levels. Future studies should focus on unraveling the consequences of Mg^{2+} deficiency on renal energy metabolism in T2D patients.

In conclusion, we showed that when dietary Mg^{2+} intake is low, feeding mice a HFD induces severe hypomagnesemia in the absence of urinary or fecal Mg^{2+} wasting. The Mg^{2+} deficiency lead to renal phospholipidosis in HFD-fed mice, whereas the HFD did not induce these effects in normomagnesemic conditions. Renal lipid accumulation should be explored as a potential novel factor influencing renal function in hypomagnesemic T2D patients. T2D patients are at risk for hypomagnesemia, and in the hypomagnesemic T2D patients, correcting blood Mg^{2+} levels could be beneficial to preserve kidney function.

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Materials and methods

Mouse study

This 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). 48 male C57BL6/J mice (Charles River, Germany) were allocated randomly into four groups of mice (n=12 per group) (38). Mice, obtained at 9-10 weeks of age, were acclimatized for two weeks in a temperature and light controlled room, six mice per cage (Eurostandard Type III). Mice had access to acidified drinking water and standard pellet chow *ad libitum* (Ssniff Spezialdiäten, GmbH, Germany). Experimental diets consisted of 10 or 60 kcal% palm oil and 0.03 or 0.21% (wt./wt.) magnesiumoxide (Ssniff Spezialdiäten, NormalMg-LFD #S9074-E0277, LowMg-LFD #S9074-E0287, NormalMg-HFD #S9074-E0297, LowMg-HFD #S9074-E0317). Researchers and animal caretakers were blinded for the content of Mg^{2+} in the experimental diets throughout the entire duration of the experiment. At day -1, 84 and 112 mice were housed individually in metabolic cages for 24 hours for the collection of urine and feces and the determination of food and water intake. Blood was collected *via* sub-mandibular vein puncture at day -1, 28, 56 and 84 and collected in Microvette tubes (Sarstedt, Germany). After coagulation samples were spun down at 3,500 g for 5 minutes and serum was collected. One mouse died unrelated to the dietary intervention and was excluded from future analyses. After 17 weeks on the diets, mice were anaesthetized by 4% v/v isoflurane and exsanguinated *via* orbital sinus bleeding. Death was confirmed by cervical dislocation. Colon was cleaned with PBS and cut into a proximal, transversal and distal part. The renal capsule was removed and colon and kidney were subsequently stored in 10% (vol./vol.) formalin or snap frozen in liquid nitrogen for future analyses.

RT-qPCR

Total RNA was extracted from half of the kidney and from the distal part of the colon using TRIzol reagent (Invitrogen, UK) according to manufacturer's protocol. The isolated RNA underwent DNase (Promega, USA) treatment and subsequently RNA concentrations were determined using the Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). RNA was reversed transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, The Netherlands). Gene expression levels were quantified by SYBR-Green (BioRad, USA) on a CFX96 real-time PCR detection system (BioRad, USA) and normalized for *Gapdh* expression. Primer sequences are provided in Table 1.

Table 1 | RT-qPCR primer sequences

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Cldn10b	GGAGTTCCCCTCCATGCT	GCAAAAATGGAACCGAAAAA
Cldn14	GTCCAGCTCCTAGGCTTCCT	CATCCACAGTCCCTTCAGGT
Cldn16	GTTGCAGGGACCACATTAC	GAGGAGCGTTCGACGTAAAC
Cldn19	GGTTCCTTTCTCTGCTGCAC	CGGGCAACTTAACAACAGG
Cpt1-l	GTGAGCCTGGCCTCGCC	TGAGTGGTGACCGAGTCTGC
Cnnm2v1	GTCTCGCACCTTTGTTGTCA	GTCGCTCCGACTGAGAGAAT
Cnnm4	TCTGGGCCAGTATGTCTCTG	CACAGCCATCGAAGGTAGG
Egf	GAGAATCTACTGGACAGACAGTGG	CTCGAGATTCTCTCCTGGATG
Gapdh	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC
Gla	CTGATGCCTGCATAAGTGAGC	TGCCCTTTGAATCCCTCTCG
Hnf1-β	CAAGATGTCAGGAGTGCGCTAC	CTGGTCACCATGGCACTGTTAC
Hmgcr	TGCGTAAGCGCAGTTCCTTC	TCACAGTCCTGGATCCTCC
Kim-1	GGAAGTAAAGGGGGTAGTGGG	AAGCAGAAGATGGGCATTGC
Klk1	ACCTCAAGCTCCTGCCTAATG	GTGGGCCTCCGCACAAG
Lpl	ATTTGCCCTAAGGACCCCTG	ATTCCCGTTACCGTCCATCC
Mrs2	TCCTGTGCCCCCTGTGATGAC	TCCTTCCGGCCTGAGGCTGTT
Slc12a3	CTTCGGCCACTGGCATTCTG	GATGGCAAGGTAGGAGATGG
Pepck1	CCTAGTGCCTGTGGGAAGAC	AGCCCTTAAGTTGCCTTGGG
Plcb1	CTTGCAACTCAAGCCCGTG	TGGAGTCATCATCCCACTTGAC
Plcg1	GCAAATTGAGAGGTGGCTCC	TCGGAGGAAGCGCATATTGG
Plcg2	GTATGATCCGATGCCCCTGG	CGTGCACCAAGAACCTTGAC
Pvalb	CGCTGAGGACATCAAGAAGG	AGCTTTCAGCCACCAGAGTG
Slc41a1	CATCCCACACGCCTTCCTGC	CGGCTGGCCTGCACAGCCAC
Smpd1	AGAGCACTCCTGTCAATGGC	AGCCCCCAATTCTTAGGGTG
Smpd2	ACAGGGCAATAAGCCTGTGC	GGGATGTCCCAGCAGTTGAG
Smpdl3a	CTAAAGGCGCAAATGCCTCC	ATGAGGTGGGCTATCCCCTG
Trpm6	CTTCACAATGAAAACCTGCCC	AAAGCCATGCGAGTTATCAGC
Trpm7	GGTTCCTCCTGTGGTGCCTT	CCCCATGTCGTCTCTGTCGT

Histology

Kidney tissue was fixed in 10% vol./vol. neutral-buffered formalin (KLINIPATH, the Netherlands) in PBS for 24 hours. Samples were dehydrated through alcohol, embedded in paraffin and cut into 4 μm sections. Sections were stained with H&E or Periodic acid Schiff (PAS) staining using standard procedures. For immunohistochemistry of the kidney sections, deparaffinization and rehydration were performed, followed by antigen retrieval using a citrate buffer at pH 6.0. Sections were blocked by a 1% wt./vol. BSA in PBS and incubated overnight at 4 °C with the primary antibodies rabbit anti-NCC (1:200, Millipore) and rat anti-BCRP (1:100, Kamiya

Biomedical Company). For detection, kidney sections were incubated for 2 hours at room temperature with Alexa-conjugated secondary antibody (1:300, Invitrogen - Molecular Probes). Images were taken using an AxioCam camera and ZEN lite software (Zeiss, Sliedrecht, the Netherlands). For the whole-slide immunohistochemistry, a Leica DMI6000 confocal microscope and LAS AF software were used. The NCC-stained area in the whole-kidney slices (Fig. 7A) was determined using a script. The analysis was performed on the annotated cortical area of the whole-kidney slices, with stain artifacts removed (Fig. 7B). First, the NCC-channel in the annotated cortical area was isolated from the picture (Fig. 7C). Otsu's method was used to automatically determine a suitable NCC-channel intensity threshold (39). An empirically chosen intensity threshold of 67 was used in cases where the automatic threshold was determined to be insufficient based on visual inspection. A closing operation was performed on the thresholded image, using a square structuring element with size ten. Components with a pixel size smaller than 200 were removed, resulting in the final image (Fig. 7D). Finally, the percentage of NCC-stained area was determined by dividing the pixels in the final image by the pixels in the annotated area. The researcher performing these analyses was blinded for the mice groups during all the steps of

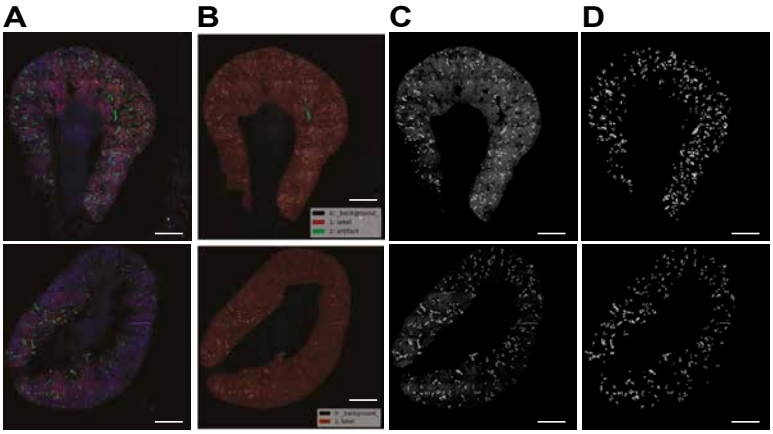

Figure 7 | Approach for automated segmentation of the NCC-stained tubuli

(**A**) Representative images from immunohistochemistry stainings of whole-kidney (scale bars, 1 mm). DAPI: blue, BCRP: red, NCC: green. (**B**) Immunohistochemistry stained image overlaid with handcrafted annotations. The area annotated with red ('1: label') was exclusively considered when calculating the NCC-stained area. (**C**) Image obtained by isolating the NCC-channel from the original RGB image. (**D**) Resulting segmentation of the green channel after applying the threshold, performing the closing operation and removing small (noisy) components.

analysis. The code for segmentation was written in Python (RRID:SCR_008394), utilizing the Scikit-image and OpenCV packages.

For the Oil Red O staining, kidney samples were snap frozen in liquid nitrogen and cut into 10 μm sections, stained with Oil Red O (Sigma-Aldrich, Missouri, USA) and counterstained with hematoxylin. For electron microscopy, we used immersion fixation. Small fragments of cortex were fixed in 2.5% wt./vol. glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C and washed in the same buffer. The tissue fragments were postfixed in palade-buffered 2 w/v% OsO4 for 1 h, dehydrated, and embedded in Epon812, Luft's procedure (Merck, Darmstadt, Germany). Semi-thin sections (1 μm) were stained with Toluidine blue 1% wt./vol. (metachromatic staining). Ultrathin sections were contrasted with 4% wt./vol. uranyl acetate for 45 min and subsequently with lead citrate for 5 min at room temperature. Sections were examined using a Jeol 1400 electron microscope (JEOL, Tokyo, Japan).

Analytical procedures

Serum, urinary and fecal Mg^{2+} concentrations were determined using a spectrophotometric assay (Roche/Hitachi, Tokyo, Japan) according to the manufacturer's protocol. The Ca^{2+} concentration was measured by the *o*-cresophthalein complexone method. Absorbance was measured using a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad laboratories, California, USA). Serum and urinary Na^{+} and K^{+} concentrations were measured by the clinical chemistry department of the Radboudumc using standardized methods.

Quantification and statistical analysis

To determine whether there was a significant interaction effect between the two main variables (dietary fat and Mg^{2+} content), a two-way ANOVA was used. If there was no significant interaction effect, significant differences between the groups were assessed using a two-way ANOVA approach with Tukey's multiple comparisons test. If the two-way ANOVA demonstrated a significant interaction effect, a multiple *t*-test approach using the Holm-Sidak correction for multiple comparisons was used. To determine statistical significance in the NCC-stained area of whole-kidney slices, an unpaired Student's *t*-test was used. Statistical significance was assessed using Graphpad Prism v7 (California, USA, RRID: SCR_002798). Differences with a *p*-value of ≤ 0.05 were considered statistically significant. Results are presented as mean $\pm$ standard error of the mean (SEM).

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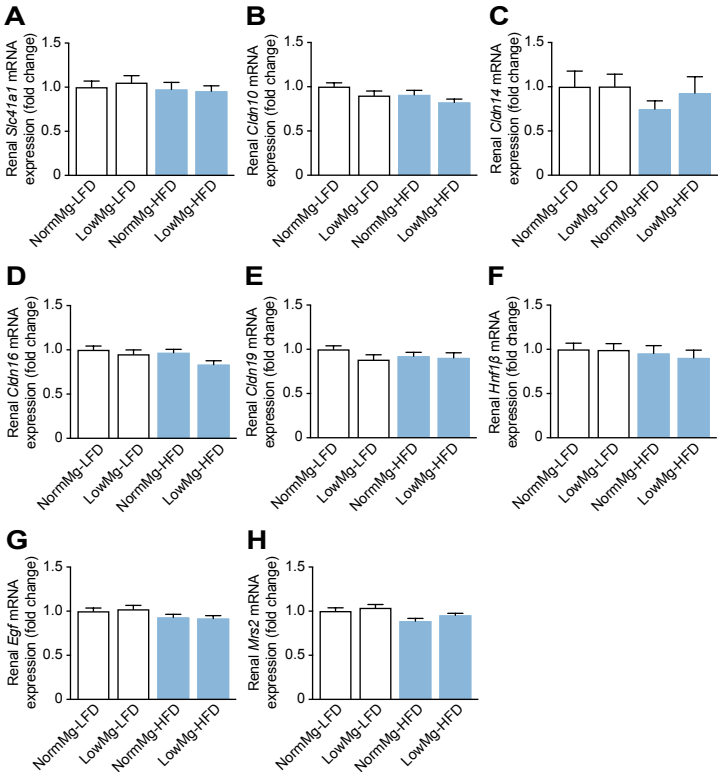
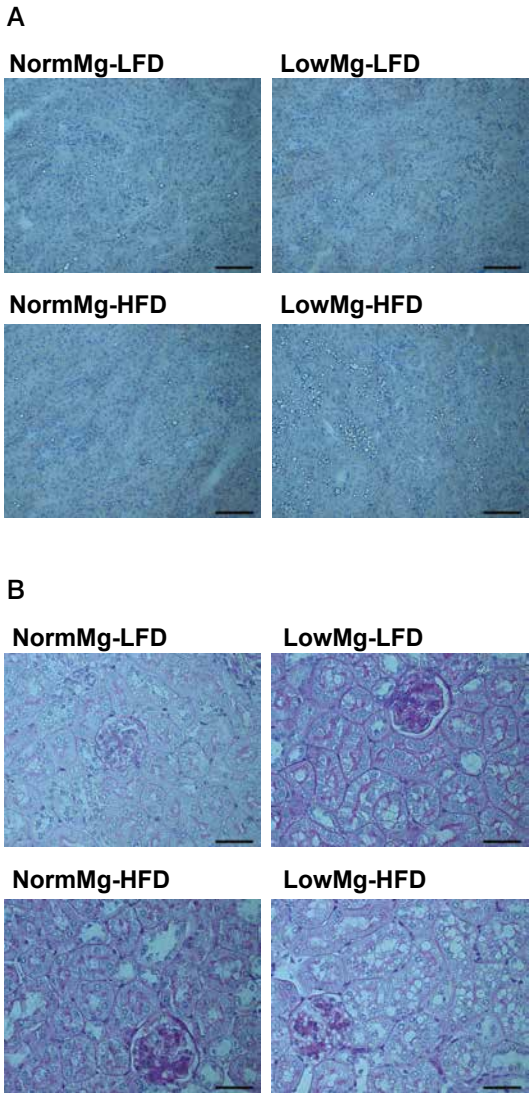
Supplementary data



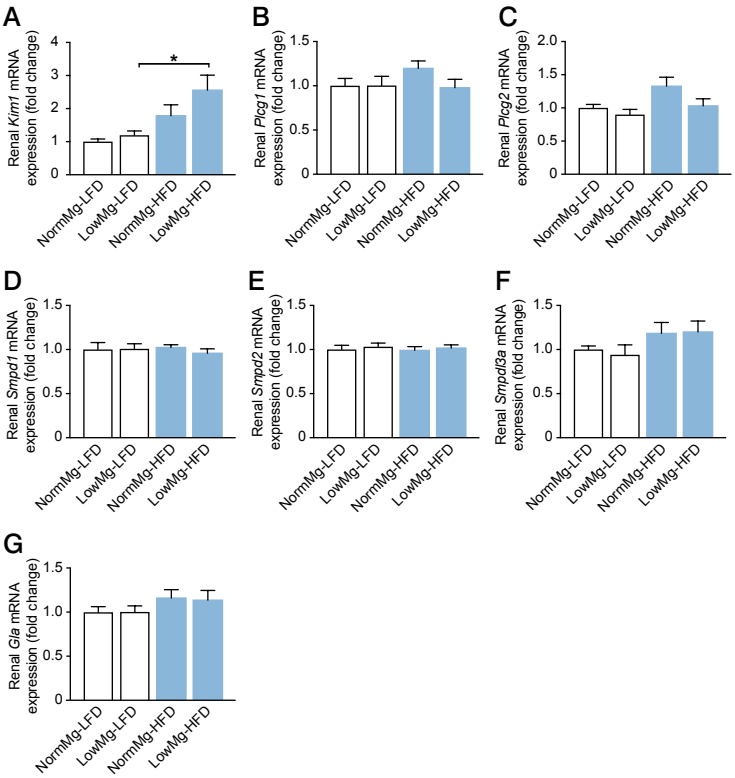
Supplementary figure 1 | No differences in renal mRNA expression of other magnesiotropic genes between any of the groups

(**A-H**) Renal mRNA expression of (**A**) *Slc41a1*, (**B**) *Cldn10*, (**C**) *Cldn14*, (**D**) *Cldn16*, (**E**) *Cldn19*, (**F**) *Hnf1β*, (**G**) *Egf* and (**H**) *Mrs2* normalized to *Gapdh* expression, relative to NormalMg-LFD. Two-way ANOVA for *Cldn10*, *Egf* and *Mrs2* indicates a significant dietary calorie effect, $p \leq 0.05$. Two-way ANOVA for *Cldn16* indicates a significant dietary Mg^{2+} effect, $p \leq 0.05$. Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates a $p \leq 0.05$.



Supplementary figure 2 | Proximal tubule vesicles are negative for PAS and Oil Red O staining

(**A**) Representative images of Oil Red O stained kidney slides (scale bars, 100 μm). (**B**) Representative images of PAS stained kidney slides (scale bars, 50 μm).



Supplementary figure 3 | Renal mRNA expression differences of genes involved in metabolism.

(**A-G**) Renal mRNA expression of (**A**) *Kim-1*, (**B**) *Plcg1*, (**C**) *Plcg2*, (**D**) *Smpd1,* (**E**) *Smpd2*, (**F**) *Smpdl3a* and (**G**) *Gla*. Two-way ANOVA for *Plcg2* indicates a significant dietary Mg^{2+} and calorie effect, $p \leq 0.05$. Two-way ANOVA for *Smpdl3a* indicates a significant dietary calorie effect, $p \leq 0.05$. Open bars, LFD. Filled bars, HFD. Data are mean ± SEM, * indicates a $p \leq 0.05$.

"Hope clouds observation"

– Frank Herbert | Dune

5

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

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Abstract

In T2D patients, treatment with metformin is associated with reduced blood Mg^{2+} levels. However, the mechanism underlying this phenomenon remains to be elucidated. Therefore, we investigated how T2D and metformin affect Mg^{2+} homeostasis.

In this study, twenty diabetic db/db mice and twenty control db/m mice were treated with metformin or placebo for four weeks. Db/m mice had a considerably lower body weight than db/db mice, which was not affected by metformin-treatment. Metformin attenuated the glycosuria of the db/db mice. 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, using genetically modified mice, we show that T2D causes hypomagnesemia. Moreover, metformin treatment has no effect on Mg^{2+} homeostasis in mice.

Keywords: Colon; db/db; kidney; magnesium; metformin; type 2 diabetes.

Introduction

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). However, the etiology and underlying mechanisms of hypomagnesemia in T2D patients remains largely unknown (5).

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 transcription (6).

The intestine and kidney collaboratively regulate Mg^{2+} balance and maintain its blood concentrations within a narrow range (7, 8). In the gut, the bulk of Mg^{2+} absorption occurs in the small intestine *via* paracellular (passive) transport (7). 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 channels (6). In the kidney, 95-99% of filtered Mg^{2+} is reabsorbed under physiological circumstances (6). 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 (9, 10). 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 reabsorbed (11). In physiological conditions, the DCT reclaims 5-10% of filtered Mg^{2+} transcellularly via TRPM6/7 channels (10, 12). 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) (10, 13-16). SNPs in TRPM6 that impair its response to insulin have been associated with an increased risk of developing T2D and gestational diabetes (15, 17).

Metformin, the first-line pharmacotherapy in T2D (18), suppresses hepatic gluconeogenesis and improves insulin sensitivity (19). Therefore, its major clinical benefit is reducing blood glucose levels with only a minimal risk of hypoglycemia (20, 21). The most common side effects of metformin treatment are lactic acidosis, nausea and diarrhea (22). Recent cohort studies showed that metformin use in T2D patients is associated with reduced blood Mg^{2+} levels (1, 23). However, the mechanism that underlies this correlation has not yet been elucidated. To investigate how T2D and metformin affect Mg^{2+} 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.

Results

Metformin reduces food intake of db/db mice without affecting body weight

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, respectively, at four weeks, $p \leq 0.05$). Metformin treatment had no effect on body weight in both db/m and db/db mice (Fig. 1A,B).

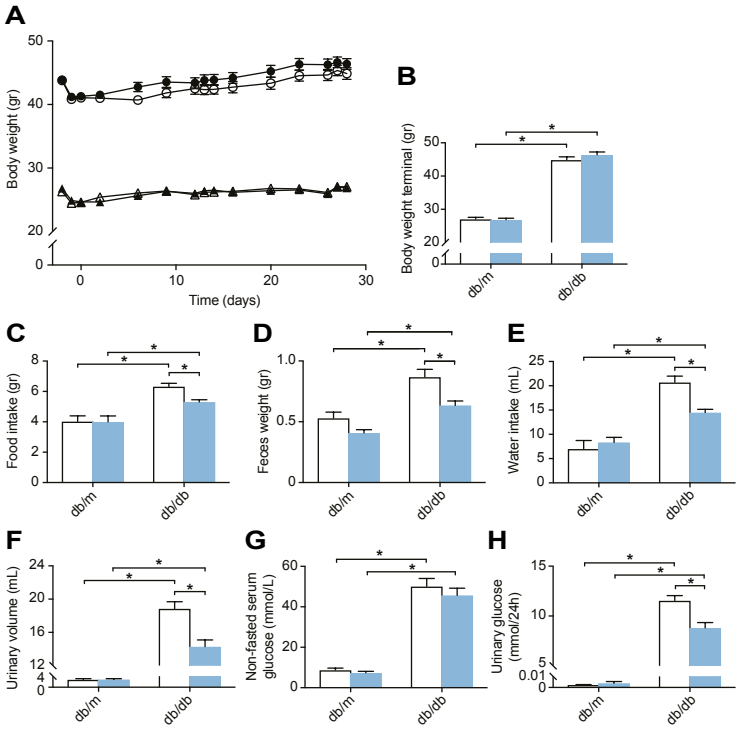

Figure 1 | Metformin treatment does not affect body weight, but reduces food intake and urinary glucose excretion in db/db mice

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. * indicates a $p \leq 0.05$.

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 magnesium 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 μmol/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 enhanced (11). 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).

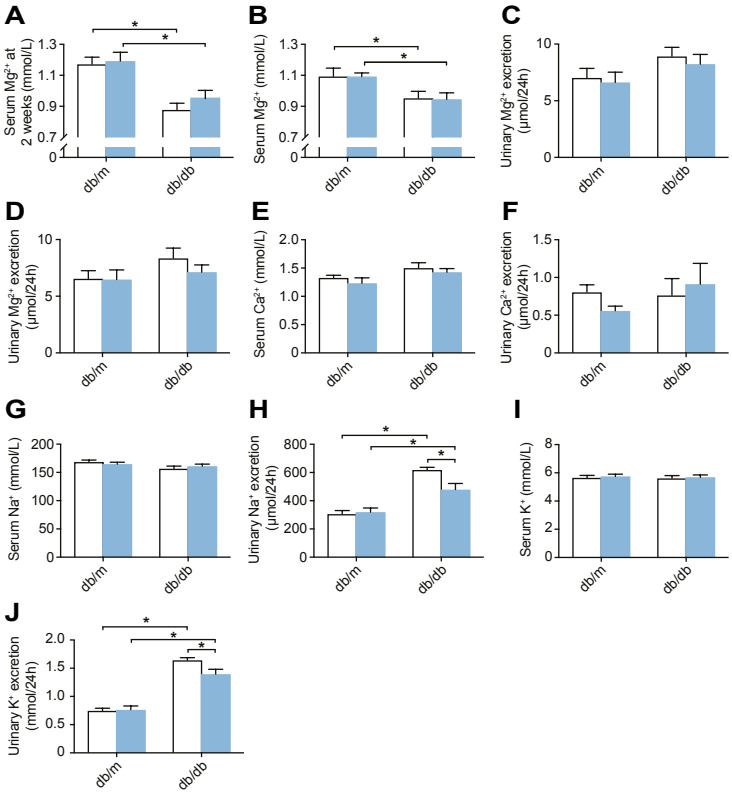

Figure 2 | Db/db mice have a lower serum Mg^{2+} concentration which is not modulated by metformin treatment

(**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/24h 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. * indicates a $p \leq 0.05$.

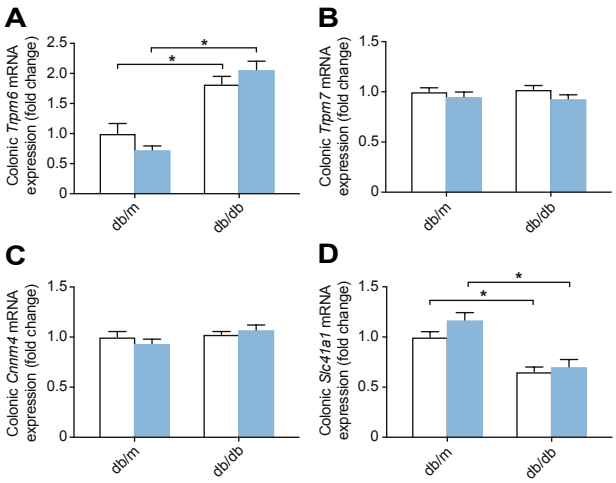

Figure 3 | Upregulation of *Trpm6* mRNA expression in the colon of db/db mice

Colonic mRNA expression of (**A**) *Trpm6*, (**B**) *Trpm7*, (**C**) *Cnnm4* and (**D**) *Slc41a1*. Open bars, placebo-treated mice; closed bars, metformin-treated mice. Data are mean ± SEM. * indicates a $p \leq 0.05$.

Db/db mice have an elevated renal expression of genes involved in magnesium 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).

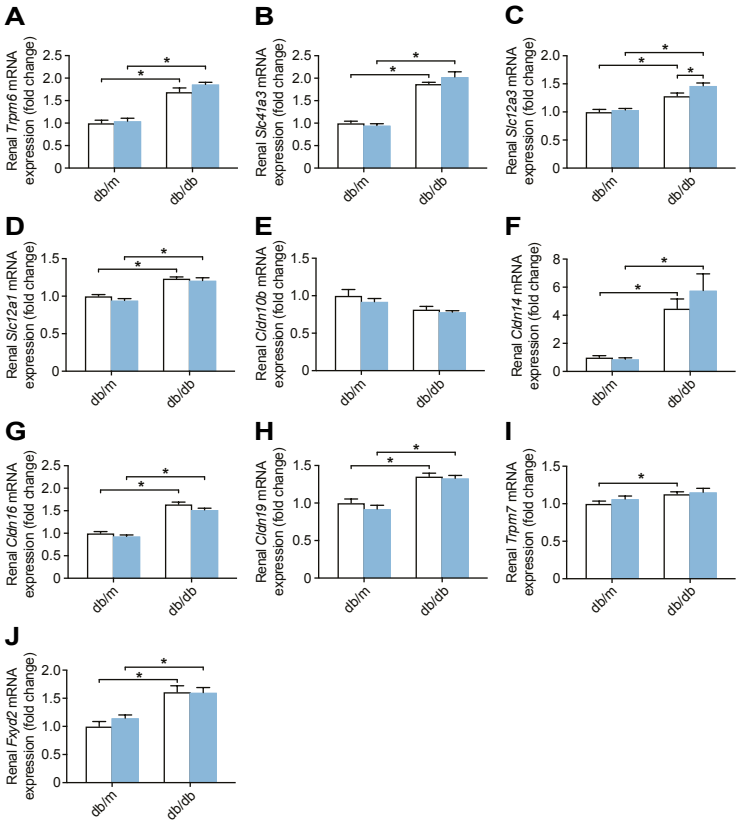

Figure 4 | Higher gene expression of essential magnesiotropic in db/db mice mRNA expression of genes involved in renal electrolyte handling

(**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 ± SEM. * indicates a $p \leq 0.05$.

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, 23). 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.

Metformin is the first-line therapy for T2D (24). 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, 23, 25-27). 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 (26). 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 (28). 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 (25).

Hypomagnesemia is prevalent in over 30% of T2D patients (29-32). A remaining question is whether hypomagnesemia is the cause or the consequence of T2D (5). In the present study, db/db mice developed hypomagnesemia, 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, 33). 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 (34, 35). Therefore, it is unlikely that glycosuria underlies hypermagnesuria-induced hypomagnesemia in T2D.

The kidneys are essential in maintaining the serum Mg^{2+} concentration within the physiological range (11). The DCT is the final segment where Mg^{2+} can be reabsorbed (6). In the DCT, regulated Mg^{2+} reabsorption takes place transcellularly *via* TRPM6 (14). Mg^{2+} uptake by TRPM6 is dependent on NCC, although the underlying mechanism remains largely unknown (36, 37). 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+} reabsorption (6). In the TAL, paracellular Mg^{2+} and Ca^{2+} reabsorption is regulated by the Cldn14/16/19 complex (38, 39). *Cldn14* mRNA expression is strongly regulated by dietary Ca^{2+} intake (40, 41). 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* expression (42). 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.

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. The expression of genes such as *Cldn10b*/*14*, *Slc12a1* and *Slc12a3* are regulated by both dietary intake and serum levels of K^{+}, Na^{+} and Ca^{2+} (37, 38, 43). 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, hypomagnesemia 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.

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Materials and 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). 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 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 synthetize 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 Mg^{2+} concentrations were determined using a spectrophotometric assay (Roche/Hitachi, Tokyo, Japan), according to manufacturer's protocol. Ca^{2+} concentrations were determined by the o-cresophthalein complexone method. Absorbance for the Mg^{2+} and Ca^{2+} 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 methods (1). 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.

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"One is always considered mad, when one discovers
something that others cannot grasp"

– Ed Wood

6

Direct binding to free fatty acid decreases blood magnesium in hypertriglyceridemic states

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Abstract

The blood triglyceride level is one of the main determinants of the blood magnesium (Mg^{2+}) concentration in patients with type 2 diabetes (T2D). Hypomagnesemia (blood Mg^{2+} concentration <0.7 mmol/L) has serious consequences as it increases the risk of developing T2D from a pre-diabetic state and accelerates the disease progression. This study aimed to determine by which mechanism triglyceride levels affect blood Mg^{2+} concentrations.

In a cohort of overweight subjects, serum Mg^{2+} levels inversely correlated with triglycerides incorporated in large very low-density lipoprotein particles. After lipid loading, we observed a post-prandial increase in plasma triglyceride and free fatty acid (FFA) levels and a reciprocal reduction in the blood Mg^{2+} concentration, both in mice and in healthy subjects. Further *in vitro* experiments revealed that the decrease of plasma Mg^{2+} is explained by direct binding of Mg^{2+} to FFAs. Moreover, the binding of Mg^{2+} to albumin is dependent on FFA, as Mg^{2+} did not bind to fatty-acid-free albumin. FFA-dependent reduction of the free Mg^{2+} concentration was not affected by the presence of physiological concentrations of other cations.

This study shows that elevated FFA and triglyceride levels directly reduce blood Mg^{2+} levels, in part explaining the high prevalence of hypomagnesemia in metabolic disorders. Our data challenge how the fractional excretion of magnesium is calculated and interpreted in the clinic.

Keywords: Albumin; free fatty acid; hypomagnesemia; hypertriglyceridemia; magnesium; magnesium deficiency; obesity; triglycerides.

Introduction

Hypomagnesemia (blood magnesium (Mg^{2+}) concentration <0.7 mmol/L) is commonly observed in patients with type 2 diabetes (T2D) or the metabolic syndrome (1-3) and can result in general complaints such as fatigue, headache and weakness (4, 5). Low oral Mg^{2+} intake and low blood Mg^{2+} levels increase the risk of developing T2D, but also accelerate the disease progression (6-8). A reduced blood Mg^{2+} is also associated with more diabetes-related complications such as cardiovascular disease and renal failure (9-12).

Blood Mg^{2+} levels are carefully maintained between 0.7 and 1.1 mmol/L by the interplay of the intestine, bone and kidney (13). In blood, approximately 27% of Mg^{2+} is bound to albumin, 8% is complexed to anions such as phosphate, bicarbonate and citrate, leaving 65% as the free fraction, which is the biologically active form (14). Although albumin-binding has been known for decades, the buffering effect of albumin on the regulation of Mg^{2+} homeostasis has been largely neglected (15).

Blood fatty acid (FA) and triglyceride levels are largely regulated by four organs: the intestine, liver, muscle and adipose tissue. In the postprandial state, the intestine absorbs dietary lipids as FAs, which are re-esterified into triglycerides and incorporated into chylomicrons that reach metabolically active tissues *via* the circulation (16). The liver can also incorporate FAs into triglyceride and secrete these as very low-density lipoprotein (VLDL) particles, which is especially important during fasting (17). Skeletal muscle stores FAs in the form of triglycerides, but also consumes large amounts of FAs during exercise (18). White adipose tissue also stores FAs as triglycerides, which can be released by lipolysis as free fatty acids (FFA) during a state of energy deprivation (19, 20). In blood, these negatively charged FFAs are bound to carrier proteins, predominantly albumin, with a non-polar interaction (21). In physiological conditions, approximately two FFA molecules are bound to a single albumin molecule (22). However, in a state of hypertriglyceridemia up to seven FFA molecules are able to bind, but with sequentially lower binding constants (21, 22).

In metabolic diseases, high blood triglycerides are associated with a lower blood Mg^{2+} concentration, but the directionality of this correlation remains unclear (1, 23, 24). Severe hypomagnesemia in animals leads to increased blood triglyceride levels, possibly by disrupting the function of the enzyme lecithin-cholesterol acyltransferase or by activating lipolysis in adipose tissue (25, 26). However, whether triglycerides can affect Mg^{2+} homeostasis has not been investigated.

In this study, we measured serum Mg^{2+} concentrations and the plasma lipoprotein concentration and composition by use of a metabolomics platform in a cohort of overweight individuals (27). To further unravel the exact relationship between hypertriglyceridemia and Mg^{2+} levels, we combined the population-based cross-sectional study with *in vivo* oral lipids loads both in men and in mice and with subsequent *in vitro* experiments.

Results

Serum magnesium levels inversely correlate with plasma triglyceride levels in overweight individuals

Factors affecting blood Mg^{2+} levels were evaluated in 285 overweight human individuals from the 300-Obesity cohort, with a BMI >27 kg/m^2 (Table 1). The average serum Mg^{2+} concentration in this cohort was 0.89 ± 0.09 (SD) mmol/L with only 2% of the individuals having hypomagnesemia (serum Mg^{2+} <0.7 mmol/L, see Fig. 1 and Table 1). Despite the fairly normal serum Mg^{2+} levels in these subjects, serum Mg^{2+} and triglyceride levels, predominantly those in VLDL particles, were inversely correlated (Table 1). The serum Mg^{2+} concentration was also inversely correlated with the HOMA-IR. As the HOMA-IR is strongly correlated with the plasma triglyceride concentration (Supplementary tables 1-2), we questioned whether insulin resistance modulated the inverse correlation between Mg^{2+} and triglyceride, and triglycerides in VLDL particles. However, the HOMA-IR did not influence these correlations in multivariable regression analyses (Supplementary tables 3-4).

To further investigate the relationship between lipoproteins and the serum Mg^{2+} level, the composition of the lipoprotein particles was investigated and also correlated to the serum Mg^{2+} concentration (Table 1). The concentration of the larger VLDL particles showed the strongest inverse correlations to the serum Mg^{2+} levels (Table 1). Interestingly, the concentration of smaller HDL particles positively correlated to serum Mg^{2+}.

There was no correlation between the serum Mg^{2+} and any of the intermediate and low-density lipoprotein (IDL and LDL) particle concentrations (Table 1). No significant correlation was observed between serum Mg^{2+} and the diameter of the VLDL, LDL or HDL particles (Table 1).

Increased triglyceride levels directly reduce plasma magnesium concentrations in mice

To unravel the underlying mechanism that explains how blood triglycerides are associated with blood Mg^{2+} concentrations, mice were subjected to an oral gavage of olive oil following an overnight fast. Plasma triglycerides and FFA levels both peaked at four hours post-gavage (Fig. 2A,B).

Interestingly, the plasma Mg^{2+} concentration showed a reciprocal decrease and reached a nadir also at four hours post-gavage (Fig. 2A,B). At basal levels (t=0 hours) no significant correlation was observed between plasma Mg^{2+} levels and FFA concentrations (Fig. 2C, p=0.27). However, when plasma FFA levels increased (t=4 and 6 hours), there was a clear inverse correlation between Mg^{2+} and FFA concentrations (Fig. 2D,E, $p \leq 0.05$). When plasma FFA levels decreased and reached a concentration comparable to baseline (t=8 hours), there was no longer a significant correlation between plasma FFA and Mg^{2+} concentrations (Fig. 2F, p=0.54).

Table 1 | Mg^{2+} and triglycerides inversely correlate in overweight individuals

Univariate analyses for the correlation of demographics, laboratory analyses and lipoprotein particle concentration of overweight individuals (300-Obesity cohort) with the serum Mg^{2+} concentration.

Variable	Correlation Coefficient	*p*-value	*n*
Demographics			
Gender (m=0, f=1)	-0.051	0.391	285
BMI (kg/m^2)	-0.063	0.291	285
Age (years)	-0.099	0.096	285
Waist circumference (cm)	-0.071	0.231	285
SBP (mmHg)	-0.016	0.791	285
DBP (mmHg)	0.079	0.186	285
Heart rate (beats/min)	-0.083	0.164	282
Laboratory analyses			
Triglycerides (mmol/L)	-0.159	0.007	284
Glucose (mmol/L)	-0.062	0.299	284
HbA_{1c} (mmol/mol)	-0.032	0.595	284
HOMA-IR	-0.123	0.038	283
Total cholesterol (mmol/L)	0.041	0.495	284
Triglycerides in VLDL (mmol/L)	-0.158	0.008	284
Triglycerides in LDL (mmol/L)	-0.026	0.667	284
Triglycerides in HDL (mmol/L)	-0.052	0.380	284
Cholesterol in VLDL (mmol/L)	-0.093	0.118	284
Cholesterol in LDL (mmol/L)	0.075	0.205	284
Cholesterol in HDL (mmol/L)	0.116	0.050	284
ApoA1 (g/L)	0.071	0.235	283
ApoB (g/L)	-0.037	0.539	283
Mean diameter VLDL (nm)	-0.095	0.111	284
Mean diameter LDL (nm)	-0.030	0.612	284
Mean diameter HDL (nm)	0.026	0.320	284
Lipoprotein particle concentration			
Concentration of Chylomicrons & EL-VLDL	-0.170	0.004	284
Concentration of VL-VLDL	-0.174	0.003	284
Concentration of L-VLDL	-0.163	0.006	284
Concentration of M-VLDL	-0.149	0.012	284
Concentration of S-VLDL	-0.108	0.068	284
Concentration of VS-VLDL	-0.004	0.942	284
Concentration of IDL	0.058	0.333	284
Concentration of L-LDL	0.060	0.313	284
Concentration of M-LDL	0.059	0.319	284
Concentration of S-LDL	0.053	0.377	284

Table 1 | Continued.

Variable	Correlation Coefficient	*p*-value	*n*
Lipoprotein particle concentration			
Concentration of VL-HDL	0.020	0.739	284
Concentration of L-HDL	0.089	0.137	284
Concentration of M-HDL	0.163	0.006	284
Concentration of S-HDL	0.179	0.002	284

m, male; f, female; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; EL-VLDL, extra large VLDL; VL-VLDL, very large VLDL; L-VLDL, large VLDL; M-VLDL, medium VLDL; S-VLDL, small VLDL; VS-VLDL, very small VLDL; IDL, intermediate-density lipoprotein; L-LDL, large LDL; M-LDL, medium LDL; S-LDL, small LDL; VL-HDL, very large HDL; L-HDL, large HDL; M-HDL, medium HDL; S-HDL, small HDL.

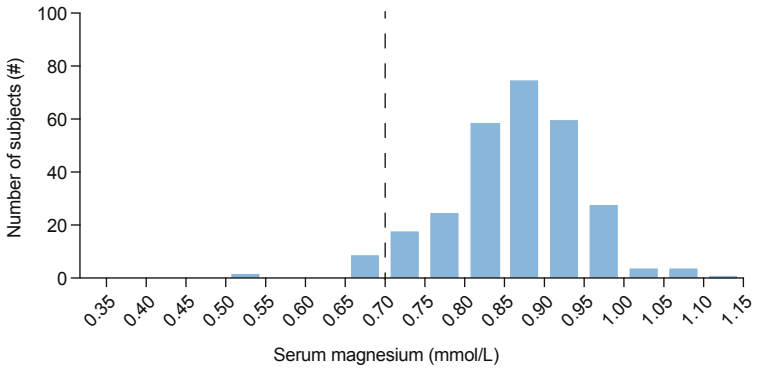

Figure 1 | The prevalence of hypomagnesemia is low in overweight subjects of the 300-Obesity cohort

Distribution of the serum Mg^{2+} concentration of overweight subjects of the 300-Obesity cohort. The dotted vertical line indicates the threshold for hypomagnesemia.

Similar correlations were observed between plasma Mg^{2+} and triglyceride levels (Supplementary Fig. 1A-D).

Increased blood lipids can enhance glucose-induced insulin secretion (28). Insulin is known to result in a compartmental shift of Mg^{2+}, decreasing blood concentration and increasing intracellular levels (29). To rule out insulin-dependent effects, the same oral gavage experiment was performed in inducible Kir6.2-p.Val59Met mice, which develop hypoinsulinemia and hyperglycemia (30). A similar reduction in plasma Mg^{2+} was observed in response to the oral gavage of olive oil. While this reduction was numerically similar, it did not reach statistical significance due to a high variation between the animals, which was substantially larger than in the initial experiment (Fig. 2G). However, a significant inverse correlation between plasma Mg^{2+} and triglycerides was still observed in these hypoinsulinemic animals (Fig. 2H, $p \leq 0.05$).

Binding of magnesium to albumin is dependent on free fatty acids

As ~30% of Mg^{2+} is bound to albumin, the predominant carrier of FFAs, we investigated whether the binding of Mg^{2+} to albumin is dependent on FFAs (14). The Mg^{2+} concentration declined over increasing levels of bovine serum albumin (BSA) dissolved in a $MgCl_2$ solution (Fig. 3A). At a near-physiological concentration of BSA (0.5 mmol/L or 33.25 g/L) Mg^{2+} concentrations decreased from 0.85 ± 0.02 to 0.64 ± 0.01 mmol/L (25% reduction, Fig. 3A). Interestingly, dissolving fatty-acid-free-BSA (FF-BSA) in a $MgCl_2$ solution abrogated this effect, in line with binding of Mg^{2+} to albumin being FFA-dependent (Fig. 3B). Linear regression analyses showed a significant inverse correlation between Mg^{2+} and BSA, of which the regression constant was approximately four times stronger for BSA than for FF-BSA (Fig. 3C). To exclude that other cations present in blood compete with the binding of Mg^{2+} to BSA, BSA was dissolved in a physiological buffer, which mimicked the concentration of other abundant blood electrolytes. This approach resulted in a similar decrease in the Mg^{2+} concentration (Fig. 3D). Again, the FF-BSA had no significant effect on Mg^{2+} levels (Fig. 3E). In the physiological buffer, BSA and FF-BSA displayed similar correlations compared to the $MgCl_2$ buffer (Fig. 3F).

Free fatty acids directly decrease magnesium concentrations

We then set out to modify the binding of Mg^{2+} to albumin by increasing the concentration of FFA. Elevating the concentration of FFA in a BSA solution directly reduced the Mg^{2+} concentration (linear regression: $y=-0.12x + 0.83$, $p \leq 0.05$, Fig. 4A). To resemble the *in vivo* setting, the experiment was repeated using fetal bovine serum (FBS) instead of BSA. Increasing the FFA levels in FBS reduced Mg^{2+} concentrations to a similar extent (linear regression: $y=-0.10x + 1.36$, $p \leq 0.05$, Fig. 4B). Interestingly, the reduction in the Mg^{2+} concentration is protein-independent, as increasing the

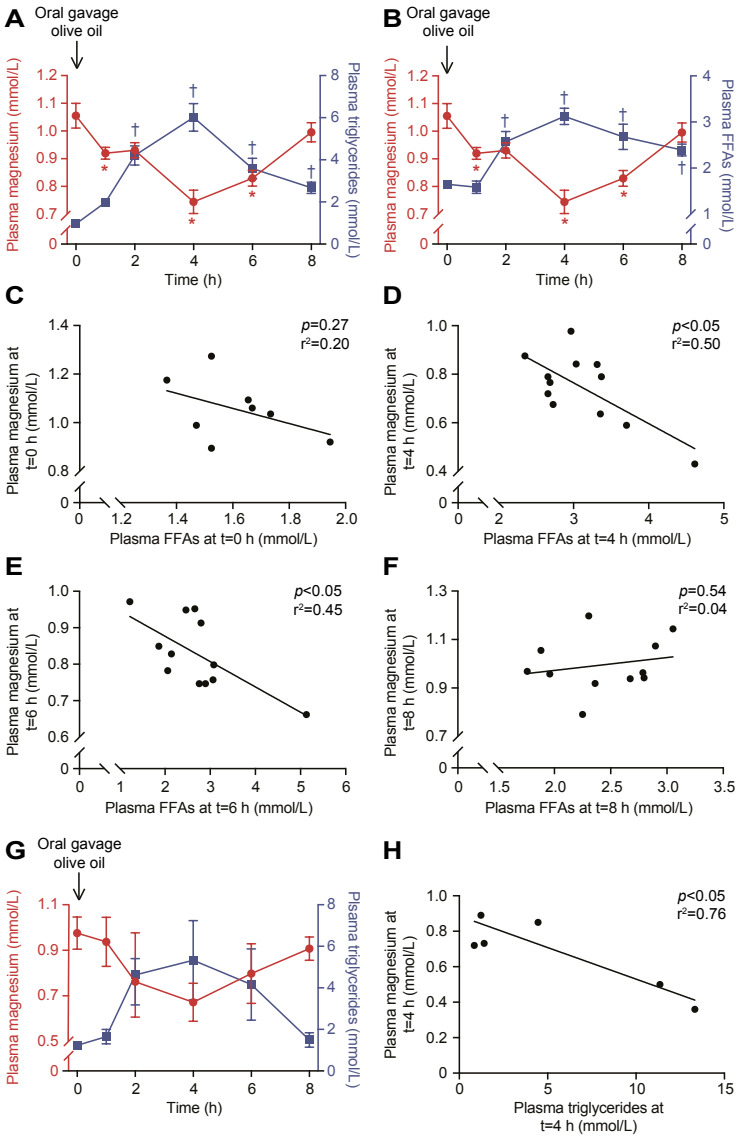

Figure 2 | Increased plasma FFA and triglyceride levels directly reduce the plasma Mg^{2+} concentration in mice

Figure 2 | Continued.

(**A**) Plasma Mg^{2+} (circles, left y-axis) and triglyceride (squares, right y-axis) concentrations before (t=0) and at 1, 2, 4, 6 and 8 hours after an oral gavage of 200 μL olive oil in wild-type mice (n=12). (**B**) Plasma Mg^{2+} (circles, left y-axis) and FFA (squares, right y-axis) concentrations before (t=0) and at 1, 2, 4, 6 and 8 hours post-gavage. (**C-F**) Linear regression analyses between plasma Mg^{2+} and FFA concentrations at t= (**C**) 0, (**D**) 4, (**E**) 6 and (**F**) 8 hours post-gavage, data from the experiment in graph A-B. Each dot represents an individual mouse. Several data points are missing due to insufficient sample availability. (**G**) Plasma Mg^{2+} (circles, left y-axis) and triglyceride (squares, right y-axis) concentrations before (t=0) and at 1, 2, 4, 6 and 8 hours after an oral gavage of 200 μL olive oil in hypoinsulinemic Kir6.2-p.Val59Met mice (n=7). Linear regression analysis between the plasma Mg^{2+} and triglyceride concentration at t=4 hours post-gavage, data from the experiment in graph **G**. Each dot represents an individual mouse. Two mice were excluded due to insufficient sample availability. Data are mean ± SEM. * Indicates a $p \leq 0.05$ of Mg^{2+} concentrations compared to t=0 hours. † Indicates a $p \leq 0.05$ of triglyceride or FFA concentrations compared to t=0 hours.

FFA levels in a $MgCl_2$ solution also lowers the Mg^{2+} concentration (linear regression: $y=-0.08x + 1.00$, $p \leq 0.05$, Fig. 4C).

Elevating triglyceride levels reduces the serum Mg^{2+} concentration in healthy subjects

To demonstrate that triglycerides and FFAs also affect the blood Mg^{2+} concentration in humans, samples obtained from 24 healthy female subjects, with a BMI >25 kg/m^2, who received an oral fat load, were investigated (31). Serum FFA and Mg^{2+} levels and plasma triglyceride concentrations were measured over a period of eight hours. Plasma triglyceride levels significantly increased from 1.18 ± 0.10 (t=0 hours) to 2.13 ± 0.18 (peak at t=4 hours) mmol/L (Fig. 5A). Serum FFA concentrations were significantly elevated from 0.38 ± 0.03 (t=0 hours) to 0.76 ± 0.04 (peak at t=6 hours) mmol/L (Fig. 5B). In accordance with our previous findings, serum Mg^{2+} levels dropped from 0.82 ± 0.01 (t=0 hours) to 0.75 ± 0.02 (nadir at t=6 hours) mmol/L (Fig. 5A,B). We then measured total serum Mg^{2+} levels using inductively coupled plasma mass spectrometry (ICP-MS) and found that the total serum Mg^{2+} concentration was not affected by the cream intake; hence only the free Mg^{2+} levels are affected (Fig. 5C).

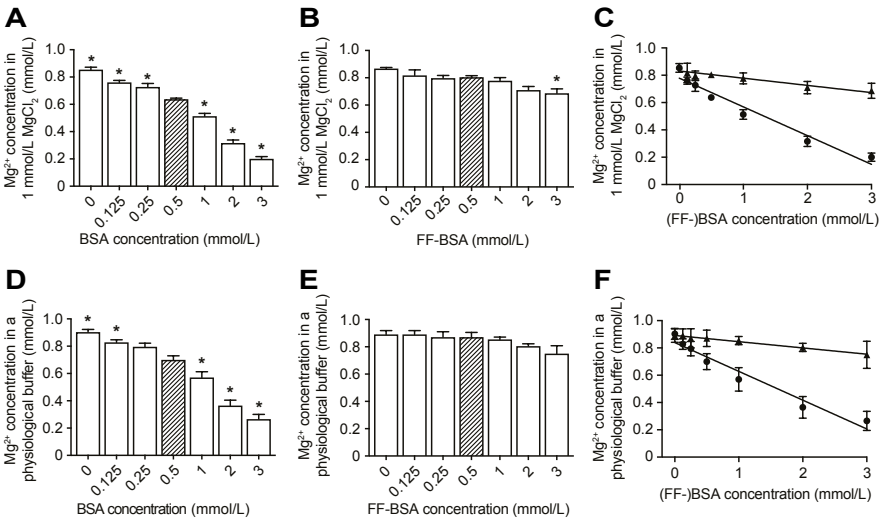

Figure 3 | Binding of Mg^{2+} to albumin is FFA-dependent

(**A-B**) The Mg^{2+} concentration in an increasing level of (**A**) BSA or (**B**) FF-BSA dissolved in a 1 mmol/L $MgCl_2$ solution. (**C**) Linear regression analyses of the Mg^{2+} concentration in increasing levels of BSA (circles, y=-0.21x + 0.78, r^2=0.96, $p \leq 0.05$) or FF-BSA (triangles, y=-0.05x + 0.83, r^2=0.90, $p \leq 0.05$), data from graph **A-B**. (**D-E**) The Mg^{2+} concentration in an increasing level of (**D**) BSA or (**E**) FF-BSA dissolved in a physiological buffer. (**F**) Linear regression analyses of the Mg^{2+} concentration in increasing levels of BSA (circles, y=-0.21x + 0.78, r^2=0.96, $p \leq 0.05$) or FF-BSA (triangles, y=-0.05x + 0.83, r^2=0.90, $p \leq 0.05$), data from graph **D-E**. The striped bars indicate a BSA/FF-BSA concentration (0.5 mmol/L) that is near the physiological range. Data are mean ± SEM of three replicate experiments. * Indicates a $p \leq 0.05$. FF-BSA: free-fatty-acid-free BSA.

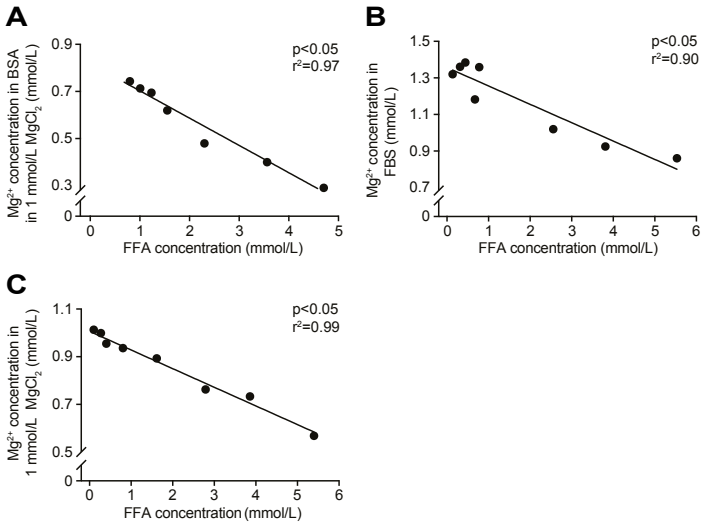

Figure 4 | FFAs directly reduce Mg^{2+} concentrations

(**A-C**) Linear regression analyses of the Mg^{2+} versus the FFA concentration in (**A**) BSA in 1 mmol/L $MgCl_2$ ($y=-0.12x + 0.83$, $r^2=0.97$, $p \leq 0.05$), (**B**) FBS ($y=-0.10x + 1.36$, $r^2=0.90$, $p \leq 0.05$) and (**C**) a 1 mmol/L $MgCl_2$ solution ($y=-0.08x + 1.00$, $r^2=0.99$, $p \leq 0.05$). Results of one representative experiment are shown. The experiment was repeated three additional times with similar results.

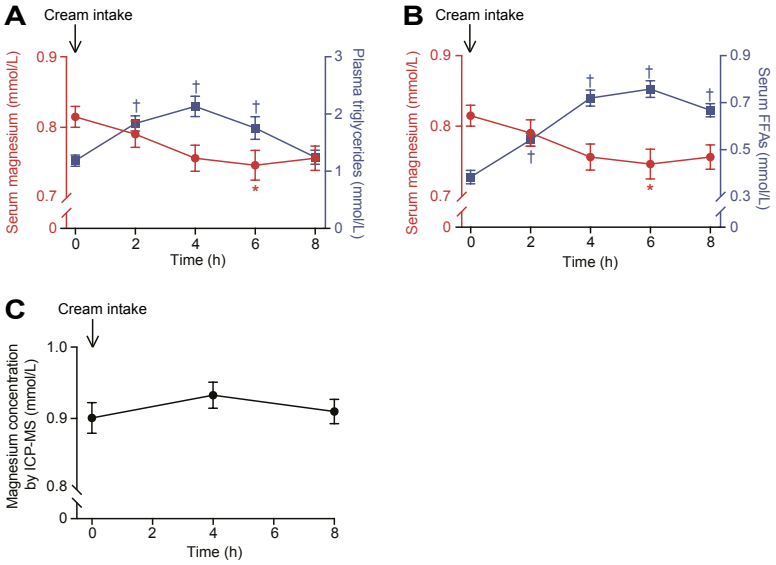

Figure 5 | Increased serum FFA and plasma triglyceride levels directly reduce the serum Mg^{2+} concentration in healthy overweight or obese female individuals

(**A**) Serum Mg^{2+} (circles, left y-axis) and plasma triglyceride (squares, right y-axis) concentrations before (t=0) and at 1, 2, 4, 6 and 8 hours after an oral lipid load in healthy overweight females (n=22). (**B**) Serum Mg^{2+} (circles, left y-axis) and FFA (squares, right y-axis) concentrations before (t=0) and at 1, 2, 4, 6 and 8 hours after an oral lipid load in healthy overweight females (n=22). (**C**) Total serum Mg^{2+} levels measured by inductively coupled plasma mass spectrometry (ICP-MS) at t=0, 4 and 8 hours after oral lipid intake. Data are mean ± SEM. * Indicates a $p \leq 0.05$ of Mg^{2+} concentrations compared to t=0 hours. † Indicates a $p \leq 0.05$ of triglyceride or FFA concentrations compared to t=0 hours.

Discussion

Hypomagnesemia is a common phenomenon in T2D. In the current study, we show that high blood FFA and triglyceride concentrations directly reduce blood Mg^{2+} levels. High FFA levels bind Mg^{2+} resulting in decreased circulating free Mg^{2+} levels.

This conclusion is based on complementary results from our *in vitro*, animal and human *in vivo* studies. First, in a large cohort of overweight individuals, the concentration of triglycerides in large VLDL particles was inversely correlated with the blood Mg^{2+} concentration. Second, a dietary lipid load directly reduced the blood Mg^{2+} concentration in both mice and man, independent of insulin action. Third, we

demonstrated that this phenomenon occurs due to direct binding of FFA molecules to Mg^{2+}. These findings demonstrate that triglycerides reduce free blood Mg^{2+} concentrations and consequently place hypertriglyceridemic patients at risk for Mg^{2+} deficiencies.

Here, we demonstrated that increased triglyceride and FFA levels reduce free Mg^{2+} concentrations, the biologically active form of Mg^{2+}, by a direct interaction between negatively charged FFA molecules and Mg^{2+} ions. This binding is highly specific for Mg^{2+}, as the presence of physiological concentrations of other cations did not affect the interaction between Mg^{2+} and FFAs. The phenomenon of reduced Mg^{2+} levels as a result of elevated FFA levels has been observed in dogs, however no underlying mechanism was suggested (32). Moreover, previous studies showed that Ca^{2+} can bind to FFAs, and that elevating FFA levels in patients reduces blood Ca^{2+} concentrations (33). However, in our *in vitro* experiments the addition of physiological concentrations of Ca^{2+} did not affect the Mg-FFA interaction, indicating a higher affinity of Mg^{2+} compared to Ca^{2+} for binding FFAs. This is likely due to the fact that a Mg^{2+} ion has a significantly higher charge density than a Ca^{2+} ion (34).

Our findings may explain why certain factors that affect circulating FFAs are associated with changes in blood Mg^{2+} concentrations. Molecules that activate lipolysis, resulting in increased blood FFA levels, such as β-adrenergic agonists, ethanol and epinephrine, are associated with reduced blood Mg^{2+} levels (1, 35-37). Indeed, an intravenous infusion of the β-adrenergic agonist terbutaline causes a reduction in the serum Mg^{2+} concentration, which is correlated to the elevated concentration of plasma FFAs, but not glucose (38). This does not exclude the possibility of additional potential mechanisms. Prolonged fasting, which induces lipolysis and increases circulating FFAs, also results in hypomagnesemia (39).

Approximately 30% of blood Mg^{2+} is bound to albumin (14). However, our data indicate that the binding of Mg^{2+} to albumin depends on the availability of FFAs. Direct binding of Mg^{2+} to albumin is minimal, because albumin depleted of FFAs showed little binding to Mg^{2+}. To correct for albumin binding, the factor of 0.7 is used when calculating the fractional excretion of Mg^{2+} (FEMg, $[(uMg \times sCr)/(sMg \times uCr \times 0.7)] \times 100$) (40). As the large majority of FFAs in blood is bound to albumin, alterations in the FFA concentration may be the determining factor in the binding of Mg^{2+} to albumin (22). This paradigm change questions the current protocol to calculate the FEMg. The use of the factor of '0.7' is accurate in physiological conditions, but will lead to inaccurate calculations in pathological conditions, such as hypertriglyceridemia.

Several limitations need to be considered. Hypertriglyceridemia in mice was achieved using olive oil, while in human subjects this was done by an oral load of cream. Olive oil contains no Mg^{2+}, while the cream used in the human study contains 3.3 mmol/L Mg^{2+}, leading to a potential underestimation of the reduction in serum

Mg^{2+} in the healthy volunteers. Moreover, in the *in vitro* experiments, FFAs extracted from BSA were used to increase FFA levels in several solutions. However, the yield of this extraction procedure was not equal in each performed experiment, making it difficult to combine the data of all experiments. Despite these differences in FFA-yield, the results were similar in all four replicate experiments. Lastly, in overweight individuals and T2D patients, Mg^{2+} inversely correlates with triglycerides (1, 23, 24). Our *in vitro* data show direct binding of Mg^{2+} to FFA molecules, which, in contrast to triglyceride molecules, contain a negative charge. It is unlikely that Mg^{2+} binds to uncharged triglyceride molecules. However, in humans the blood triglycerides and FFA levels strongly correlate, meaning that most patients with hypertriglyceridemia also have elevated FFA levels, which would underlie the inverse correlation between Mg^{2+} and triglycerides (41-44).

This study has several strengths. Our data extend from molecule to population and have clinical implications. Moreover, we demonstrated the directionality of the inverse association between triglycerides and Mg^{2+}, which could explain why hypomagnesemia is so prominent in diseases such as T2D. Our data do not rule out that changes in Mg^{2+} concentrations could also influence lipid levels.

In conclusion, we show that elevated blood FFA and triglyceride levels directly reduce the blood Mg^{2+} concentration by binding of Mg^{2+} ions to FFA molecules. Our data explain the high prevalence of hypomagnesemia in several metabolic diseases characterized by elevated triglyceride levels (1-3). In these patient groups, hypertriglyceridemic patients are at particular risk for hypomagnesemia, and therefore, blood Mg^{2+} levels should be routinely measured and monitored in these patients.

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Materials and methods

300-Obesity cohort

302 Individuals aged 55 to 80 were enrolled in the 300-Obesity cohort study at the Radboud university medical center in the period between 2014 and 2016 (45). All subjects had a BMI above 27 kg/m^2. Subjects with a recent cardiovascular event (MI, transient ischemic attack, stroke <6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral subcutaneous anti-coagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium were excluded. Blood samples were taken in the morning following an overnight fast. Blood glucose, triglycerides, total cholesterol and high density lipoprotein cholesterol were measured using standard laboratory procedures. HOMA-IR was calculated by the formula: (fasting plasma glucose × fasting plasma insulin)/22.5. High-throughput nuclear magnetic resonance (NMR) metabolomics platform (Nightingale's Biomarker Analysis Platform) (27) was used for the quantification of 231 lipid and metabolite measures. The metabolites were measured in a single experiment setup for the quantification of different metabolite groups. In this paper we focus on the lipoproteins: total lipid concentrations of 14 lipoprotein subclasses, lipoprotein particles sizes, apolipoproteins and cholesterol. The NMR metabolomics platform has previously been used in various epidemiological studies (46, 47). Details of the experimentation have been described previously (27).

Oral gavage of olive oil in mice

This 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). 12 Male C57BL6/J mice (Charles River, Germany) were obtained at an age of 9-10 weeks, and were acclimatized for two weeks in a temperature- and light-controlled room, with six mice per cage (Eurostandard Type III) and were allowed *ad libitum* access to acidified tap water and standard pellet chow (Ssniff Spezialdiäten, GmbH, Germany). After the acclimatization period, mice received experimental chow containing 18.3% protein (wt/wt), 4.1% crude fat (wt/wt), 25.1% starch (wt/wt) and 33.6% sugar (wt/wt) (Ssniff Spezialdiäten, E15000-04). After 2 weeks on the synthetic diet, mice were fasted overnight, from 9:00 PM to 9:00 AM. Mice received 200 μL intragastric olive oil (extra virgin, Carbonell, Cordoba, Spain) *via* oral gavage. Blood was drawn *via* tail-bleed, using chilled Na-heparin capillaries (Praxisdienst, Longuich, Germany) coated with paraoxon (Sigma, St. Louis, MO, USA), before the gavage (t=0 hours) and at 1, 2, 4, 6 and 8 hours post-gavage. The capillaries were spun down at 3000 g and plasma was separated.

Oral gavage of olive oil in mice with diabetes

All experimental procedures were conducted in compliance with the UK Animals Scientific Procedures Act (1986) and University of Oxford ethical guidelines. In four male and four female C57BL6 mice expression of a Kir6.2-p.Val59Met transgene was induced using a 400 μL tamoxifen (0.02 g/mL corn oil) subcutaneous injection. This inducible mouse model recapitulates the phenotype of neonatal diabetes and develops diabetes by impaired insulin secretion (48). Three days after induction, mice were fasted overnight from 5:00 PM to 9:00 AM. Successful induction was validated by measuring fasted glucose levels. One female mouse did not have elevated fasting glucose levels and was excluded from subsequent analyses. The subsequent experimental setup of the oral gavage and blood drawing was identical to the above-described experiment in C57BL6/J wild-type mice.

Bovine serum albumin (BSA) and fatty-acid-free-BSA (FF-BSA) dissolved in a $MgCl_2$ solution or a physiological buffer

BSA (Sigma Aldrich) and FF-BSA (Sigma Aldrich) were separately dissolved at several concentrations in a 1 mmol/L $MgCl_2$ (Merck Millipore, Darmstadt, Germany) solution or a physiological buffer, both set at pH 7.5 by adding NaOH. The physiological buffer contained 27 mmol/L $NaHCO_3$ (Merck Millipore), 112 mmol/L NaCl (Merck Millipore), 5 mmol/L KCl (Merck Millipore), 1 mmol/L $MgCl_2$, 1 mmol/L Na_2HPO_4 (VWR International, USA), and 2.5 mmol/L $CaCl_2$ (Merck Millipore) dissolved in Milli-Q.

Increasing FFA levels in BSA, fetal bovine serum (FBS) and $MgCl_2$ solutions

To extract endogenous FFAs, BSA (0.2 g/mL) dissolved in Milli-Q was mixed (1:2 (vol./vol.)) with ice-cold ethanol:diethyl ether (3:1 (vol./vol.)). The lipid phase was evaporated overnight at room temperature. In order to remove trace amounts of BSA from the solution, it was centrifuged three times with an Amicon® 50 kDa filter for 15 minutes at 2500 g to clog the protein in the filter. Extracted FFAs were added to 250 μl of FBS (Biowest, South America), 1 mmol/L $MgCl_2$, or 0.5 mmol/L BSA in the amounts of 0, 25, 50, 100, 200, 350, 500, and 700 μL. Dilution factors were accounted for when measuring the concentrations of Mg^{2+} and FFA.

Analytical measurements

Protein (Pierce, Thermo Scientific, Massachusetts, USA), FFA (WAKO Diagnostics, Delfzijl, The Netherlands), triglycerides (Roche Molecular Biochemicals, Indianapolis, USA) and Mg^{2+} (Roche/Hitachi, Tokyo, Japan) concentrations were measured using a spectrophotometric assay according to the manufacturer's protocols. The Mg^{2+} calorimetric assay is based on a Xylidyl Blue-I method and was measured at 600 nm. FFAs were measured at 546 nm, triglycerides at 500 nm, and protein at 562 nm on a

Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad laboratories, California, USA). For the inductively coupled plasma mass spectrometry (ICP-MS) measurements, serum samples were dissolved in HNO_3 (>65%, Sigma), diluted, and subjected to ICP-MS.

Oral lipid load in human subjects

A total of 24 female volunteers underwent an oral fat loading test. The study was approved by the Institutional Review Board of the Franciscus Gasthuis & Vlietland Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam (31). The study was registered at ClinicalTrials.gov under clinical trial number NCT01967459, which aimed to study the effect of vitamin D supplementation on postprandial leukocyte activation. Samples from the baseline oral cream load (before the vitamin D treatment) were used in this study to measure serum Mg^{2+} and FFA concentrations.

Inclusion criteria were an age above 18 years, a premenopausal status, a body mass index (BMI) of 25 kg/m^2 or above and vitamin D deficiency. Exclusion criteria were the use of any kind of medication, except for oral contraceptives, smoking, pregnancy, participation in a clinical study less than 6 months before inclusion, and the use of vitamin supplements. All subjects visited the hospital after a 10 hours overnight fast. A fasting venous blood sample was obtained. Subject received an oral fat load using fresh cream (Albert Heijn, Zaandam, the Netherlands) in a dose of 50 grams of fat per square meter body surface calculated by the Mosteller formula. During the oral fat loading test participants were not allowed to eat or to drink except water and they were asked to refrain from physical activity. Venous blood sampling was repeated at a two-hour interval until eight hours and serum Mg^{2+} and FFA levels and plasma triglyceride levels were measured. Two patients were excluded due to insufficient sample availability.

Statistical analyses

Results are presented as mean ± standard error of the mean (SEM), unless stated otherwise. Variables of overweight individuals were correlated univariately to serum Mg^{2+} levels using Pearson's correlation analyses using SPSS for Windows (V22.0.0.1 IBM). Based on the initial animal experiment in wild-type mice, the sample size for the experiment in Kir6.2-p.Val59Met mice was calculated using a one-way ANOVA statistic (with Dunnett's correction for multiple comparison), to detect an effect size of 0.3 (SD 0.13) with a power of 80% and alpha level of 5%, a total of 6 animals are required per group. The sample size of the oral cream load study in healthy individuals was assessed using a one-way ANOVA (with Dunnett's correction for multiple comparison), to detect an effect size of 0.1 (SD 0.1) with a power of 80% and an alpha of 5%, a total of 23 individuals are required. Significance of Mg^{2+}, triglyceride and

FFA concentrations compared to t=0 hours were evaluated using a one-way ANOVA with Dunnett's correction for multiple comparisons. Direct correlations between Mg^{2+} and triglyceride or FFA concentrations were assessed using linear regression analyses. A *p*-value of ≤0.05 was considered statistically significant. All statistical analyses were performed using Graphpad Prism v7.

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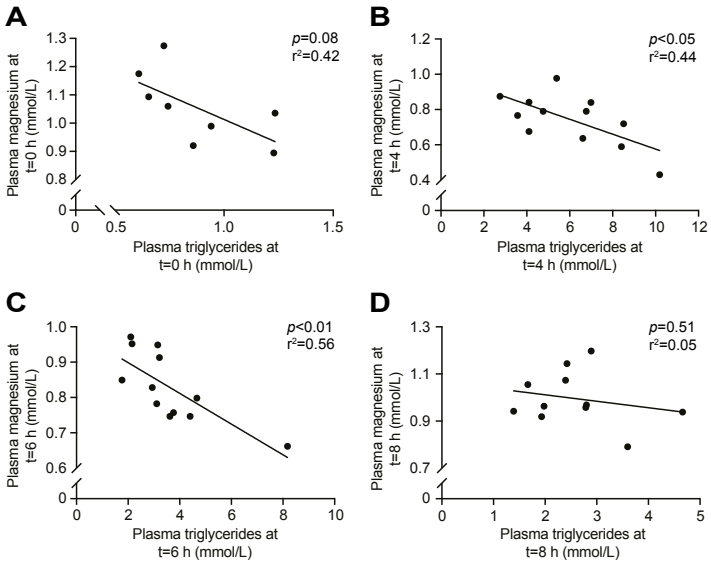
Supplementary data



Supplementary figure 1 | Plasma Mg^{2+} and triglyceride concentrations inversely correlate in hypertriglyceridemic states

(**A-D**) Linear regression analyses between serum Mg^{2+} and triglyceride concentrations at t = (**A**) 0, (**B**) 4, (**C**) 6 and (**D**) 8 hours post-gavage from the experiment in Figure 2A-B. Each dot represents an individual mouse. Several mice were excluded due to insufficient sample availability.

Supplementary table 1 | Characteristics of the Overweight Individuals from the 300-Obesity Cohort.

Variable	300-Obesity cohort mean ± SD
Demographics	
Gender (m:f, %)	45:55 (m:f)
BMI (Kg/m^2)	30.7 ± 3.4
Age (years)	67 ± 5
Waist circumference (cm)	107 ± 10
SBP (mmHg)	130 ± 14
DBP (mmHg)	80 ± 9
Heart rate (beats/min)	63 ± 10
Laboratory analyses	
Magnesium (mmol/L)	0.89 ± 0.09
Triglycerides (mmol/L)	1.8 ± 1.0
Glucose (mmol/L)	5.7 ± 1.3
HOMA-IR	13.7 ± 19.1
HbA_{1c} (mmol/mol)	41.8 ± 8.0
Total cholesterol (mmol/L)	6.3 ± 1.1
Triglycerides in VLDL (mmol/L)	1.23 ± 0.72
Triglycerides in LDL (mmol/L)	0.23 ± 0.07
Triglycerides in HDL (mmol/L)	0.15 ± 0.04
Cholesterol in VLDL (mmol/L)	1.14 ± 0.37
Cholesterol in LDL (mmol/L)	2.25 ± 0.64
Cholesterol in HDL (mmol/L)	1.31 ± 0.29
ApoA1 (g/L)	1.59 ± 0.01
ApoB (g/L)	1.19 ± 0.02
Mean diameter VLDL particle (nm)	36.6 ± 1.2
Mean diameter LDL particle (nm)	23.5 ± 0.1
Mean diameter HDL particle (nm)	9.8 ± 0.2

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; DBP, diastolic blood pressure; f, female; HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; m, male; SBP, systolic blood pressure; VLDL, very-low-density lipoprotein.

Supplementary table 2 | Univariate regression analysis of HOMA-IR, as dependent variable, and triglycerides

Variable	Correlation coefficient	*p*-value	*n*
Triglycerides (mmol/L)	0.245	0.000	284

Supplementary table 3 | Univariate regression analysis of HOMA-IR, as dependent variable, and triglycerides in VLDL

Variable	Correlation coefficient	*p*-value	*n*
Triglycerides in VLDL	0.236	0.000	284

Supplementary table 4 | Multivariate regression analysis of HOMA-IR and triglycerides with the serum Mg^{2+} concentration as dependent variable

Variable	Correlation coefficient	*p*-value	*n*
HOMA-IR	-0.089	0.143	283
Triglycerides (mmol/L)	-0.137	0.024	284

HOMA-IR, homeostatic model assessment of insulin resistance

Supplementary table 5 | Multivariate regression analysis of HOMA-IR and triglycerides in VLDL particles with the serum Mg^{2+} concentration as dependent variable

Variable	Correlation coefficient	*p*-value	*n*
HOMA-IR	-0.092	0.128	283
Triglycerides in VLDL	-0.137	0.024	284

HOMA-IR, homeostatic model assessment of insulin resistance

"Without the element of enjoyment,
it is not worth trying to excel at anything"
– Magnus Carlsen

7

Summary

Summary

Hypomagnesemia in type 2 diabetes, cause or consequence?

Magnesium (Mg^{2+}) is an essential ion in the functioning of over 600 enzymes and has a wide variety of functions throughout the body. Hypomagnesemia (blood Mg^{2+} concentration <0.7 mmol/L) can result in clinical symptoms including, but not limited to, muscle spasms, arrhythmias, migraines, depression, tiredness and generalized weakness. Therefore, the body maintains the blood Mg^{2+} concentration in the physiological range, between 0.70-1.05 mmol/L, by the complex interplay of the kidneys, intestine and bone. Hypomagnesemia is a common clinical finding in patients with type 2 diabetes (T2D). The causal relationship and main mechanisms underlying the hypomagnesemia in these patients is still unknown despite numerous hypotheses described in the literature. Recently, lower Mg^{2+} levels have been associated with an increased risk of developing T2D in the general population. In T2D patients, lower blood Mg^{2+} levels result in a more rapid disease progression and more severe T2D-related complications, including a quicker renal function decline. How reduced Mg^{2+} levels affect energy and lipid metabolism, leading to this increased risk and disease progression, has not been investigated in detail. Therefore, this thesis aimed to establish the causal relationship and the molecular mechanism(s) underlying hypomagnesemia in T2D.

Which factors contribute to the hypomagnesemia in type 2 diabetes patients?

Identifying which T2D-related factors contribute to reductions in the blood Mg^{2+} concentration of T2D patients will facilitate more focused future research. As T2D is a complex disease with a wide array of different comorbidities, polypharmacy is unavoidable when treating T2D. Several of these medications are known to influence the Mg^{2+} balance and could, therefore, explain the Mg^{2+} deficiency of T2D patients.

To determine which factors affect the Mg^{2+} balance, the plasma Mg^{2+} concentration of 395 T2D patients of the PARELSNOER cohort was measured. As described in **Chapter 2**, in total 31% of these patients had hypomagnesemia. To investigate which factors contributed to this hypomagnesemia, changes in the plasma Mg^{2+} concentration were correlated to patient characteristics, laboratory results and medication use. Plasma glucose and triglyceride levels showed the strongest inverse correlations with the plasma Mg^{2+} concentration, whereas the use of medication was not responsible for major changes. However, treatment with metformin was associated with a lower plasma Mg^{2+} level, independent of the fasting glucose concentration. The findings presented in this chapter indicate that hypomagnesemia is associated with factors that are intrinsic to T2D. This study has established the prevalence of hypomagnesemia in a large cohort of T2D patients and has identified which of the factors are most strongly associated with changes in plasma Mg^{2+} levels.

Metabolic consequences of hypomagnesemia

From this cohort study, the causal relationship between T2D and hypomagnesemia cannot be established. Recently, in collaboration with the group of Prof. Hoorn of the Erasmus MC in Rotterdam, it was found that lower Mg^{2+} levels were associated with an increased risk of developing T2D in the general population.

To identify how a Mg^{2+} deficiency affects energy and lipid metabolism in T2D, mice were fed a low or high fat diet (LFD, HFD), combined with a low or normal Mg^{2+} food content. A low dietary Mg^{2+} intake resulted in less HFD-induced weight gain, described in **Chapter 3**. The reduced body weight of the Mg^{2+} deficient animals was associated with improved insulin sensitivity, absent liver steatosis, and lower fasting glucose concentrations. In contrast, these animals had elevated serum triglyceride and free fatty acid (FFA) levels, caused by increased lipolysis in white adipose tissue, resulting in a lower fat mass. Lipolysis is a process that is regulated by the β_3-adrenergic receptor, which was increased in expression in the white adipose tissue of the Mg^{2+} deficient HFD-fed mice. Brown adipose tissue is an important organ in metabolism as it consumes energy to produce heat. The activity of brown adipose tissue, which is also regulated by the β_3-adrenergic receptor, was enhanced in the Mg^{2+}-deficient HFD-fed mice, resulting in a higher body temperature. However, no difference was observed in total energy expenditure between the two HFD-fed groups. Taken together, our data show that Mg^{2+} deficiency results in disturbances in lipid and energy homeostasis, indicative of a catabolic phenotype, which is possibly a result of activation of the β-adrenergic system. This highlights the essential role of Mg^{2+} in lipid metabolism and, therefore, Mg^{2+} deficient T2D patients could be at additional risk for dyslipidemia.

In Mg^{2+} deficient mice on a HFD, lipid metabolism was also disturbed in the kidney, which is reported in **Chapter 4**. The mice had massive lysosomal accumulations of charged lipids, such as phospholipids or sphingolipids, in the cells of the proximal tubule. The exact lipid composition and underlying mechanism was not identified. However, the extent of the damage proposes a novel mechanism to explain the association between reduced Mg^{2+} levels and renal function decline.

Mice with type 2 diabetes develop hypomagnesemia

To address the other side of the coin, namely whether hypomagnesemia can be a consequence of T2D, two different T2D animal models were analyzed in this thesis, a HFD model and a genetic diabetes model (db/db). The results of these studies are described in **Chapter 4** and **5**. Mice on a HFD had reduced serum Mg^{2+} levels already after 4 weeks on the diet; predominantly in the mice receiving a diet low in Mg^{2+}. The intestines and kidneys are the major organs regulating blood Mg^{2+} levels. Despite the hypomagnesemia in the HFD-fed mice, there was a reduced intestinal expression of an epithelial Mg^{2+} channel, *Trpm6*. However, no fecal Mg^{2+} loss was observed. Also

in the kidney, the expected upregulation of *Trpm6* was not present in the hypomagnesemic HFD-fed mice. There was no urinary Mg^{2+} loss, indicative of intact renal compensation despite the lower *Trpm6* expression. Db/db mice, a genetic model for T2D, also developed hypomagnesemia. As described in **Chapter 5**, in db/db mice the compensatory upregulation in *Trpm6* is present both in kidney and intestine. This is in contrast to the HFD-fed mice, which had reduced gene expression of *Trpm6* in colon and kidney (**Chapter 4**). However, as in the HFD-fed mice, no renal Mg^{2+} wasting occurred in the db/db mice, despite massive glucosuria. Treating the db/db mice with metformin, which was associated with a lower plasma Mg^{2+} concentration in the PARELSNOER cohort, did not affect the Mg^{2+} homeostasis. This indicates that the lower plasma Mg^{2+} level in metformin users is likely mediated by indirect factors.

Together, these data clearly show that hypomagnesemia is a consequence of T2D, which is not mediated by metformin use. As no renal or intestinal Mg^{2+} loss was observed, it remains unclear what the exact underlying cause of the hypomagnesemia in these mice is.

Free fatty acids reduce blood magnesium levels

As no renal or intestinal loss of Mg^{2+} was observed in the animal models, despite the development of hypomagnesemia, we hypothesized other causes for reduced Mg^{2+} levels. Plasma triglycerides are one of the major determinants of changes in the blood Mg^{2+} concentration both in T2D patients (**Chapter 2**) and overweight individuals (**Chapter 6**).

To investigate whether triglycerides could affect the blood Mg^{2+} concentration, hypertriglyceridemia was induced in mice using an oral gavage of olive oil (**Chapter 6**). Indeed, increasing concentrations of plasma triglycerides caused a severe and immediate reduction in the plasma Mg^{2+} concentration. Interestingly, when plasma triglyceride and FFA levels returned to baseline, plasma Mg^{2+} levels also returned to normal. Performing a lipid load of cream in healthy volunteers yielded similar results, indicating that the changes observed in mice also occur in humans. As the plasma Mg^{2+} concentration was almost perfectly inversely correlated with plasma triglyceride and FFA levels in hypertriglyceridemic mice, we suspected direct binding of Mg^{2+} to negatively charged FFA molecules. Adding increasing amounts of FFAs to Mg^{2+} containing solutions *in vitro*, reduced ionized Mg^{2+} concentrations in a dose-dependent manner. These findings add FFAs as an important contributor in Mg^{2+} homeostasis and explain the molecular mechanism underlying the inverse correlation between blood triglycerides and Mg^{2+} concentrations in metabolic disorders.

Perspectives

The studies performed in this thesis expand our knowledge on the etiology and metabolic consequences of hypomagnesemia in T2D. The data open up new research directions on the role of Mg^{2+} in both physiology and pathophysiology.

In the PARELSNOER cohort of T2D patients we have shown that hypomagnesemia is correlated to factors intrinsic to the disease, namely plasma glucose and triglycerides concentrations. Some factors that were not included in this study could also impact the Mg^{2+} status of T2D patients, such as dietary intake, genetic differences and the presence of comorbidities. Also, it would be interesting to collect data of these patients after several years, to be able to draw conclusions regarding causality. Do lower Mg^{2+} levels lead to a worse disease progression and mortality? Including these analyses in future research would provide essential information regarding the causes of hypomagnesemia, and its metabolic consequences, in T2D patients.

In this cohort of T2D patients there was a strong inverse correlation between plasma triglyceride and Mg^{2+} concentrations. From the animal data it became apparent that Mg^{2+} deficiency results in high blood triglyceride levels. On the other hand, inducing hypertriglyceridemia in mice using an oral gavage resulted in a rapid decrease in the blood Mg^{2+} concentration. This indicates that severely hypertriglyceridemic T2D patients are at extra risk of developing hypomagnesemia. An interesting approach is to measure Mg^{2+} levels in patients with primary hypertriglyceridemia, who do not have T2D. This could differentiate the effects of triglycerides and insulin resistance on Mg^{2+} homeostasis.

In this thesis, we unraveled that the effect of triglycerides on Mg^{2+} is due to direct binding of Mg^{2+} to FFA molecules. Interestingly, several factors that induce increases in blood FFA levels are also associated with hypomagnesemia, such as β-adrenergic agonists, fasting, acute stress and ethanol use. These findings will open new research opportunities for understanding the etiology of hypomagnesemia in conditions such as asthma, admittance to intensive care, metabolic syndromes and alcohol dependence or withdrawal. In this thesis only the short-term effects of hypertriglyceridemia on Mg^{2+} homeostasis have been investigated. Does the binding of Mg^{2+} to FFA also induce a chronic hypomagnesemia? Potentially, reducing blood triglyceride and FFA levels is a new way to normalize blood Mg^{2+} levels.

Mice on Mg^{2+} deficient high fat diet had increased expression of the β_3-adrenergic receptor, which was associated with a higher lipid breakdown and heat production. This thesis has only focussed on the effects on the β_3-adrenergic receptor, but it was not investigated whether the Mg^{2+} deficiency also caused other downstream effects of the β-adrenergic system (via the β_1 or β_2 receptors). In literature Mg^{2+} has been described to suppress the effects of adrenaline on heart rate. Studying how Mg^{2+} can inhibit the β-adrenergic system will be relevant for both physiology and for stress-related conditions.

In clinical practice, blood Mg^{2+} levels are not routinely measured in T2D patients, despite the high prevalence of hypomagnesemia, occurring in almost one in three patients with T2D. Several new mechanisms and factors that contribute to the hypomagnesemia in T2D patients have been identified in this thesis. Now that we better understand why T2D patients develop hypomagnesemia, which factors are involved in this process and what metabolic consequences this has, it is important that Mg^{2+} will receive more attention both in the clinic and in the general population.

"It's the job that's never started as takes longest to finish"

– J.R.R. Tolkien | The Lord of the Rings

8

Discussion and clinical implications

Discussion and clinical implications

Magnesium (Mg^{2+}) plays an important role in human physiology in general and energy metabolism in particular. Hypomagnesemia (blood Mg^{2+} concentration <0.7 mmol/L) is a common clinical finding in T2D patients (1). However, little is known regarding the metabolic consequences of hypomagnesemia (2). It is still unclear whether hypomagnesemia in T2D patients contributes towards the development of the disease, or that hypomagnesemia is merely a consequence of T2D (3). This thesis contains a number of studies that further unravel the metabolic consequences and the underlying cause of hypomagnesemia in T2D.

Hypomagnesemia in type 2 diabetes patients

We determined the prevalence of hypomagnesemia and the extent of urinary Mg^{2+} loss in a cohort of patients with advanced T2D (PARELSNOER), and investigated clinical and laboratory factors associated with low plasma Mg^{2+} levels. The prevalence of hypomagnesemia in the PARELSNOER cohort of 395 T2D patients was 31% (**Chapter 2**), which matches the prevalence numbers of previous studies (Table 1 in **Chapter 1**).

Knowing which T2D-related parameters are associated with hypomagnesemia will aid in identifying the key risk factors, will provide insight into the underlying mechanisms and will contribute to the awareness of hypomagnesemia in T2D patients in the clinics. The major factors that correlated with reduced plasma Mg^{2+} levels in the PARELSNOER cohort of T2D patients were elevated plasma concentrations of glucose and triglycerides (**Chapter 2**). Multiplying plasma glucose by triglycerides levels is known as the 'triglyceride glucose index' (TyG index), which is a marker of insulin resistance (4, 5). Hypomagnesemia was not a prominent feature of overweight individuals from the 300-Obesity cohort (hypomagnesemia in 5 out of 285 individuals, 2%, **Chapter 6**), who do not have severe insulin resistance, suggesting that insulin resistance is key in the development of hypomagnesemia in metabolic disorders. This is substantiated by observational cohort studies that identified an inverse correlation between the serum Mg^{2+} concentration and the 'homeostatic model assessment of insulin resistance' (HOMA-IR) score, the clinical measure of insulin resistance (6, 7).

These data suggest that patients with the most severe T2D are at the highest risk to develop hypomagnesemia. It cannot be concluded whether this is a direct effect of insulin resistance on Mg^{2+} homeostasis, or whether this is mediated by factors that are secondary to the insulin resistance, including hyperglycemia and hypertriglyceridemia, or a combination of these factors (8). By using animal studies, such as dietary interventions or genetically modified mice, the effect of these factors on the Mg^{2+} balance can be individually investigated.

Potential causes of hypomagnesemia in type 2 diabetes

Many T2D-related factors have been proposed to contribute to the development of hypomagnesemia, including, but not limited to, medication use, urinary Mg^{2+} wasting, reduced dietary intake and intestinal malabsorption (9).

The effect of medication use on magnesium homeostasis

Due to the complexity of T2D, its wide range of complications and associated multimorbidity, polypharmacy is common when treating T2D patients (10, 11). Some of the medication extensively used among T2D patients, such as proton pump inhibitors (PPIs) and thiazide diuretics, are known to induce hypomagnesemia (12-14). Therefore, medication use was expected to be a major contributor to the etiology of hypomagnesemia in T2D patients. However, the contribution of PPIs and thiazides towards a reduction in plasma Mg^{2+} was minimal, substantiating the notion that hypomagnesemia is intrinsic to T2D, and not merely a consequence of medication use **(Chapter 2)**.

Metformin use correlated with a reduced plasma Mg^{2+} level (**Chapter 2**), which was also reported in other observational cohort studies (15, 16). In a small short-term intervention study in fourteen T2D patients metformin treatment resulted in a mild reduction in plasma Mg^{2+} levels after 2-4 weeks (from 0.72 to 0.70 mmol/L, $p<0.05$), despite substantially improving blood glucose levels (17). Possibly, the effect of metformin on Mg^{2+} homeostasis is indirect, as metformin treatment can result in chronic diarrhea, leading to intestinal malabsorption (18). An indirect effect of metformin is in line with our animal data, in which metformin treatment did not affect Mg^{2+} homeostasis in either db/db or db/m mice (**Chapter 5**). These findings are in accordance with a study that observed equal serum Mg^{2+} levels when treating type 1 diabetic and control rats with metformin (19). It is, therefore, likely that the association between metformin use and lower serum Mg^{2+} levels in T2D patients is caused by factors that were not included in the analyses of these cohort studies. It would be interesting to measure Mg^{2+} levels in blood samples of T2D patients receiving metformin versus placebo to conclude whether metformin directly affects Mg^{2+} homeostasis in T2D patients.

Triglycerides and free fatty acids

Elevated blood triglyceride concentrations are associated with reduced blood Mg^{2+} levels in T2D patients and overweight individuals **(Chapter 2 and 6)**. Inducing hypertriglyceridemia *via* an oral lipid load in mice and healthy individuals resulted in a rapid reduction in blood Mg^{2+} levels, showing that a high fat intake diminishes blood Mg^{2+} levels (**Chapter 6**). As triglycerides consist of three free fatty acid (FFA) molecules, plasma triglycerides concentrations strongly correlate with plasma FFA levels (**Chapter 6**) (20-22). These negatively charged FFA molecules bind to Mg^{2+}, reducing the free Mg^{2+} concentration in the blood (**Chapter 6**).

The major organs regulating the blood Mg^{2+} level are the intestine, bone and kidney (23). The data of our studies postulate that FFAs should be considered as a novel player in the blood Mg^{2+} axis. The finding that FFAs directly influence blood ionized Mg^{2+} concentrations provides new directions for the field of Mg^{2+} research and proposes novel potential mechanisms for hypomagnesemia observed in conditions such as asthma, critical illness and alcohol addiction (24-28). Activation of lipolysis in white adipose tissue (WAT) is regulated primarily by the β_3-adrenergic receptor (29). Several factors that affect FFA levels also influence blood Mg^{2+} concentrations (Table 1). For instance, β-adrenergic agonists are associated with reduced blood Mg^{2+} concentrations (24, 30-32) (**Chapter 2**). Certain β-adrenergic agonists, such as isoproterenol and salbutamol, are commonly subscribed to asthma patients, and interestingly, hypomagnesemia is frequently observed in asthma patients (24, 45-47). One of the factors contributing to the reduction in serum Mg^{2+} levels in asthma patients is the use of β-adrenergic agonists (48). Moreover, in a small intervention study the administration of the β-adrenergic agonist terbutaline lowered blood Mg^{2+} levels, which significantly correlated with increased blood FFA levels, underlining the role of FFAs in β-adrenergic-agonist-induced hypomagnesemia (49). The use of β-adrenergic agonists may be the underlying cause of hypomagnesemia in asthma patients, by increasing blood FFA levels and thereby reducing the ionized Mg^{2+} concentration. The novel notion that FFAs affect Mg^{2+} homeostasis could contribute

Table 1 | Interventions that cause changes in the blood FFA level induce opposite changes in the blood Mg^{2+} concentration.

Intervention	Context	Change in blood FFA	Change in blood Mg^{2+}	Reference
Isoproterenol	Treatment for bradycardia, heart block or asthma	↑	↓	(33)
Salbutamol	Treatment for asthma or COPD	↑	↓	(34)
Epinephrine	Stress/critical illness	↑	↓	(34-36)
Lipid load	High fat meal	↑	↓	**Chapter 6**
Fasting	Generally performed before blood drawing in human studies	↑	↓	(37)
Ethanol	Alcohol addiction or withdrawal	↑	↓	(38-40)
Cold exposure		↑	↓	(41-44)
Nicotinic acid (vitamin B_3)	Blocks lipolysis and lowers triglyceride levels	↓	↑	(38)

COPD: chronic obstructive pulmonary disease

to the etiology of hypomagnesemia in metabolic disorders, alcohol addiction, stress-related disorders and asthma. From these data it remains unclear if the FFA-Mg binding, leading to a reduced blood Mg^{2+} concentration, should be considered as a 'pseudo-hypomagnesemia'. Is the binding merely affecting the spectrophotometric assay, or do the reduced ionized Mg^{2+} levels also have clinical effects and implications that are similar to a Mg^{2+} depletion?

Urinary magnesium wasting

In T2D patients of the PARELSNOER cohort over 40% of the patients displayed hypermagnesuria (**Chapter 2**), defined as a fractional excretion of Mg^{2+} (FEMg) >4% (50). Interestingly, FEMg did not differ between hypomagnesemic and normomagnesemic patients. Possibly, some hypermagnesuric patients were able to compensate for the urinary Mg^{2+} loss by increasing intestinal uptake or by releasing Mg^{2+} from storage compartments, such as the bone, and thereby maintaining normal plasma Mg^{2+} levels.

Which mechanism can be responsible for the high FEMg in T2D patients? An important hypothesis for hypermagnesuria in T2D patients is glucosuria. Because of its osmotic effect glucose may reduce tubular Mg^{2+} reabsorption by preventing the pre-urine from being concentrated and, thereby, lowering its Mg^{2+} concentration (9, 51-53). However, when feeding mice a high fat diet (HFD) severe hypomagnesemia developed in the absence of glucosuria (**Chapter 4**). Moreover, sodium-glucose transporter 2 (SGLT2) inhibitors induce massive glucosuria, but lead to a moderate increase in serum Mg^{2+} levels, making it unlikely that glucosuria underlies the hypermagnesuria and hypomagnesemia observed in T2D patients (54, 55).

Another potential mechanism is that insulin stimulates the Mg^{2+} channel, TRPM6, essential for Mg^{2+} reabsorption in the DCT (56). Therefore, insulin resistance could reduce Mg^{2+} reabsorption by TRPM6 and lead to hypermagnesuria. Colonic and renal mRNA expression of *Trpm6* was decreased in hypomagnesemic HFD-fed mice, but no fecal or urinary Mg^{2+} wasting was observed (**Chapter 4**).

Kidney damage with associated proteinuria is present in 20-40% of T2D patients (57, 58). As ~30% of blood Mg^{2+} is bound to albumin, proteinuria is associated with hypomagnesemia (59). In contrast to the current paradigm, Mg^{2+} does not bind albumin directly, but *via* an interaction with FFAs attached to the albumin molecule (**Chapter 6** and Figure 1). Therefore, when more FFAs are attached to an albumin molecule it will increase the number of bound Mg^{2+} ions (Figure 1). Possibly, hypertriglyceridemia can exacerbate the albuminuria-induced urinary Mg^{2+} wasting.

The urinary Mg^{2+} loss is determined by calculating the fractional excretion of Mg^{2+} (FEMg) according to the formula (60):

$$\frac{[\text{urinary magnesium}] \times [\text{serum creatinine}]}{[\text{serum magnesium}] \times [\text{urinary creatinine}] \times 0.7} \times 100\%$$

The factor of 0.7 is used in this formula to correct for the 30% bound Mg^{2+} fraction in physiological conditions. As the amount of Mg^{2+} bound to an albumin molecule is dependent on the number of FFAs bound to the albumin, the aforementioned 30% of bound Mg^{2+} would not be a fixed percentage, but would fluctuate depending on the lipid status of the individual. Moreover, an increased bound fraction of Mg^{2+} in the blood will reduce the filterable fraction, reducing the FEMg. On the other hand, in hypertriglyceridemic states the measured serum Mg^{2+} concentration will also be lower, resulting in a higher FEMg. The fact that triglyceride and FFA levels have a strong influence on the calculated FEMg could explain the equal distribution of urinary Mg^{2+} wasting between normomagnesemic and hypomagnesemic patients (61).

The metabolic consequences of hypomagnesemia

As ATP requires Mg^{2+} for its phosphoryl transfer reactions, Mg^{2+} is required for the proper functioning of over 600 enzymes (2, 23). Mg^{2+} deficiency has been linked to defects in several metabolic pathways, including glucose handling, mitochondrial

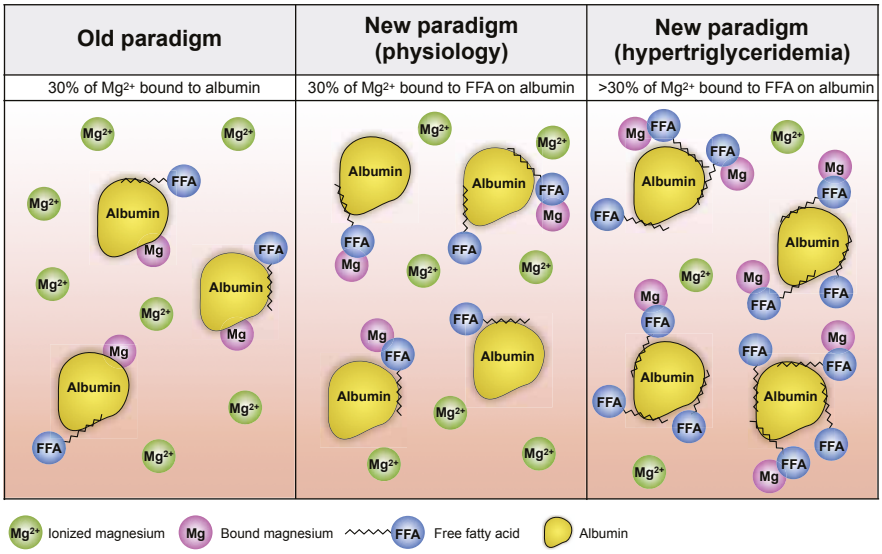


Figure 1 | Mg^{2+} binds to albumin *via* FFAs

In the old paradigm, Mg^{2+} was thought to bind directly to albumin. The results from this thesis have elucidated that Mg^{2+} is bound to albumin by an interaction with FFAs. In hypertriglyceridemic states this will lead to a larger fraction of the blood Mg^{2+} to be bound to FFAs attached to the albumin molecules.

function and lipid metabolism (62-66). However, it remains unclear how reduced blood Mg^{2+} levels could contribute to the development of T2D and its associated complications.

Magnesium on body weight

Obesity is one of the major risk factors for the development of T2D (67). Several patient studies have identified a univariate inverse correlation between the blood Mg^{2+} concentration and BMI (**Chapter 2**) (68, 69). However, multivariate regression analyses revealed that this correlation is confounded by other variables, such as plasma triglyceride levels (**Chapter 2**) (70). In T2D patients, a higher dietary intake of Mg^{2+} does not affect body weight (71). In several animal studies hypomagnesemia resulted in a reduced body weight gain, without differences in energy expenditure or food intake (**Chapter 3** and Table 2). However, in these animals, the hypomagnesemia was more severe than the mild hypomagnesemia commonly observed in T2D patients (**Chapter 2**).

The effect of magnesium on insulin sensitivity

Insulin resistance is one of the hallmarks of T2D and strongly correlates with obesity (72-74). There is a discrepancy between animal and patient data regarding the role of Mg^{2+} on insulin sensitivity.

In humans a lower Mg^{2+} intake and reduced serum Mg^{2+} levels are correlated with a decreased insulin sensitivity (6, 75, 76). In a population study, lower serum Mg^{2+} levels increased the risk of developing T2D, which was mediated for 29% through insulin resistance (76). However, the effect of oral Mg^{2+} supplementation on insulin sensitivity in subjects with the metabolic syndrome or T2D has provided conflicting results (Table 2 in **Chapter 1**).

Animal and *in vitro* studies have unraveled that Mg^{2+} is essential for the tyrosine-kinase activity of the insulin receptor (79, 85-87). Mg^{2+} is thus required for insulin-stimulated glucose uptake, whereas it does not affect basal glycolysis (88). In contrast, different animal models of Mg^{2+} deficiency have shown improvements on whole-body insulin sensitivity (Table 2). As Mg^{2+} also affected the body weight of these animals, it is difficult to determine whether the effects of Mg^{2+} on insulin sensitivity are a direct effect, or merely mediated by differences in body weight. This finding makes these animal models less ideal to investigate the direct effects of Mg^{2+} deficiency on insulin sensitivity.

Hypomagnesemia enhances β-adrenergic signaling

Depending on the tissue type, activation of β-adrenergic receptors leads to different downstream effects (**Figure 2**). In mice, Mg^{2+} deficiency resulted in an increase in β_3-adrenergic receptor expression in white adipose tissue (WAT), where it induces

Table 2 | Animal studies that have assessed the role of Mg^{2+} deficiency on body weight and/or insulin sensitivity.

Study	Setup	Body weight Mg^{2+} deficient animals	Insulin sensitivity of Mg^{2+} deficient animals	Insulin sensitivity assessment
Reis *et al*. (77)	Rats, six-week low Mg^{2+} diet	↓	↑	↑ pIR ↑ pIRS ↑ IVITT
Bertinato *et al*. (78)	Rats, fourteen-week high fat & energy diet combined with moderately low or normal Mg^{2+}	↓	↔	↔ Plasma glucose ↔ OGTT
Chubanov *et al*. (64)	Mice, *Trpm6* knockout	↓	↑	↑ OGTT
Suarez *et al*. (79)	Rats, four-day low Mg^{2+} diet	↔	↓	↑ Plasma glucose ↓ IVGTT ↓ pIR
Murasato *et al*. (80)	Rats, three-week low Mg^{2+} diet	↓	No data	No data
Legrand *et al*. (81)	Rats, eight-week low Mg^{2+} diet	↓	Mixed	↑ IVGTT ↑ IVITT ↓ OGTT ↓ Insulin secretion
Chaudhary *et al.* (82)	Rats, three-month high/normal sucrose combined with low or normal Mg^{2+}	↓	↓	↓ Liver & muscle glucose uptake
Kimura *et al*. (83)	Rats, eight-week low Mg^{2+} diet	↓	↑	↓ Plasma glucose ↓ OSTT ↓ Insulin
Kurstjens *et al*. (84) (**Chapter 3**)	Mice, seventeen-week low Mg^{2+} diet combined with high fat	↓	↑	↓ Plasma glucose ↑ IPGTT ↑ IPITT

IPGTT: intraperitoneal glucose tolerance test, IPITT: intraperitoneal insulin tolerance test, IVGTT: intravenous glucose tolerance test, IVITT: intravenous insulin tolerance test, OGTT: oral glucose tolerance test, OSTT: oral sucrose tolerance test, pIR: insulin receptor phosphorylation, pIRS: insulin receptor substrate phosphorylation.

lipolysis (**Chapter 3**). In these hypomagnesemic mice the downstream effects of enhanced β_3-adrenergic receptor signaling were also elevated, including lipolysis, brown adipose tissue activity, body temperature and gluconeogenesis (**Chapter 3 and 4**). In this animal study it was not investigated whether the effects of Mg^{2+} were specific to the β_3-adrenergic receptors, or if the β_1 and β_2 receptors were also affected. However, other studies indicated that Mg^{2+} suppresses β-adrenergic signaling in general, both *in vitro* and *in vivo* (89-91). In the heart, Mg^{2+} suppressed the β_1-adrenergic receptor by inhibiting the $Ca_v1.2$ channel, thereby reducing the L-type Ca^{2+} current (91). In rats lower blood Mg^{2+} levels enhanced the effects of epinephrine on heart rate and cardiac output, which was mediated by the β_1-adrenergic receptor (92).

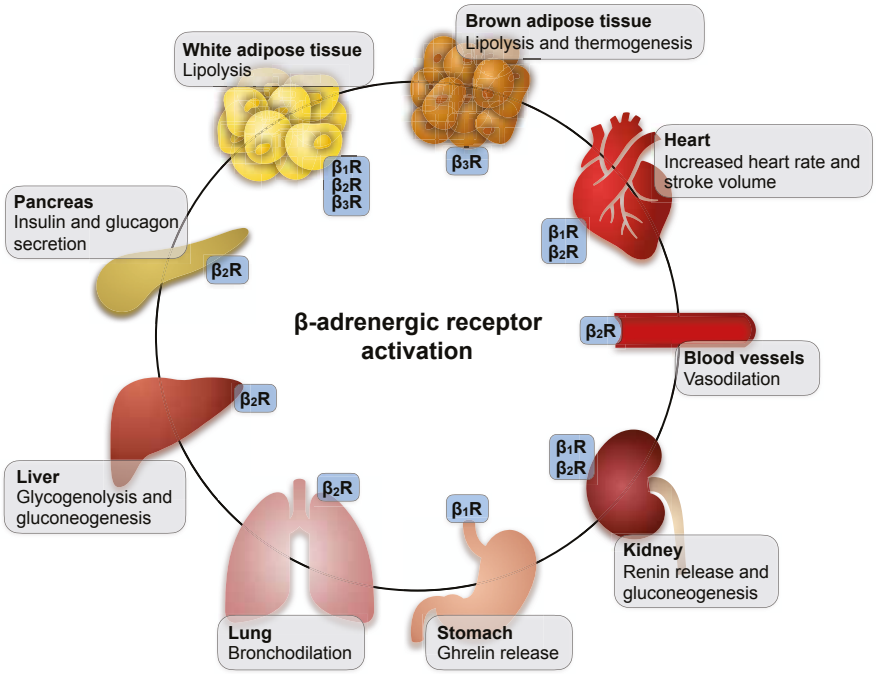


Figure 2 | Downstream effects of the activation of β-adrenergic receptors in different tissues

In white adipose tissue, the β_3R is dominant, whereas in kidney and heart the β_1R is dominant. β_nR: β_n-adrenergic receptor.

Besides suppressing β-adrenergic signaling, Mg^{2+} also reduced epinephrine secretion by blocking Ca^{2+} channels, and Mg^{2+} infusion in patients inhibited catecholamine release (93-95). Interestingly, supplying Mg^{2+} to animals before slaughter suppresses their stress levels (96, 97), whereas Mg^{2+} deficient animals have elevated serum and urine concentrations of catecholamines (80, 98). However, no significant differences in catecholamine levels were observed in our experiments (**Chapter 3**).

More mechanistic knowledge is required to understand how Mg^{2+} affects β-adrenergic receptor signaling and the secretion of catecholamines. Enhanced activation of the sympathetic nervous system is associated with obesity and insulin resistance, which are major risk factors for developing T2D (99-101). Could reduced blood Mg^{2+} levels increase the risk of T2D due to a higher activity of the sympathetic nervous system?

Serum magnesium and complications of type 2 diabetes

Reduced blood Mg^{2+} levels in T2D patients have been associated with an increased prevalence of comorbidities, including diabetic nephropathy, retinopathy, neuropathy and micro- and macrovascular disease, (69, 102-104). It is, however, unclear whether the higher prevalence of T2D-related complications is a direct consequence of lower blood Mg^{2+} levels or whether they are merely mediated by increased T2D disease severity.

In T2D patients, lower serum Mg^{2+} levels are an independent predictor of increased renal function decline (105, 106). Moreover, the serum Mg^{2+} concentration has been inversely associated with albuminuria, a marker of kidney disease (104). Inducing hypomagnesemia in mice using a restricted Mg^{2+} diet resulted in HFD-induced renal phospholipidosis (**Chapter 4**). These massive accumulations can result in proximal tubular damage, which could be an explanation for the correlation between serum Mg^{2+} levels and renal function decline. The enzymes that break down charged lipids, such as phospholipases for phospholipid breakdown, and neutral sphingomyelinases for the hydrolysis of sphingolipids, require Mg^{2+} for their function (107-109). Mg^{2+} deficiency could thereby disrupt the breakdown of charged lipids, resulting in lysosomal accumulation. Interestingly, sphingolipid metabolism was also disturbed in transient receptor melastatin 6 (*Trpm6*) knockout mice, which have severe hypomagnesemia, and elevated serum and liver levels of sphingolipids (110). No research, either *in vitro* or *in vivo,* has yet focused on the role of Mg^{2+} in sphingolipid metabolism.

Clinical implications

The data presented in this thesis have further substantiated the high prevalence of hypomagnesemia in T2D patients. Hypomagnesemia was demonstrated to be intrinsic to T2D, and not merely a consequence of polypharmacy (**Chapter 2**). These

studies have expanded our knowledge on both the development and consequences of hypomagnesemia in T2D, which can have several important clinical implications.

Multiple cohort studies have confirmed the importance of Mg^{2+} in the development of T2D. Increased dietary intake of Mg^{2+} was linked to a reduced risk of developing T2D in several large population-based cohort studies (111-113). Meta-analyses of these cohort studies demonstrated a relative risk for the development of T2D of 0.75-86 per increment of 100 mg/day Mg^{2+} intake (114-116). In addition, lower serum Mg^{2+} levels have been associated with an increased risk of developing T2D, with individuals in the lowest sextile of serum Mg^{2+} having a two-fold increased risk of developing T2D compared to patients in the highest sextile (117). Recently, a population-based study showed a hazard ratio of 1.18 for the development of T2D per 0.1 mmol/L decrease in serum Mg^{2+}, which was partially attributed to SNPs in key magnesiotropic genes, including CNNM2 and TRPM6 (76). Interestingly, feeding a HFD to Mg^{2+}-deficient mice reduced the expression of these genes (**Chapter 4**). Increasing the dietary intake of Mg^{2+}-rich foods and correcting blood Mg^{2+} levels could reduce the incidence of T2D in the population.

Lowering blood lipid levels could be a novel method of improving blood ionized Mg^{2+} levels. Hypertriglyceridemia, which is a common lipid abnormality in T2D patients, reduced Mg^{2+} levels by direct binding of Mg^{2+} to FFA molecules (**Chapter 6**). As hypertriglyceridemia induces hypomagnesemia, Mg^{2+} levels should be more closely monitored in severely hypertriglyceridemic T2D patients. Combining Mg^{2+} supplementation with lipid-lowering medication could be a more effective strategy to restore blood Mg^{2+} levels in hypertriglyceridemic patients.

The direct binding of Mg^{2+} to FFAs has implications for the calculation of the FEMg. The data presented in this thesis indicate that the fraction of ionized Mg^{2+} could fluctuate based on the lipid status of the patient. An increased blood FFA concentration could result in an erroneously calculated FEMg. Therefore, future clinical studies should investigate whether the lipid status of the patient has to be taken into consideration when calculating and interpreting the FEMg in hypertriglyceridemic T2D patients. Both clinicians and researchers have to be aware about these limitations when interpreting the FEMg in hypertriglyceridemic patients.

The data from this thesis clearly show that hypertriglyceridemia induces hypomagnesemia and add FFA as a new player in Mg^{2+} homeostasis. This knowledge will open up new research directions and could explain the cause of hypomagnesemia in different diseases (e.g. asthma, alcohol addiction and metabolic disorders), which should be a major focus of further clinical investigations. Recent population studies have indicated an increased risk for developing T2D with decreasing serum Mg^{2+} levels; underlining the clinical necessity of maintaining physiological Mg^{2+} levels. However, the molecular mechanisms underlying this increased risk remain largely unknown. The results presented in this thesis identify

dyslipidemia as one of the potential contributing factors. Supplementing T2D patients with oral Mg^{2+} has yielded conflicting findings (Table 2 in **Chapter 1**). Intervention trials should focus on supplementing only the hypomagnesemic T2D patients, which could yield stronger positive effects. As the intervention studies have only been performed in small populations, large clinical trials are required to investigate the potential of Mg^{2+} to reduce T2D disease progression and complications. Mg^{2+} supplementation would provide a cheap and safe treatment and prevention strategy.

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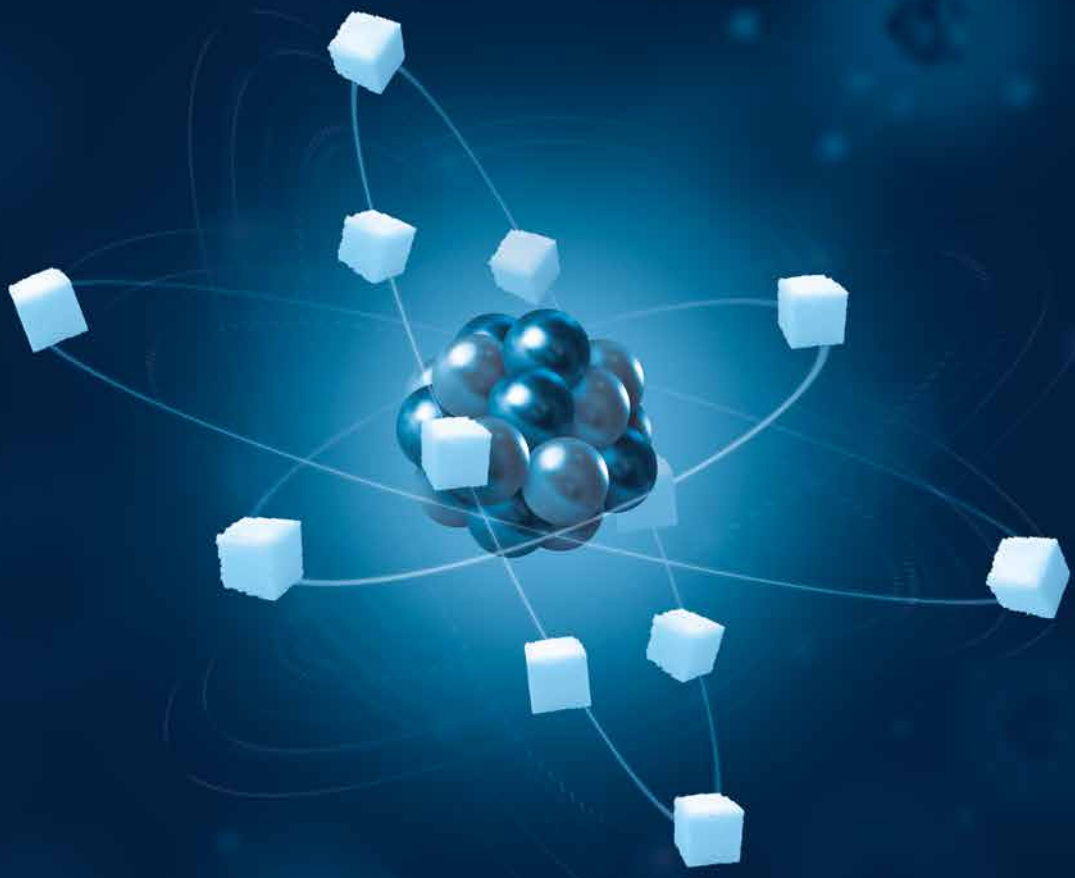


"One of the principal functions of a friend is to suffer the punishments (in a milder and symbolic form) that we should like, but are unable, to inflict upon our enemies"

– Aldous Huxley | Brave New World

9

Samenvatting

Samenvatting

Magnesiumtekort bij diabetes mellitus type 2: oorzaak of gevolg?

Magnesium (Mg^{2+}) is een mineraal dat essentieel is voor het functioneren van elke cel in het lichaam. Om een normale concentratie Mg^{2+} in het bloed te behouden werken drie organen nauw samen: de nieren, de darmen en de botten. Als er onvoldoende Mg^{2+} wordt opgenomen uit het voedsel door de darmen, of de nieren teveel Mg^{2+} verliezen via de urine, kan er een Mg^{2+}-tekort in het lichaam ontstaan. Dit heet hypomagnesiëmie (Mg^{2+}-concentratie in het bloed lager dan 0.7 mmol/L). De meest bekende symptomen van hypomagnesiëmie zijn spierkrampen, moeheid, hoofdpijn en hartritmestoornissen. Aangezien deze klachten vrij algemeen van aard zijn, worden de symptomen vaak niet toegeschreven aan een Mg^{2+}-tekort. Mede hierdoor blijft een hypomagnesiëmie vaak onopgemerkt bij de patiënt.

Hypomagnesiëmie komt vaak voor bij patiënten met diabetes mellitus type 2 (T2D), in de volksmond beter bekend als 'ouderdomssuikerziekte'. Het is echter niet duidelijk wat de oorzaak hiervan is. Bovendien is het grotendeels onbekend wat de gevolgen van een Mg^{2+}-tekort zijn voor het ziekteverloop en het ontstaan van comorbiditeiten in T2D patiënten. Het doel van het onderzoek in dit proefschrift is om de oorzaak van hypomagnesiëmie bij patiënten met T2D op te helderen. Verder is in deze studies uitgezocht wat voor effecten dit Mg^{2+}-tekort heeft op de energiehuishouding en op de stofwisseling van vetten in de context van T2D. Beide aspecten zijn bestudeerd op het niveau van moleculen, cellen, muizen en patiënten.

Welke factoren dragen bij aan de hypomagnesiëmie in type 2 diabetes patiënten?

Om gericht vervolgonderzoek mogelijk te maken is eerst uitgezocht welke T2D-gerelateerde factoren bijdragen aan een verlaagde bloed Mg^{2+}-concentratie. Aangezien T2D een complexe ziekte is met veel verschillende comorbiditeiten, komt polyfarmacie veel voor in de behandeling van T2D. Verschillende medicijnen kunnen de Mg^{2+}-balans beïnvloeden en kunnen daardoor mogelijkerwijs het Mg^{2+}-tekort in T2D patiënten verklaren. Om te onderzoeken welke factoren de Mg^{2+}-balans beïnvloeden is in **hoofdstuk 2** gebruik gemaakt van een observationeel cohort, het PARELSNOER cohort, bestaande uit 395 T2D patiënten met nauwkeurig beschreven kenmerken. Bij 31% van de patiënten werd een hypomagnesiëmie vastgesteld. Bovendien had meer dan 40% van de patiënten teveel Mg^{2+} in de urine (fractionele excretie van Mg^{2+} >4%), onafhankelijk van de concentratie van Mg^{2+} in het bloed.

Om te identificeren welke factoren bijdragen aan veranderingen in de bloed Mg^{2+}-concentratie van de patiënten is gebruik gemaakt van multivariabele regressieanalyses. Door middel van dit statistisch model kan worden achterhaald welke factoren het meest samenhangen met een verlaagde Mg^{2+}-concentratie in het

bloed, en dat bleek de concentratie van glucose en triglyceriden (een soort vetmolecuul) in het bloed te zijn. Het gebruik van een aantal geneesmiddelen, namelijk metformine, een glucoseverlagend geneesmiddel bij T2D, maagzuurremmers uit de groep van protonpompremmers en geneesmiddelen die de β-adrenerge receptor stimuleren waren ook geassocieerd met een lagere bloed Mg^{2+}-concentratie. De totale bijdrage van medicatie aan de hypomagnesiëmie in T2D was echter minimaal. Deze bevindingen tonen aan dat hypomagnesiëmie direct samenhangt met T2D, en dus niet verklaard kan worden door het gebruik van medicatie.

Wat zijn de gevolgen van een magnesiumtekort?

Om te onderzoeken wat de rol van Mg^{2+} is in de energiehuishouding en het vetmetabolisme is in **hoofdstuk 3** gebruik gemaakt van een muismodel representatief voor T2D. Muizen kregen gedurende 17 weken een dieet dat veel vet bevatte om de ontwikkeling van T2D na te bootsen. Het dieet werd gecombineerd met een lage of normale hoeveelheid Mg^{2+} in het voedsel om te onderzoeken wat de invloed is van een Mg^{2+}-tekort op de stofwisseling en op het ontstaan van T2D.

De muizen op een Mg^{2+}-beperkt dieet werden minder dik door het hoog vetdieet dan de muizen op een normaal Mg^{2+} dieet. Het lagere lichaamsgewicht ging gepaard met meerdere secundaire positieve aspecten, waaronder een verbeterde insuline-gevoeligheid, verminderde leververvetting en een lager glucosegehalte van het bloed. De Mg^{2+}-deficiënte muizen hadden echter een verhoogde triglyceriden-concentratie in het bloed, dat een gevolg was van een versnelde afbraak van vet in het vetweefsel. De afbraak van het vetweefsel wordt voornamelijk gereguleerd door activatie van de β_3-adrenerge receptor. De genexpressie van deze receptor was verhoogd in het vetweefsel van de Mg^{2+}-deficiënte muizen op een hoog vetdieet. De β_3-adrenerge receptor reguleert tevens de activiteit van het bruine vetweefsel, dat betrokken is bij de regulatie van de lichaamstemperatuur. Het bruine vet was actiever in de muizen, wat resulteerde in een verhoogde lichaamstemperatuur. Er was echter geen verschil in het energieverbruik tussen de muizen met een normale of lage hoeveelheid Mg^{2+} in het dieet.

Naast een verhoogde concentratie van vet in het bloed waren er ook uitgebreide stapelingen van vetten in de nier waarneembaar, hetgeen is beschreven in **hoofdstuk 4**. Deze vetzuurstapeling, genaamd fosfolipidose, kan een andere verklaring zijn waarom hypomagnesiëmie geassocieerd is met nierfalen in T2D. Deze bevindingen suggereren dat T2D patiënten met een lage bloed Mg^{2+} waarde een extra risico hebben op het ontwikkelen van dyslipidemie en nierschade.

Wat is het effect van type 2 diabetes op de magnesiumbalans?

Om het effect van T2D op de Mg^{2+}-balans te onderzoeken zijn twee verschillende muismodellen gebruikt. In **hoofdstuk 4** is er gebruik gemaakt van de hierboven beschreven muizen die een hoog vetdieet kregen. Al binnen vier weken daalde de bloed Mg^{2+}-concentratie van de muizen op een hoog vet dieet. Als de bloed Mg^{2+}-concentratie daalt kan de darm meer Mg^{2+} uit het dieet opnemen, en kan de nier meer Mg^{2+} uit de voorurine terugresorberen. Het belangrijkste kanaal voor gereguleerd Mg^{2+}-(re)absorptie is *Trpm6*. Ondanks de hypomagnesiëmie van de muizen was de expressie van *Trpm6* in de darm en nier niet verhoogd. Er werd echter geen verlies van Mg^{2+} waargenomen *via* de urine of de feces.

In **hoofdstuk 5** is gebruik gemaakt van een genetisch muismodel voor T2D, de db/db muis. Ook in dit model voor T2D onwikkelden de muizen een verlaagd bloed Mg^{2+}-niveau. De lagere bloed Mg^{2+}-concentratie zorgde in de db/db muizen voor een compensatoire verhoging in de genexpressie van *Trpm6* in zowel de darmen als nieren. Ook in deze muizen werd er geen groot verlies van Mg^{2+} in de urine geobserveerd, ondanks de hoge concentratie van glucose in de urine. Het behandelen van db/db muizen met metformine, dat geassocieerd was met lagere Mg^{2+}-waardes in de T2D patiënten van het PARELSNOER cohort, had geen effect op de Mg^{2+}-balans. Samenvattend laten de bevindingen zien dat hypomagnesiëmie een gevolg is van T2D, maar het blijft onduidelijk op welke wijze het Mg^{2+} exact verloren gaat.

De binding tussen magnesium en vrije vetzuren

De concentratie van triglyceriden in het bloed bepaalt voor een belangrijk deel de verlaagde bloed Mg^{2+}-concentratie, zowel bij T2D patiënten (**hoofdstuk 2**) als bij mensen met overgewicht (**hoofdstuk 6**). In **hoofdstuk 6** is aangetoond dat het induceren hoge vetwaardes in het bloed van muizen, door middel van een orale toediening van olijfolie, zorgde voor een directe daling van de concentratie Mg^{2+} in het bloed. Zodra de vetwaardes in het bloed weer daalden, herstelde de bloed Mg^{2+}-concentratie weer naar het oorspronkelijke niveau. Om te laten zien dat deze resultaten ook relevant zijn voor mensen, hebben we de Mg^{2+}-concentratie bepaald in het bloed van gezonde vrouwen die een portie slagroom hadden geconsumeerd. Hetzelfde fenomeen kon worden waargenomen: zodra het vetgehalte in het bloed steeg, daalde de concentratie Mg^{2+} in het bloed. In de muizen was een omgekeerd verband tussen de serumconcentraties van vrije vetzuren en Mg^{2+}, hetgeen suggereert dat Mg^{2+} direct bindt aan de negatief geladen vrije vetzuren. Inderdaad, zodra toenemende hoeveelheden vrije vetzuren werden toegevoegd aan verschillende Mg^{2+}-bevattende oplossingen, nam de concentratie vrij Mg^{2+} in deze oplossingen af. Deze resultaten maken duidelijk waarom er een negatieve correlatie bestaat tussen triglyceriden en Mg^{2+} in T2D patiënten en kunnen het vaak voorkomen van hypomagnesiëmie deels verklaren.

Perspectieven

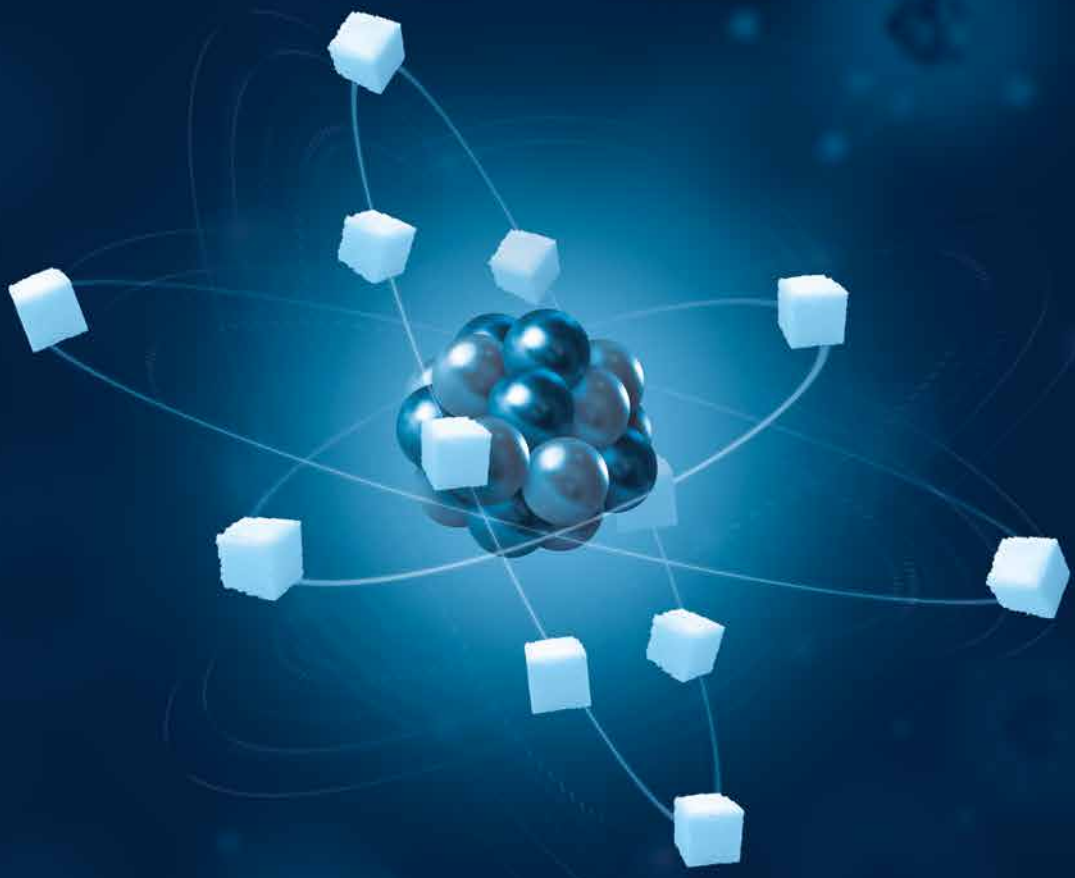
Dit proefschrift heeft onze kennis over het ontstaan van hypomagnesiëmie, en de gevolgen hiervan, verbreed in de context van T2D. Het opent nieuwe wegen voor onderzoek naar de functie van Mg^{2+} in de fysiologie alsmede de pathofysiologie.

Analyses in het PARELSNOER cohort van T2D patiënten toonden aan dat hypomagnesiëmie intrinsiek is aan T2D, en niet enkel het gevolg van medicatiegebruik. Bij deze analyse zijn een aantal belangrijke elementen niet geïncludeerd, namelijk: genetische variatie, de aanwezigheid van comorbiditeiten en de hoeveelheid Mg^{2+} in het dieet. Ook zouden de bloed Mg^{2+}-concentraties van de patiënten gemeten moeten worden gedurende meerdere jaren, zodat er conclusies kunnen worden getrokken aangaande causaliteit. Deze analyses zouden belangrijke inzichten geven over het ontstaan van hypomagnesiëmie, en de gevolgen hiervan op de stofwisseling, in T2D patiënten.

In dit cohort van T2D patiënten was er een sterk negatief verband tussen de concentratie van Mg^{2+} en triglyceriden in het bloed. De resultaten van dit proefschrift laten zien dat een Mg^{2+}-tekort zorgt voor hoge triglyceridewaardes in muizen op een hoog vetdieet. Aan de andere kant leiden hoge triglyceride– en vrije vetzuurconcentraties ook tot een sterke verlaging van de bloed Mg^{2+}-concentratie. Het zou interessant zijn om Mg^{2+}-concentraties te meten in mensen zonder T2D maar met hoge triglyceridenconcentratie in het bloed, om de effecten van hoge triglyceriden op de Mg^{2+}-balans te onderscheiden van insulineresistentie. Er zijn verschillende ziektebeelden en factoren die zorgen voor een verhoging in de vrije vetzuurconcentraties in het bloed, zoals overmatig alcoholgebruik, stress en medicijnen die de β-adrenerge receptoren stimuleren (behandeling voor astma). Een verlaagde bloed Mg^{2+}-concentratie wordt vaak gezien bij de voorgenoemde factoren. De bevinding dat Mg^{2+} bindt aan vrije vetzuren zou een verklaring kunnen zijn voor de hypomagnesiëmie in verschillende ziektebeelden. In dit proefschrift zijn echter enkel de korte termijneffecten van hypertriglyceridemie op de Mg^{2+}-concentratie bestudeerd. Of de binding van Mg^{2+} aan vrije vetzuren ook een verklaring vormt voor een chronische hypomagnesiëmie is in dit proefschrift niet onderzocht.

Muizen op een Mg^{2+}-beperkt dieet hadden een verhoogde genexpressie van de β_3-adrenerge receptor in het vetweefsel, dat gepaard ging met een verhoogde vetafbraak en vetverbranding. We hebben echter niet bestudeerd of de effecten specifiek waren voor de β_3-adrenerge receptor of dat het Mg^{2+}-tekort ook invloed had op andere β-adrenerge receptoren (de β_1 en β_2 receptoren). In de literatuur is beschreven dat Mg^{2+} de effecten van adrenaline, een stimulator van β-adrenerge receptoren, onderdrukt. Het zal relevant zijn te bestuderen hoe Mg^{2+} het β-adrenerge systeem kan onderdrukken in zowel fysiologie als in verscheidene stress-gerelateerde omstandigheden.

De onderzoeken die worden beschreven in dit proefschrift laten zien dat een Mg^{2+}-tekort zeer vaak voorkomt bij patiënten met T2D. In dit proefschrift zijn een aantal nieuwe factoren en mechanismen blootgelegd welke bijdragen aan de hypomagnesiëmie in patiënten met T2D. Bovendien is duidelijk geworden dat een Mg^{2+}-tekort diverse ingrijpende consequenties heeft op het metabolisme en de lipidenhuishouding. Dit proefschrift benadrukt het belang van Mg^{2+} in de context van T2D.



"Yesterday I was clever, so I wanted to change the world.
Today I am wise, so I am changing myself"

– Path of Exile | Rumi of the Vaal

10

List of abbreviations
List of publications
Curriculum vitae
Research data management
RIMLS portfolio

List of abbreviations

A

ACE	Angiotensin-converting enzyme
ADRB3	Adrenergic receptor beta 3
ANOVA	Analysis of variance
AQP2	Aquaporin-2
ATC	Anatomic therapeutic chemical
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
APOA1	Apolipoprotein A1
APOB	Apolipoprotein B
AUC	Area under the curve

B

BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin

C

Ca^{2+}	Calcium ion
CCD	Central commission for animal experiments
cDNA	Complementary DNA
CKD	Chronic kidney disease
CLDN	Claudin
CNNM2	Cyclin M2
CNNM4	Cyclin M4
CNT	Connecting tubule
CPT1-L	Carnitine palmitoyltransferase liver type
CPT1-M	Carnitine palmitoyltransferase muscle type

D

DBP	Diastolic blood pressure
DCT	Distal convoluted tubule
DKD	Diabetic kidney disease
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid

E

EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
eGFR	Estimated glomerular filtration rate
EGTA	Ethylene glycol tetraacetic acid
EL-VLDL	Extra large VLDL
EM	Electron microscopy
ESRD	End stage renal disease
eWAT	Epididymal white adipose tissue

F

FABP1	Fatty acid binding protein 1
FBS	Fetal bovine serum
FEMg	Fractional excretion of magnesium
FA	Fatty acid
FFA	Free fatty acid
FF-BSA	Free fatty acid-free bovine serum albumin
FPKM	Fragments per kilobase million

G

GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GLA	Alpha galactidose
GTT	Glucose tolerance test
GLUT	Glucose transporter
GO-term	Gene ontology term

H

HbA_{1c}	Glycated hemoglobin
HDL	High-density lipoprotein
H&E	Haematoxylin & eosin
HFD	High fat diet
HNF1B	Hepatocyte nuclear factor 1 homeobox B
HPLC	High-performance liquid chromatography
HR	Heart rate
HSL	Hormone-sensitive lipase

I

IDL	Intermediate-density lipoprotein
IHC	Immunohistochemistry
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue

K

K^+	Potassium ion
KDa	Kilo Dalton
KIM1	Kidney injury molecule
KLK1	Kallikrein 1

L

LCFA	Long-chain fatty acid
L-HDL	Large high-density lipoprotein
L-LDL	Large low-density lipoprotein
L-VLDL	Large very low-density lipoprotein
LDL	Low-density lipoprotein
LFD	Low fat diet
LPL	Lipoprotein lipase

M

MDRD	Modification of diet in renal disease
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesiumchloride
M-HDL	Medium high-density lipoprotein
M-LDL	Medium low-density lipoprotein
M-MLV	Murine leukemia virus
MQ	Milli-Q water
mRNA	Messenger ribonucleic acid
MRS2	Magnesium transporter 2
M-VLDL	Medium very-low density lipoprotein

N

Na^+	Sodium ion
NCC	The thiazide-sensitive NaCl cotransporter
NKCC2	Sodium-potassium-chloride cotransporter
NMR	Nuclear magnetic resonance

P

PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PEPCK1	Phosphoenuylpyruvate carboxykinase 1
PK-M	Pyruvate kinase muscle type
PLIN2	Perilipin 2
PPAR	Peroxisome proliferator-activated receptor
PPI	Proton pump inhibitor
PT	Proximal tubule
PVALB	Parvalbumin
PLC	Phospholipase C

R

RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RAAS	Renin-angiotensin-aldosterone system
RER	Respiratory exchange ratio
RPM	Round per minute
RT	Reverse transcriptase
RT-qPCR	Real time quantitative PCR

S

SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
S_{crea}	Serum creatinine
SLC12A3	Solute carrier family 12 member 3
SLC41A1	Solute carrier family 41 member 1
S-HDL	Small high-density lipoprotein
S-LDL	Small low-density lipoprotein

S_{Mg}	Serum magnesium
SMPD	Sphingomyelin phosphodiesterase
S-VLDL	Small very low-density lipoprotein
T	
T2D	Type 2 diabetes
TAL	Thick ascending limb
TRPM	Transient receptor melastatin member
U	
UCP1	Uncoupling protein 1
U_{crea}	Urinary creatinine
U_{Mg}	Urinary magnesium
V	
VLDL	Very low-density lipoprotein
VL-HDL	Very large high-density lipoprotein
VL-VLDL	Very large very low-density lipoprotein
VS-VLDL	Very small very low-density lipoprotein
W	
WAT	White adipose tissue
WHO	World health organization

List of publications

1. Kurstjens S, de Baaij JHF, Bouras H, Bindels RJM, Tack CJJ, Hoenderop JGJ. Determinants of hypomagnesemia in patients with type 2 diabetes mellitus. *Eur. J Endocrinol.* 176:11-19; 2017.
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*Authors contributed equally to this work

Curriculum vitae

Steef Kurstjens was born in Nijmegen, the Netherlands, on 10 July 1991. After graduating from high school in 2009 at the Stedelijk Gymnasium Nijmegen, he obtained his bachelor in Biomedical Sciences at the Radboud University Nijmegen in 2013. He then started his master Biomedical Sciences, majoring Human Pathobiology with a specialization-track Infectious Diseases. Steef performed his first master-internship at the department of Physiology at the Radboudumc, in the group of Prof. dr. P.M.T. Deen. Here, he investigated the molecular cause of diuresis and loss of aquaporin-2 expression in polycystic kidney disease and nephronophthisis. His second master-internship was based on a new collaboration between the department of Pediatric Infectious Disease, Radboudumc, group of Prof. dr. P.W.M. Hermans, and the department of Organic Chemistry, Radboud University, group of Prof. dr. ir. J.C.M. van Hest. Here, he focused on setting up a novel FACS-based method to isolate antigen-specific B-cells. After obtaining his master-degree in 2014, he joined the department of Physiology, Radboudumc, as a PhD candidate under the supervision of Prof. dr. J.G.J. Hoenderop, Prof. dr. C.J.J. Tack, Prof. dr. R.J.M. Bindels and dr. J.H.F. de Baaij. In his PhD project, he investigated the causal relationship between hypomagnesemia and type 2 diabetes mellitus. Steef presented his findings by oral and poster presentations at national conferences such as the Annual Dutch Diabetes Research Meeting in Oosterbeek, the Netherlands, and the New Frontiers Symposia of the Radboudumc in Nijmegen, the Netherlands. Moreover, he was awarded a travel grant by the RIMLS to attend and present a poster at the PhD symposium of the IRB institute of Barcelona. He also received an internalization grant by the Radboud University to attend and give a poster and oral presentation at the Experimental Biology meeting in Chicago, where he was nominated for the Campbell award in the Endocrinology and Metabolism section. He successfully completed the PhD training program of the RIMLS and supervised 9 undergraduate students from Biomedical Sciences, Medicine and Medical Biology studies.

Research data management

The data obtained in this thesis are archived according to the Findable, Accessible, Interoperable and Reusable (FAIR) principles.[1] Both the raw and processed data that were generated during my PhD were stored on Labguru, a digital lab book, which is centrally stored and backed up daily. All data on Labguru are accessible by the associated senior staff members (view only). Moreover, the data were directly stored on servers of the Radboud University, which are also backed up daily. The animal studies described in **Chapters 3,4, 5** and **6** were approved by the animal ethics board of the associated universities. The human studies presented in **Chapters 2** and **5** were conducted according to the principles expressed in the Declaration of Helsinki. The studies were approved by the Medical Ethics Committee of the Radboudumc and by the Institutional Review Board of the Franciscus Gasthuis & Vlietland Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam. The RNA-SEQ data from **Chapter 3** is available through the public functional genomics data repository GEO (gene expression omnibus). Data generated in this thesis are part of published articles and its materials and files are available from the corresponding authors upon reasonable request. To ensure general accessibility of the data, all filenames, primary and secondary data and descriptive files used to provide the final results are documented according to the protocol of the department of Physiology.

1. Wilkinson MD, Dumontier M, Aalbersberg IJ, *et al.* The FAIR Guiding Principles for scientific data management and stewardship. *Sci Data* 2016; 3: 160018

PhD portfolio

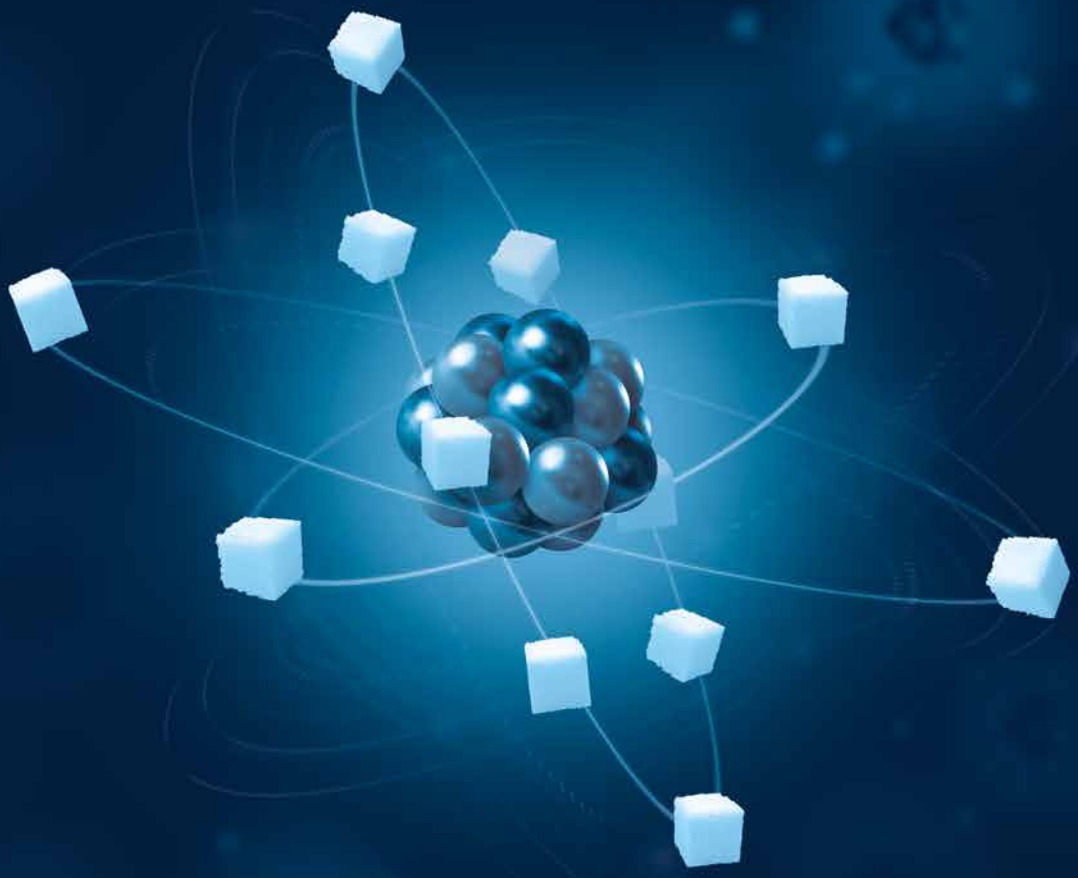
Institute for Molecular Life Sciences
Radboudumc

Radboud Institute for Molecular Life Sciences

	PhD period:
Name PhD student:	*01-11-2014 – 01-11-2018*
Steef Kurstjens	Promotors:
Department:	*Prof. dr. J.G.J. Hoenderop*
Physiology	*Prof. dr. C.J.J. Tack*
Research School:	Co-promotor:
Radboud Institute for Molecular Life Sciences	*Dr. J.H.F. de Baaij*

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops	2014	2
- RIMLS Graduate Course	2014	1
- Winterschool Nierstichting	2015	1
- Scientific Integrity course		
b) Seminars & lectures		
- RIMLS Radboud Research Rounds / Lecture Series	2015-2018	1.4
- RIMLS Seminars	2015-2018	1.5
- RIMLS Spotlights / Kidney Theme Meetings	2015-2018	1.9
- RIMLS Technical Forum on statistics	2016	0.1
- RIMLS Meet the expert: Seahorse	2018	0.2
c) (Inter)national Symposia & Congresses		
- RIMLS Radboud New Frontiers #	2014-2017	2
- RIMLS PhD retreat *#	2015-2018	4
- Radboud Science Day *	2015	0.7
- Internal Medicine Science Day *	2015	0.5
- Experimental Biology Meeting *#	2017	2
- Annual Dutch Diabetes Research Meeting *	2016-2017	1
d) Other		
- Peer review scientific articles	2015-2018	0.3
- Mentor/supervising of foreign PhD student	2016	0.5
- Meeting international professors	2017	0.5
TEACHING ACTIVITIES		
e) Lecturing		
- Teaching Medical course: Capita Selecta	2015	0.5
- Open House University: Biomedical Sciences study	2016	0.2
- Open House RIMLS	2016	0.2
- Mentor first year students	2018	0.1
- Teaching Biomedical Science: Gordon Syndrome	2016-2017	0.4
- Teaching Biomedical Science: learning community	2016	0.2
- Teaching Biomedical Science: supervise practical	2017	0.2

TEACHING ACTIVITIES		
f) Students		
- Supervision Marlies Fennis (Master Medicine)	2015	2
- Supervision Frank Krewinkel (Master Medicine)	2016	1
- Supervision Mares Voet (Master Biomedical Sciences)	2016-2017	2
- Supervision Nikki van Hooft (Bachelor Biomedical Sciences)	2017	1
- Supervision Veronica Garzero (Master Biomedical Sciences)	2017-2018	2
- Supervision Albert Ruiz Llombart (Master Biomedical Sciences)	2017-2018	2
- Supervision Olivier Schäffers, Joep van Walsum and Myrthe Swart	2016-2017	1.5
- Supervision of 'meet-the-PhD' -students	2017	2
TOTAL		**35.9**



"Our envy of others devours us most of all.
Rub your eyes and purify your heart
- and prize above all else in the world
those who wish you well.
Do not hurt them or scold them,
and never part from any of them in anger"

– Aleksandr Solzhenitsyn | The Gulag Archipelago

11

Dankwoord | Acknowledgments

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