Metformin

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Metformin

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Chapter 1.

Introduction

INTRODUCTION

Metformin is a fascinating medicine. Since publication of the major results of the United Kingdom Prospective Diabetes Study (UKPDS) in 1998,¹ metformin is the preferred initial pharmacological treatment for patients with type 2 diabetes.² Metformin has favourable effects on glycaemia control, insulin requirements, weight development and cardiovascular outcomes.^{1,3} Last year, we celebrated the 60th anniversary of the first clinical use of metformin for diabetes (figure 1). Even after sixty years, there is still much to discover about mechanisms of action and clinical effects of metformin.

Figure 1. Diabetologia dedicated a special issue to 60 years of metformin use (September 2017)

METFORMIN: HISTORICAL PERSPECTIVE

Metformin is an oral glucose-lowering drug from the biguanide class. The history of biguanides is linked to herbal medicine. In medieval Europa, *Galega officinalis* (a.k.a. goat's rue or French lilac; figure 2), a plant rich in guanidine, was ascribed benefits against worms, plague, snake bites and fever.

In 1772, John Hill recommended *Galega* to treat conditions now attributed to diabetes, like thirst and frequent urination.⁴ In the beginning of the 20th century, guanidine was reported to reduce blood glucose in animals. However, because of toxicity, guanidine was not suitable for human use.⁵ In 1922, Werner and Bell synthesized metformin by combining two guanides into a biguanide (dimethylbiguanide), but it was more than 25 years later after a combination of circumstances that the real potential of the drug was discovered. In the 1940s, the guanidine-based drug proguanil (paludrine, component of Malarone) was

Figure 2. *Galega officinalis* (H. Zell/Wikimedia Commons)

developed as an antimalarial treatment. In search for other antimalarial drugs, metformin was reported not only to be helpful in treating an influenza outbreak in the Philippines, but also to lower blood glucose in some of the patients.⁵ In 1957, dr. Jean Sterne was the first to publish a study the effect of metformin on glucose levels in animals.⁶ Soon after, metformin became available in the UK. Because of the risk of lactic acidosis, especially with phenformin, biguanides were taken off the market in the USA. Although the incidence of lactic acidosis amongst users of metformin was much lower, it was not until 1994 that the U.S. Food and Drug Administration (FDA) approved metformin for clinical use.⁵ Only four years later, metformin became the number one choice in the treatment of type 2 diabetes, after publication of the UKPDS.¹

Metformin: mechanisms of action

Metformin has several well established effects. It lowers blood glucose levels by reducing the hepatic glucose output by inhibiting hepatic gluconeogenesis and it improves insulin sensitivity. In addition, it reduces insulin requirements and prevents weight gain.³ A recent study showed that metformin reduces gluconeogenesis by inhibiting mitochondrial redox reactions.⁷ Activation of the cell's principle energy sensor, AMP-activated protein kinase (AMPK), by metformin improves the insulin sensitivity. Metformin Inhibits complex I of the respiratory chain in the mitochondria, which results in an increase in ADP and AMP levels.⁸ This altered cellular energy charge is detected by AMPK. AMPK has several favorable effects, like reduction of lipogenesis, which improves insulin sensitivity.⁹ Apart from

the liver, metformin has less pronounced effects in muscle and fat tissue, by different mechanisms.¹⁰ Furthermore, novel evidence shows that a significant part of the effect of metformin is caused by action in the intestines. Furthermore, novel evidence shows that a significant part of the effect of metformin is caused by direct action in the intestines. Metformin is absorbed in the duodenum and jejunum with a bioavailability of approximately 50%. However, a study showed that a delayed-release metformin formulation targeting the ileum, a region of the gut where the absorption of metformin is low, was as effective at lowering glucose concentrations as the regular immediate-release metformin, despite achieving lower metformin plasma concentrations.¹¹ Furthermore, metformin may alter the intestinal microbiome.¹² A limitation of the mentioned studies is that most of them are not studies in human, but in vitro studies or animal studies with doses varying from therapeutic to supratherapeutic. Therefore, although progress has been made, the exact mechanisms of metformin are still not fully understood.

Metformin: clinical effects

Metformin is the first choice as treatment in patients with type 2 diabetes who are not able to achieve glycaemic targets despite lifestyle interventions.² It is effective, both as single treatment as in combination with other glucose-lowering drugs. Metformin is generally well tolerated, cheap, has a good safety profile and prevents weight gain. In type 2 diabetes, metformin reduces mean HbA1c by 1.0 to 1.5%-point.¹³ In addition, metformin prevents development of type 2 diabetes, when added to lifestyle intervention in nondiabetic persons with elevated fasting and post-glucose load plasma glucose concentrations.¹⁴ The Hyperinsulinemia: the Outcome of its Metabolic Effects (HOME) study showed that the use of metformin was associated with prevention from macrovascular disease with a number needed to treat to prevent one macrovascular end point of 16.1 (95% CI, 9.2-66.6).³ However, a recent meta-analysis did not find a significant effect on cardiovascular disease, possibly due to a paucity of additional randomised controlled trials.¹⁵ Therefore, uncertainty remains whether metformin really does reduce the risk of cardiovascular disease in type 2 diabetes.

Metformin: side effects

In the HOME study, 15% of individuals randomised to metformin did not finish the 4.3 year study because of side effects, as compared to 8% in the placebo group (Figure 3). The most common side effects were gastrointestinal complaints. Possible causes are a reduction of

Figure 3. Design of the HOME trial (A) and flow diagram of recruitment and retention of patients during the study (B).

ileal absorption of bile acids by metformin or accumulation of metformin in the intestine.¹⁶ Vitamin B12 deficiency is another side effect of metformin, although the real clinical impact has yet to be sorted out. Finally, for many years the most feared side effect was the aforementioned lactic acidosis. However, several studies and a meta-analysis showed that the risk of lactic acidosis with metformin was negligible, especially in patients with an estimated glomerular filtration rate (eGFR) ≥ 30 ml/min.^{17,18}

Metformin & pharmacogenetics: influence of genetic variation on the effects of metformin

Therapeutic response to metformin is highly variable. During the last decade, numerous studies of the influence of pharmacogenetic variation on the effects of metformin have been published.¹⁹ At first, research mostly focused on variation in genes involved in the transport of metformin, with several candidate gene studies. However, results remained inconclusive, because of lack of power and our incomplete understanding of the mechanism and actions of metformin. Analyses with genome-wide association studies look more promising, but larger samples are needed. A promising initiative was the foundation of the Metformin Genetics (MetGen) Consortium, an international collaboration with a collection of numerous cohorts with over 10,000 individuals treated with metformin, in whom metformin response can be defined.

AIMS OF THIS THESIS

This thesis describes results of four post-hoc analyses of the HOME trial and results of two analyses of the cohorts of the MetGen Consortium. The HOME trial is the largest randomised controlled trial investigating the long-term effects of metformin versus placebo in patients with type 2 diabetes treated with insulin. Main results of the trial showed that metformin versus placebo, when added to insulin in patients with type 2 diabetes, not only improved glycaemic control, reduced insulin requirements and prevented weight gain, but also protected against the development of macrovascular disease.³ Figure 3 shows the trial design and recruitment plus retention of patients.

The following research questions are addressed in subsequent chapters:

- a. *Does long-term treatment with metformin increase serum levels of methylmalonic acid (MMA) and is this increase of MMA (if any) associated with onset or progression of neuropathy?*
Several trials and observational studies show that the use of metformin is associated with lower serum levels of vitamin B12 (B12) which is thought to be caused by impaired B12 absorption.²⁰ Although the literature on metformin treatment and serum vitamin B12 is abundant, we do not know whether this results in B12 deficiency at the tissue level – this is in fact extremely controversial. Current guidelines recognize B12 deficiency as a disadvantage of metformin, and are waiting for more clinical evidence to make new recommendations on the detection and prevention

of vitamin B12 deficiency.² Therefore, we analysed, in the HOME trial, the effects of metformin vs placebo on serum levels of MMA, the gold standard biomarker for tissue B12 deficiency, and on neuropathy.

Chapter 2: Long-term treatment with metformin in type 2 diabetes and methylmalonic acid

- b. *Does treatment with metformin decrease serum levels of vitamin D?*

Metformin reduces ileal absorption of bile acids.¹⁶ This may contribute to several effects of metformin like prevention of weight gain, gastrointestinal complaints and the development of B12 deficiency. It is unknown whether metformin may cause deficiency of fat-soluble vitamins like vitamin D due to malabsorption of bile acids. Vitamin D deficiency occurs frequently in older patients up to more than 50% of cases, and increases the risk of osteoporosis and related fractures.²¹ Therefore, we analysed the effects of metformin versus placebo on serum levels of vitamin D on the short and longer term.

Chapter 3: Long-term treatment with metformin in type 2 diabetes and vitamin D levels

- c. *Does treatment with metformin affect caloric intake?*

A major side effect of insulin therapy in type 2 diabetes is weight gain, which is associated with increasing insulin resistance and cardiovascular risk.²² Metformin prevents weight gain in type 2 diabetes patients.³ However, the mechanisms involved are still unknown. Less energy intake, malabsorption and metabolic effects may contribute. Studies of the effects of metformin on energy intake in human are sparse and inconclusive. Small sample sizes, study designs with short term follow-up or without control groups and/or low doses of metformin used, all together, limit definite conclusions to be drawn. Therefore, we investigated whether treatment with metformin affected energy intake in patients in the HOME trial.

Chapter 4: Metformin-associated prevention of weight gain in insulin-treated type 2 diabetic patients cannot be explained by decreased energy intake

- d. *Does genetic variation of genes involved in the transport or pharmacokinetics of metformin influence the effect of metformin?*

The response to metformin is highly variable, even after adjusting for adherence to medication. Despite the progress in the understanding of the pharmacokinetics and pharmacodynamics of metformin, the variability in response remains only partly understood. Variation in the expression of genes involved in the transport and action of metformin may influence the response of metformin. Over the past few years, numerous studies showed associations between variation of genes involved in the transport or pharmacokinetics of metformin and the response to metformin. However, the results of most studies on polymorphisms are still inconclusive, as several studies failed to replicate the associations. Small sample sizes, study designs without control groups, the low doses of metformin used and/ or variety in ethnicity, all together, limit definite conclusions to be drawn. Therefore, we analysed the association between genetic variations and the effect of metformin in a post hoc analysis of the HOME trial.

Chapter 5: A gene variant near ATM affects the response to metformin and metformin plasma levels

- e. *Is it possible to identify new genetic variants that have impact on the response to metformin with a GWAS approach in patients of the MetGen Consortium?*

Hypothesis-driven studies of pharmacokinetic variants have shown no consistent results. The only previous GWAS with metformin showed an association with rs11212617 near the ATM locus.²³ With this study, we extended the GWAS with the patients of the MetGen Consortium.

Chapter 6: Variation in the glucose transporter gene SLC2A2 is associated with glycaemic response to metformin

- f. *Does genetic variation of genes involved in the transport of metformin influence the effect of metformin? A meta-analysis.*

Therapeutic response to metformin is highly variable, in part likely due to genetic factors. To clarify the significance of variants of metformin transporter genes in glycaemic response to metformin in T2D, we performed a large-scale meta-analysis across the cohorts of the MetGen Consortium.

Chapter 7: Variants in Pharmacokinetic Transporters and Glycaemic Response to Metformin

Chapter 8 discusses the present findings, limitations and implications of the study and offers a perspective to future research.

REFERENCES

1. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet*. 1998;352(9131):854-65.
2. American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2018. *Diabetes Care*. 2018;41(Suppl 1):S73-85.
3. Kooy A, de Jager J, Leher P, et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch Intern Med*. 2009;169(6):616-25.
4. Hill J. The vegetable system. London: ; 1772. p.54.
5. Bailey CJ. Metformin: historical overview. *Diabetologia*. 2017;60(9):1566-76.
6. Sterne J. Du nouveau dans les antidiabétiques. La NN diméthylamine guanyl guanidine (N.N.D.G.). *Maroc Med*. 1957;36:1295-6.
7. Madiraju AK, Erion DM, Rahimi Y, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature*. 2014;510(7506):542-6.
8. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001;108(8):1167-74.
9. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia*. 2017;60(9):1577-85.
10. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. *Cell Metab*. 2014;20(6):953-66.
11. Buse JB, DeFronzo RA, Rosenstock J, et al. The Primary Glucose-Lowering Effect of Metformin Resides in the Gut, Not the Circulation: Results From Short-term Pharmacokinetic and 12-Week Dose-Ranging Studies. *Diabetes Care*. 2016;39(2):198-205.
12. Forslund K, Hildebrand F, Nielsen T, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature*. 2015;528(7581):262-6.
13. Sanchez-Rangel E, Inzucchi SE. Metformin: clinical use in type 2 diabetes. *Diabetologia*. 2017;60(9):1586-93.
14. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346(6):393-403.

15. Griffin SJ, Leaver JK, Irving GJ. Impact of metformin on cardiovascular disease: a meta-analysis of randomised trials among people with type 2 diabetes. *Diabetologia*. 2017;60(9):1620-9.
16. McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. *Diabetologia*. 2016;59(3):426-35.
17. Salpeter SR, Greyber E, Pasternak GA, Salpeter EE. Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus. *Cochrane Database Syst Rev*. 2010;(4):CD002967. doi(4):CD002967.
18. Inzucchi SE, Lipska KJ, Mayo H, Bailey CJ, McGuire DK. Metformin in patients with type 2 diabetes and kidney disease: a systematic review. *JAMA*. 2014;312(24):2668-75.
19. Florez JC. The pharmacogenetics of metformin. *Diabetologia*. 2017;60(9):1648-55.
20. de Jager J, Kooy A, Lehert P, et al. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. *BMJ*. 2010;340:c2181.
21. Halfon M, Phan O, Teta D. Vitamin D: a review on its effects on muscle strength, the risk of fall, and frailty. *Biomed Res Int*. 2015;2015:953241.
22. DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care*. 1991;14(3):173-94.
23. GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group, Wellcome Trust Case Control Consortium 2, Zhou K, et al. Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. *Nat Genet*. 2011;43(2):117-20.

Chapter 2.

Long-term treatment with metformin in type 2 diabetes and methylmalonic acid

LONG-TERM TREATMENT WITH METFORMIN IN TYPE 2 DIABETES AND METHYLMALONIC ACID: POST HOC ANALYSIS OF A RANDOMIZED CONTROLLED 4.3 YEAR TRIAL

Mattijs Out, Adriaan Kooy, Philippe Lehert, Casper A Schalkwijk, Coen D A Stehouwer

ABSTRACT

Aims: Metformin treatment is associated with a decrease of serum vitamin B12, but whether this reflects tissue B12 deficiency is controversial. We studied the effects of metformin on serum levels of methylmalonic acid (MMA), a biomarker for tissue B12 deficiency, and on onset or progression of neuropathy.

Methods: In the HOME trial, 390 insulin-treated patients with type 2 diabetes were treated with metformin or placebo for 52 months. In a post hoc analysis, we analyzed the association between metformin, MMA and a validated Neuropathy Score (NPS).

Results: Metformin vs placebo increased MMA at the end of the study (95%CI: 0.019 to 0.055, $p=0.001$). Mediation analysis showed that the effect of metformin on the NPS consisted of a beneficial effect through lowering HbA1c (-0.020 per gram year) and an adverse effect through increasing MMA (0.042 per gram year), resulting in a non-significant net effect (0.032 per gram year, 95% CI: -0.121 to 0.182 , $p=0.34$).

Conclusion: Metformin not only reduces serum levels of B12, but also progressively increases serum MMA. The increase of MMA in metformin users was associated with significant worsening of the NPS. These results provide further support that metformin-related B12 deficiency is clinically relevant. Monitoring of B12 in users of metformin should be considered.

INTRODUCTION

Metformin is the cornerstone of pharmacological treatment in patients with type 2 diabetes.¹ Several intervention studies have shown favourable effects of metformin on glycemic control, weight development, insulin requirements and cardiovascular outcomes.^{2,3} In

addition, metformin use is associated with a decreased risk of cancer.⁴ Nevertheless, metformin also has side effects, notably gastrointestinal complaints. Furthermore, several trials and observational studies show that the use of metformin is associated with lower serum levels of vitamin B12 (B12)^{5,6} which is thought to be caused by impaired B12 absorption.⁷

In the *Hyperinsulinaemia: the Outcome of its Metabolic Effects* (HOME) study (ClinicalTrials.gov identifier NCT00375388), the largest randomized controlled trial investigating the long-term effects of metformin versus placebo in patients with type 2 diabetes treated with insulin, we showed that metformin versus placebo, when added to insulin in patients with type 2 diabetes, improved glycemic control, reduced insulin requirements and prevented weight gain.³ However, the HOME study also showed that metformin is associated with a lowering of serum B12 that is progressive over time, and accompanied by an increase in serum homocysteine, which is suggestive of tissue B12 deficiency.^{8,9} B12 deficiency may cause irreversible neuropathy,¹⁰ which may mimic diabetic neuropathy.¹¹ Studies of the effects of metformin on B12 levels and neuropathy are sparse and inconclusive,¹²⁻¹⁶ possibly because it may be difficult to prove any B12 deficiency-related neuropathic effects of metformin, as metformin may have neuroprotective effects through its antihyperglycemic actions.¹⁷ Current guidelines do recognize the risk of B12 deficiency as a disadvantage of metformin.¹ However, they just advise to consider periodic measurement of B12 levels, without making strong recommendations, as it remains unknown to what extent the effect of metformin on B12 may contribute to the development of clinically relevant endpoints, such as neuropathy and anemia.¹⁸

To further investigate these safety issues of the use of metformin, we analyzed, in the HOME trial, serum levels of methylmalonic acid (MMA), a more specific biomarker for tissue B12 deficiency than homocysteine,¹⁹ and studied whether an increase of MMA (if any) was associated with onset or progression of neuropathy.

MATERIALS AND METHODS

Patients

The HOME trial included 390 patients aged 30–80 years with type 2 diabetes who were receiving treatment with insulin, as previously described.^{3,20} Figure 1 shows the trial design and recruitment plus retention of patients.^{3,20}

Figure 1. Trial design (a) and flow diagram of recruitment and retention of patients in both groups (b).
*Including one patient with B12 deficiency at baseline.

Study design, randomization, interventions and follow-up

The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, the Netherlands). All participants provided written informed consent before inclusion. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial was conducted in accordance with Good Clinical Practice (CPMP/ICH/135/95; 1996) and with the Declaration of Helsinki (revised version, 2000).

Patients were randomly allocated to either placebo or metformin (in identically looking boxes). Either metformin 850 mg or placebo (one to three times daily) was added to insulin therapy. No other glucose-lowering drugs were used, allowing comparison of metformin with placebo in patients receiving insulin but no other antidiabetic agents.

The trial consisted of a 12 week pre-randomization phase, in which patients were treated with insulin only and concomitant medication was discontinued and a 4.3 year long-term treatment phase, at the beginning of which patients were randomized to receive either metformin or placebo in addition to insulin therapy (figure 1). Patients were recruited between November 1998 and July 1999; follow-up ended in 2003.

Visits and data collection

Patients visited the clinics at the start of the pre-randomization phase (three months before randomization), at baseline (for randomization to metformin or placebo), one month after baseline (to check tolerance of drug titration), and subsequently every three months until the end of the trial. During these visits, a physical examination was carried out, a medical history was taken, and laboratory investigations were performed. At every visit, patients were asked whether their medication had changed and whether they used over-the-counter drugs or supplements. Polyneuropathy was evaluated every three months by two well-trained medical doctors. For evaluation of neuropathy, the Valk Score was used, a validated clinical neuropathy score, with which neuropathy was scored as absent (0 points); mild (1–9 points); moderate (10–18 points) or severe (19–33 points; the score model is described in the Appendix Table A.1).²¹

LABORATORY INVESTIGATIONS

Sample preparation

Blood samples were drawn at baseline and after 4, 16, 28, 40, and 52 months (4.3 year), and stored at -80°C until analysis. Before analysis, plasma samples were thawed and mixed thoroughly. To provide an internal standard, 25 μl plasma was mixed with 10 μl [d3]-MMA (2.56 $\mu\text{mol/l}$) and deproteinized with 100 μl ice-cold 0.25% (v/v) formic acid in acetonitrile. Samples were subsequently centrifuged for 15 min at 14000 rpm at a temperature of 4°C . Ten microliter supernatant was injected for Ultra-Performance Liquid Chromatography tandem Mass-Spectrometry (UPLC/MSMS) analysis.

UPLC tandem MS

MMA was analyzed by UPLC (Acquity UPLC, Waters, Milford, USA) and detected in Electrospray Ionization negative Multiple Reaction Monitoring (ESI negative MRM) mode using a Xevo TQ MS (Waters, Milford, USA). MMA was analyzed on a Hydrophilic Interaction Liquid Chromatography column (Atlantis HILIC Silica, 50 x 2.1 mm, 3 μm , Waters, Milford, USA) using an isocratic separation of 10 mmol/l ammonium formate in water/acetonitrile (20/80, v/v%) at a flow rate of 300 $\mu\text{l/min}$. The injection volume was 10 μl and the column temperature was set at 30°C . Quantification of MMA was performed by calculating the peak area ratio of the unlabeled peak area to the internal standard peak area ([d3]-MMA). The MRM transitions for MMA and [d3]-MMA were respectively $117.0 > 73.0$ and $120.0 > 76.0$. Electrospray ionization was done at a capillary voltage of 0.25 kV, a source temperature of 150°C and a desolvation temperature of 600°C . For qualitative and quantitative analysis Masslynx software (V4.1, SCN 644, Waters, Milford, USA) was used.

Method validation

Linearity was determined by adding standard solution of MMA to water and to 10 different plasma samples. A six-point calibration curve was prepared for MMA (0–1 $\mu\text{mol/l}$). The peak area ratio of MMA and [d3]-MMA multiplied by the concentration of the internal standard were plotted as a function of the concentration. Calibration curves for MMA were linear over the described concentration levels ($r^2 > 0.99$). Mean slope (response factor) for MMA was 0.8466 (CV, 3.9%). For the evaluation of the inter- and intra-assay variation, a pooled plasma sample was analyzed on 14 different days (inter-assay) and 10 times on the same

day (intra-assay). Inter- and intra-assay variation for MMA was 5.1% and 3.6%, respectively. Concentrations of B12 were determined in serum by an electrochemiluminescence immunoassay (ECLIA) using the competition principle, as described previously.⁹

STATISTICAL ANALYSIS

Our main objective was to assess the effect of metformin on MMA. Our main selection was the set of all randomized patients with exclusion of patients with B12 deficiency at baseline ($\text{B12} < 150 \text{ pmol/l}$ and $\text{MMA} > 0.270 \mu\text{mol/l}$ ¹⁹; $n=7$) and patients on B12 supplementation from the time-point at which they received it ($n=9$). The main analysis was conducted through a mixed model assessing the effects of time, treatment and their interactions on MMA, with imputation of missing data (details are described in the appendix A.1.2 and A.1.3). In separate mixed models, we tested the supplementary main effects of age, sex, duration of diabetes, body mass index, estimated glomerular filtration rate (eGFR, according to the CKD-EPI equation),²² glycated haemoglobin (HbA1c, %) or use of co-medication (calcium supplements and proton pump inhibitors),^{23,24} and their interaction with the effect of metformin on MMA. In addition, we assessed the effect of the cumulative treatment dosage (gram x years) on MMA to provide further insight in the possible effects of treatment dosage and time.

We conducted three separate secondary analyses for sensitivity purposes (results are shown in the appendix section A.2) (1): We performed a t-test on the final values of MMA without baseline adjustment (2). We tested the treatment effect on the final values of MMA, adjusting for baseline values (analysis of covariance; ANCOVA) (3). As the exclusion of patients with B12 deficiency at baseline and patients on B12 supplementation may affect the intention to treat analysis, we repeated our mixed model analyses on the full set of all randomized patients without exclusion.

Our second objective was the assessment of metformin effect on neuropathy score. We hypothesized that metformin may on the one hand protect against neuropathy by its glucose-lowering effects, and on the other hand may cause neuropathy through the development of B12 deficiency. We tested this putative combined mediation effect of glucose lowering effects and B12 deficiency on neuropathy with a Multivariate Mediation effect, implemented by a Structural Equation Modeling (SEM) approach (details are described in the appendix section A.1.4).

The analysis of mixed models was carried out with R release 3.03 package; structural equation modeling was conducted with AMOS (release 19.0, IBM/SPSS). Two-sided P-values < 0.05 were considered statistically significant; “±” denotes standard deviation.

Sample Size and Power Analysis

Original sample size calculations for this trial were based on expected differences in the occurrence of disease-related endpoints, as described previously²⁰ Post-hoc power calculations for MMA and neuropathy score showed that with our sample size, observed standard deviations of 0.10 µmol/l for MMA and of 5 for the neuropathy score, and observed correlations between baseline and final values approximated to R=0.6 for MMA and to R=0.5 for the neuropathy score, differences between the two groups of 0.033 µmol/l for MMA and of 2 for the neuropathy score should be detectable at a two-sided 0.05 confidence level with a power of at least 0.95.

RESULTS

General trial results

A total of 390 individuals provided written informed consent and enrolled into the trial; 196 individuals were randomly assigned to receive metformin and 194 to receive placebo. Patients in the metformin group were slightly older than those receiving placebo (63.6 ± 9.6 vs. 59.1 ± 11.0 years) and had a more extensive cardiovascular disease history (1.17 vs. 0.92). All other characteristics were comparable between the treatment groups (Table 1).

Of the 390 included patients, 277 (71%) completed the HOME trial. The main outcomes of this trial have been reported previously, including the effects of metformin on HbA1c values and serum B12 levels.^{3,9} Those who did and did not complete the study did not differ with respect to duration of diabetes, previous occurrence or severity of cardiovascular disease, age or weight. 1880 laboratory samples were available for measurement of MMA (915 metformin, 965 placebo). At the final visit, laboratory samples were available for 242 patients (118 metformin, 124 placebo). The mean dose in the metformin group was 2050 mg during the trial. There was no difference between the groups in the use of proton pump inhibitors, levodopa, anti-neuropathic medication or supplementation of B12 or calcium.

	Placebo (n=184)	Metformin (n=191)
Demographic Characteristics, mean (SD)		
Men/ women, No.	97/97	81/115
Age (years)	59 (11)	64 (10)
Duration of diabetes (years)	12 (8)	14 (9)
Insulin treatment (years)	6 (6)	7 (8)
Current smoking, No. (%)	59 (30)	38 (19)
Use of alcohol (U/day)	0.7 (1.2)	0.7 (1.2)
Concomitant medication (n (%))		
Acetylsalicylic acid	82 (42)	78 (40)
Lipid lowering drugs	31 (16)	31 (16)
Blood pressure-lowering drugs	76 (39)	92 (47)
B12 supplementation	5 (2.6)	3 (1.5)
Calcium supplements	2 (1.0)	3 (1.5)
Levodopa	0 (0)	0 (0)
Proton pump inhibitors	3 (1.5)	7 (3.6)
Anti-neuropathic medication	4 (2.1)	5 (2.6)
Metabolic variables, mean (SD)		
Weight (kg)	87 (15)	85 (16)
Body mass index (kg/m2)	30 (5)	30 (5)
Plasma HbA1c (%)	7.9 (1.2)	7.9 (1.2)
HbA1c (mmol/mol)	63 (13)	63 (13)
Daily dose of insulin (IU/day)	64 (25)	62 (29)
eGFR (ml/min)	72 (15)	69 (14)
Vitamin B12 (pmol/l)	384 (126)	380 (121)
Methylmalonic acid (µmol/l)	0.198 (0.098)	0.194 (0.094)
B12 deficiency at baseline* (n (%))	5 (2.6)	2 (1.0)
Folate (nmol/l)	18.8 (7.8)	18.6 (6.9)
Homocysteine (µmol/l)	13.7 (4.7)	13.4 (3.7)
Diabetic complications (n (%))		
Cardiovascular	21 (11)	24 (12)
Cardiovascular intervention	17 (9)	27 (14)
Amputation	3 (2)	5 (3)
Neuropathy score**	7.6 (5.3)	8.3 (6.4)
Neuropathy		
Absent	32 (16)	29 (15)
Mild	98 (51)	92 (47)
Moderate	57 (29)	61 (31)
Severe	7 (4)	14 (7)

Table 1. Baseline Values Abbreviations: HbA1c, haemoglobin A1C; eGFR, estimated glomerular filtration rate
*B12 < 150 pmol/ml and MMA > 0.270 µmol/l.
**Valk Score: absent (0 points); mild (1–9 points); moderate (10–18 points) or severe (19–33 points).²¹

Main analysis

During the 4.3 years of placebo treatment, mean MMA increased from 0.185±0.081 to 0.200±0.074 µmol/l. During metformin treatment, MMA increased from 0.185±0.073 to 0.222±0.100 µmol/l (figure 2).

The main effect of metformin on MMA compared with placebo was significant (difference at the end of the study of 0.039 µmol/l; 95%CI: 0.019 to 0.055, Interaction test, p=0.001). As expected, eGFR had an inverse association with MMA levels, i.e. lower eGFR was associated with higher MMA levels (−0.004; 95%CI −0.005 to −0.003, p < 0.001). None of the associations were influenced by the use of calcium supplements or proton pump inhibitors. MMA increased with cumulative dose (gram x years) of metformin (figure 3). Per gram year of exposure to metformin, MMA adjusted for baseline levels increased by 0.006 µmol/l (summary mean; 95% CI 0.003 to 0.009, t-test, p < 0.001 compared to placebo).

Figure 2. Concentrations of serum MMA during the trial (unadjusted geometric mean values of all randomized patients with 95% CI). The number of patients in each treatment group is indicated.

Figure 3. Effect of cumulative treatment on MMA Association of exposure to metformin and placebo (in gram years) and serum levels of MMA (geometric mean values with standard error).

During the study, MMA did not differ significantly between treatment groups when stratified for B12 concentration, showing that metformin did not affect the biological relation between B12 and MMA (Appendix Figure A.1).

HbA1c and B12 mediating effects of metformin on neuropathy score

Metformin, as compared to placebo, had no significant effect on the neuropathy score (an increase of 0.032 per gram year of metformin, 95% CI: −0.12 to 0.18, p=0.34). As described earlier, the mean difference in the summary mean for HbA1c between the metformin and placebo groups was −0.40 percentage point (−0.55 to −0.25; p < 0.001).³ To assess the combined mediation effects of MMA (as marker of tissue B12 deficiency) and HbA1c (as marker of glycemic control) on neuropathy, we developed a bivariate mediation model. Our hypothesized model was constituted by the potential mediation effects of HbA1c and MMA added to a residual direct effect of treatment dosage (metformin gram years), and

baseline patient variables with a potential effect on neuropathy score (Appendix Fig. A.2). As shown by the mediation model, the resulting non-significant effect of metformin on neuropathy score can in fact be explained by a beneficial mediating effect through lowering of HbA1c (figure 4: a decrease of the neuropathy score of $0.04 \times 0.50 = 0.020$ per metformin gram year) and an adverse effect through increasing MMA (an increase of the neuropathy score of $0.04 \times 1.06 = 0.042$ per metformin gram year). No additional variable was detected through modification indices. All the paths retained and shown in figure 4 were significant, except for the direct (residual) path metformin → neuropathy score (0.00, $p=0.939$). The model satisfied the goodness-of-fit test (Chi-square=4.02, degree of freedom $df=2$, $p=0.13$, ratio Chi-square/ df : CMIN/ $df=2.01$, Normated Fit Index NFI=0.98).

DISCUSSION

The present data of the HOME trial are the first to show that long-term treatment with metformin, as compared to placebo, is associated with an increase of serum MMA in patients with type 2 diabetes treated with insulin. This effect of metformin increases over time and is dependent on the cumulative dose of metformin during treatment. These results are in line with our earlier findings showing a progressive decrease of serum levels of B12 in patients treated with metformin, which was associated with an increase in homocysteine levels.⁹ Taken together, these data suggest that metformin-associated decreases in B12 can, over time, lead to tissue damage. Furthermore, the increase in serum MMA by the use of metformin was associated with a small increase of a validated clinical neuropathy score. However, overall, metformin did not increase neuropathy, as compared to placebo. A possible explanation may be that the harmful effects of B12 deficiency are counteracted by the protective effects of metformin by improvement of the glucose regulation, as our analysis suggested.

Metformin and B12

An association between low serum B12 and metformin treatment was first reported in 1969.²⁵ Several trials and observational studies have since confirmed this association.^{5,6} Furthermore, reports have been published of patients with macrocytic anemia or neuropathy attributed to a metformin-induced B12 deficit, which responded to parenteral treatment with B12.^{26–28} Such case reports are suggestive, but definitive evidence has been lacking that metformin-associated decreases in B12 are biologically and clinically meaningful. In

Figure 4. Effect of MMA on neuropathy score through HbA1c (%) and MMA
The structural equation model of the effects of metformin total dose on the neuropathy score. Each variable is represented by a rectangle. A directional arrow symbolizes a relationship from an independent to a dependent variable. The numbers on the arrows are the beta of the regression and indicate the strength of the relationship. The coefficients show that, on the one hand, metformin protects against neuropathy by its glucose-lowering effects (through lowering HbA1c), while, on the other hand, the metformin-induced increase of MMA is associated with an increase of the neuropathy score. *Analyzed baseline variables were age, sex, duration of diabetes and body mass index. The full parameterized model is included in the appendix (Fig. A.3).

this respect, the HOME trial was the first to show that the metformin-associated decrease of B12 is progressive over time, can lead to B12 levels often considered clinically relevant (<150 pmol/l),^{9,18} and is accompanied by an increase in serum homocysteine, which is suggestive of tissue B12 deficiency.⁹ Nevertheless, the significance of these findings has been disputed, as current B12 assays measure not only B12 bound to transcobalamin, its cellular delivery protein (~20%), but also to haptocorrin, a protein of unknown function.¹⁸ It has been suggested that metformin affects B12 bound to haptocorrin, but not that bound to transcobalamin.^{29,30}

Metformin and MMA

The sensitivity and specificity of MMA as a biomarker for B12 deficiency are superior to those of homocysteine and B12 assays.¹⁹ Several studies have not only shown an association between MMA and B12 levels, but also with clinical symptoms of B12 deficiency, such

as neuropathy^{31,32} and lower cognitive function scores.³³ Three short-term studies examined the effect of metformin on both B12 and MMA.^{12,34,35} All studies confirmed that metformin treatment was associated with a lower total serum B12, but only one study¹² described a significant change in the serum concentrations of MMA.

Metformin and neuropathy: a summation of opposing effects

The prognosis of B12 deficiency without macrocytic anemia or neuropathy is unknown.³⁶ Therefore, it is important to investigate whether the metformin-induced B12 deficit is associated with clinical signs and (or) symptoms, such as neuropathy. Studies of the effects of metformin on neuropathy are scarce and contradicting. A case-control study of patients with type 2 diabetes and symptomatic peripheral neuropathy showed that metformin users had increased concentrations of MMA and that peripheral neuropathy was more severe in metformin users.¹² Two observational study also showed an association between the use of metformin, B12 deficiency and neuropathy.^{13,16} In addition, a secondary analysis of a long-term open-label study showed that the prevalence of neuropathy (abnormal monofilament examination) was higher in metformin users with low B12 levels compared to those with normal or borderline vitamin B12 levels.⁵ However, this finding was based on a very small number of cases of neuropathy and unfortunately no blood samples were available for measurement of MMA. In addition, a trial in 214 patients with type 2 diabetes and neuropathy showed that patients on metformin or with increased MMA benefited the most from high dose vitamin supplementation.³⁷ On the other hand, two observational studies could not show an increased risk of anemia or neuropathy in metformin users with lower B12 compared to non-users.^{14,15} As metformin may have neuroprotective effects (through its antihyperglycemic actions^{17,38} and through direct neuroprotective effects through inhibition of apoptotic cell death related to oxidative stress³⁹), any B12 deficiency-related neuropathic effects of metformin may be difficult to ascertain.

As our study had a relatively long period of follow-up, in which blood samples were frequently collected and neuropathy scores were frequently obtained, the data were suitable for SEM analysis, allowing us to differentiate between the possible neuroprotective and neuropathic effects of metformin. The results showed that, on the one hand, metformin may protect against neuropathy by its glucose-lowering effects, while, on the other hand, metformin-induced tissue B12 deficiency – estimated by MMA – is associated with a significant deterioration of the neuropathy score. As the increase in neuropathy score at

the end of the study was small, the clinical relevance of this finding remains uncertain. However, analyses showed that MMA levels increase over time. Therefore, it is possible that longer treatment with metformin may be associated with clinically relevant neuropathy in some individuals. As B12 deficiency may cause irreversible neuropathy,¹⁰ which may mimic diabetic neuropathy,¹¹ prevention or early identification of B12 deficiency is important.

Taken together, our data on B12, homocysteine,⁹ MMA and neuropathy suggest that monitoring of B12 and, when available, MMA, in long-term users of metformin should be considered.

Mechanism

Several mechanisms by which metformin may induce a B12 deficit have been suggested⁷: diminished absorption by changes in bacterial flora, interference with the intestinal absorption of the intrinsic factor-B12 complex (and) or alterations in intrinsic factor levels. Another potential explanation for metformin-induced vitamin B12 malabsorption and deficiency is that metformin has an effect on calcium-dependent membrane function in the terminal ileum.²³ However, the increase of MMA in the present study was not affected by the use of calcium supplements, proton pump inhibitors or levodopa.

Strengths and weaknesses of the study

Although our study was a post hoc safety analysis of a possible side effect of metformin, the strengths of our study are determined by the randomized, placebo-controlled, double-blind design and its relatively long period of follow-up (4.3 years), as well as the frequent serum collection and frequent testing for neuropathy.

Our study has also some limitations. First, the power analysis of the HOME trial was primarily based on the aggregation of mortality and macrovascular and microvascular endpoints.²⁰ However, this does not invalidate the current findings. Second, we did not assess other B12-related potential outcomes, notably macrocytic anemia and cognition. These issues require further study. Third, for the evaluation of neuropathy, we did not perform electrophysiological examinations, but instead used a clinically validated semi quantitative neuropathy score. The use of such a neuropathy score may have led to an underestimation of the severity of neuropathy, as the sensitivity of physical examination to determine neuropathy is lower than electrophysiological examinations.⁴⁰ However, as we did find positive results, we feel that a relatively low sensitivity does not invalidate

findings of our secondary endpoint. Finally, we studied middle-aged, Caucasian, insulin-treated individuals with type 2 diabetes who received regular dietary advice. The risk of metformin-associated B12 deficiency may be higher in older individuals and those with poor dietary habits.

Conclusion

This long-term placebo-controlled trial is the first study to show that, in patients with type 2 diabetes, metformin not only reduces serum levels of B12, but also progressively increases serum MMA (present study) as well as serum homocysteine (as earlier reported⁹). In addition, the increase of serum MMA in metformin users was associated with a significant worsening of a validated clinical neuropathy score. These results provide further evidence that metformin-related B12 deficiency is biologically and potentially clinically relevant. These data suggest that monitoring of B12 in long-term users of metformin should be considered.

ARTICLE INFORMATION

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Conflict of interest

All authors declare that there is no duality of interest associated with their contribution to this paper.

Author contributions

AK and CS designed the study. MO, AK and CS did literature searches. MO, AK and PL re-searched the data; MO and PL performed the statistical analyses; MO, AK and CDS wrote

the article. PL contributed to the discussion and reviewed and edited the article. CAS performed the analysis of serum levels of MMA and B12 and wrote the section about laboratory investigations. MO, AK, PL and CDS are guarantors. All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

Trial registration

ClinicalTrials.gov identifier NCT00375388

REFERENCES

1. American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment. *Diabetes Care*. 2017;40(Suppl 1):S64-74.
2. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet*. 1998;352(9131):854-65.
3. Kooy A, de Jager J, Lehert P, et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch Intern Med*. 2009;169(6):616-25.
4. Pernicova I, Korbonits M. Metformin--mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol*. 2014;10(3):143-56.
5. Aroda VR, Edelstein SL, Goldberg RB, et al. Long-term Metformin Use and Vitamin B12 Deficiency in the Diabetes Prevention Program Outcomes Study. *J Clin Endocrinol Metab*. 2016;101(4):1754-61.
6. Chapman LE, Darling AL, Brown JE. Association between metformin and vitamin B12 deficiency in patients with type 2 diabetes: A systematic review and meta-analysis. *Diabetes Metab*. 2016;42(5):316-27.
7. Zdilla MJ. Metformin With Either Histamine H2-Receptor Antagonists or Proton Pump Inhibitors: A Polypharmacy Recipe for Neuropathy via Vitamin B12 Depletion. *Clin Diabetes*. 2015;33(2):90-5.
8. Wulfele MG, Kooy A, Lehert P, et al. Effects of short-term treatment with metformin on serum concentrations of homocysteine, folate and vitamin B12 in type 2 diabetes mellitus: a randomized, placebo-controlled trial. *J Intern Med*. 2003;254(5):455-63.
9. de Jager J, Kooy A, Lehert P, et al. Long term treatment with metformin in patients with type

- 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. *BMJ*. 2010;340:c2181.
10. Saperstein DS, Barohn RJ. Peripheral Neuropathy Due to Cobalamin Deficiency. *Curr Treat Options Neurol*. 2002;4(3):197-201.
 11. Bell DS. Nondiabetic neuropathy in a patient with diabetes. *Endocr Pract*. 1995;1(6):393-4.
 12. Wile DJ, Toth C. Association of metformin, elevated homocysteine, and methylmalonic acid levels and clinically worsened diabetic peripheral neuropathy. *Diabetes Care*. 2010;33(1):156-61.
 13. Singh AK, Kumar A, Karmakar D, Jha RK. Association of B12 deficiency and clinical neuropathy with metformin use in type 2 diabetes patients. *J Postgrad Med*. 2013;59(4):253-7.
 14. Chen S, Lansdown JA, Moat SJ, et al. An observational study of the effect of metformin on B12 status and peripheral neuropathy. *Br J Diabetes Vascular Dis*. 2012;12:189-93.
 15. de Groot-Kamphuis DM, van Dijk PR, Groenier KH, Houweling ST, Bilo HJ, Kleefstra N. Vitamin B12 deficiency and the lack of its consequences in type 2 diabetes patients using metformin. *Neth J Med*. 2013;71(7):386-90.
 16. Hansen CS, Jensen JS, Ridderstrale M, Vistisen D, Jorgensen ME, Fleischer J. Vitamin B12 deficiency is associated with cardiovascular autonomic neuropathy in patients with type 2 diabetes. *J Diabetes Complications*. 2017;31(1):202-8.
 17. Pop-Busui R, Lu J, Brooks MM, et al. Impact of glycemic control strategies on the progression of diabetic peripheral neuropathy in the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) Cohort. *Diabetes Care*. 2013;36(10):3208-15.
 18. Stabler SP. Clinical practice. Vitamin B12 deficiency. *N Engl J Med*. 2013;368(2):149-60.
 19. Carmel R. Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*. 2011;94(1):348S-58S.
 20. Wulfele MG, Kooy A, Lehert P, et al. Combination of insulin and metformin in the treatment of type 2 diabetes. *Diabetes Care*. 2002;25(12):2133-40.
 21. Valk GD, Nauta JJ, Strijers RL, Bertelsmann FW. Clinical examination versus neurophysiological examination in the diagnosis of diabetic polyneuropathy. *Diabet Med*. 1992;9(8):716-21.
 22. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150(9):604-12.
 23. Bauman WA, Shaw S, Jayatilleke E, Spungen AM, Herbert V. Increased intake of calcium reverses vitamin B12 malabsorption induced by metformin. *Diabetes Care*. 2000;23(9):1227-31.
 24. Abraham NS. Proton pump inhibitors: potential adverse effects. *Curr Opin Gastroenterol*. 2012;28(6):615-20.
 25. Berchtold P, Bolli P, Arbenz U, Keiser G. Disturbance of intestinal absorption following metformin therapy (observations on the mode of action of biguanides. *Diabetologia*. 1969;5(6):405-12.
 26. Bell DS. Metformin-induced vitamin B12 deficiency presenting as a peripheral neuropathy. *South Med J*. 2010;103(3):265-7.
 27. Tung ML, Tan LK. Long term use of metformin leading to vitamin B 12 deficiency. *Diabetes Res Clin Pract*. 2014;.
 28. Callaghan TS, Hadden DR, Tomkin GH. Megaloblastic anaemia due to vitamin B12 malabsorption associated with long-term metformin treatment. *Br Med J*. 1980;280(6225):1214-5.
 29. Leung S, Mattman A, Snyder F, Kassam R, Meneilly G, Nexo E. Metformin induces reductions in plasma cobalamin and haptocorrin bound cobalamin levels in elderly diabetic patients. *Clin Biochem*. 2010;43(9):759-60.
 30. Greibe E, Trolle B, Bor MV, Lauszus FF, Nexo E. Metformin lowers serum cobalamin without changing other markers of cobalamin status: a study on women with polycystic ovary syndrome. *Nutrients*. 2013;5(7):2475-82.
 31. Solomon LR. Diabetes as a cause of clinically significant functional cobalamin deficiency. *Diabetes Care*. 2011;34(5):1077-80.
 32. England JD, Gronseth GS, Franklin G, et al. Practice Parameter: evaluation of distal symmetric polyneuropathy: role of laboratory and genetic testing (an evidence-based review). Report of the American Academy of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine, and American Academy of Physical Medicine and Rehabilitation. *Neurology*. 2009;72(2):185-92.
 33. McCracken C, Hudson P, Ellis R, McCaddon A, Medical Research Council Cognitive Function and Ageing Study. Methylmalonic acid and cognitive function in the Medical Research Council Cognitive Function and Ageing Study. *Am J Clin Nutr*. 2006;84(6):1406-11.
 34. Carlsen SM, Folling I, Grill V, Bjerve KS, Schneede J, Refsum H. Metformin increases total serum homocysteine levels in non-diabetic male patients with coronary heart disease. *Scand J Clin Lab Invest*. 1997;57(6):521-7.
 35. Obeid R, Jung J, Falk J, et al. Serum vitamin B12 not reflecting vitamin B12 status in patients with type 2 diabetes. *Biochimie*. 2013;95(5):1056-61.
 36. Carmel R. Diagnosis and management of clinical and subclinical cobalamin deficiencies: why controversies persist in the age of sensitive metabolic testing. *Biochimie*. 2013;95(5):1047-55.
 37. Fonseca VA, Lavery LA, Thethi TK, et al. Metanx in type 2 diabetes with peripheral neuropathy:

a randomized trial. Am J Med. 2013;126(2):141-9.

38. Ismail-Beigi F, Craven T, Banerji MA, et al. Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial. Lancet. 2010;376(9739):419-30.

39. El-Mir MY, Daille D, Villanueva G, et al. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. J Mol Neurosci. 2008;34(1):77-87.

40. Dyck PJ, Overland CJ, Low PA, et al. Signs and symptoms versus nerve conduction studies to diagnose diabetic sensorimotor polyneuropathy: CI vs. NPhys trial. Muscle Nerve. 2010;42(2):157-64.

APPENDIX

A.1. METHODS

A.1.1. The Valk Score

	Score	
Light touch, dorsum of foot	0 – 4	0 = normal
Pinprick sense, dorsum of foot	0 – 4	2 = impaired
Vibration sense of ankle	0 – 4	4 = absent
Position sense of hallux	0 – 4	
Strength of extensor hallucis longis	0 – 4	
Strength of extensor gastronomicus	0 – 4	
Ankle jerks	0 – 4	
Light touch, score level where abnormality begins	0 – 5	0 = normal; up to 5 = knee
Total	0 – 33	

Table A.1. The Valk Score ¹

A.1.2 Statistical Analysis - Imputation of missing data

The non-missing values at the last visit are 242 patients. The HOME study constitutes one of the longest studies with such a sample size, while being fully randomized and controlled. A 4.3 year study remains a very difficult task; moreover an important proportion of drop-outs is caused by illness aggravation, including death and cardio-vascular critical events, and therefore was anticipated and allowed. All our analyses were based on missing data handling. We did not conduct pattern mixture missing imputation, but we performed a simplified multiple imputation technique where we compared results based on the following techniques considered by our clinician experts as appropriate to the specific studied question: worst case value when illness aggravation, Last Observation Carried Forward, model predicting and non-missing imputation (NMI). In observing that the maximum relative error observed on the main estimate over these four techniques was less than 2.5%, we concluded that Missing At Random (MAR) assumption was reasonable, in particular as all the results related with imputed data were very close to NMI results. Thus our results seem quite stable with regard to missing data and our main technique of missing data

imputation was finally used to report the final results. In the statistical analysis section, we simply refer to the model predicting imputation, as this was our main analysis as planned in the SAP. These calculations were done with R, capturing and comparing the estimates related with the various missing data handling techniques.

A.1.3 Statistical Analysis - Mixed model analyses

We used a mixed model for assessment of the time-treatment first-order interaction with centre as a random variable, taking into account potential interaction effects between metformin and age, sex, duration of diabetes, body mass index, estimated glomerular filtration rate (eGFR, according to the CKD-EPI equation), glycated haemoglobin (HbA1c) or use of co-medication (calcium-supplements and proton pump inhibitors).² The following models of within-patients correlations in time were tested: compound symmetry, autocorrelation of level 1 and 2, autocorrelation and moving average (ARMA, various parameters), the final choice being based on minimization of the Akaike Information criterion. In previous analyses of this HOME trial, we observed that in assuming an unconstrained within patient time correlation matrix, we lost important information. In particular the AR(1) autoregressive model was found the best option among all the ARMA models. In our statistical analysis plan, we privileged the AR(1) model. However, we did compare the unconstrained, AR(1), and various ARMA models by comparing the AIC/BIC, through which the likelihood of AR(1) was confirmed.

A.1.4 Statistical Analysis - SEM analysis

SEM is a powerful extension of the general linear model that makes it possible to test a set of regression equations simultaneously.³⁻⁵ First step is the building of a model based on a presumed mechanism. The next step is the testing of the model, making use of all data available of all the visits and blood samples. Our hypothesized model was constituted by the potential mediation effects of HbA1c and MMA added to a residual direct effect of treatment dosage (metformin gram years), and baseline patient variables with a potential effect on neuropathy score. Before conducting the analysis, we observed that treatment effect had no effect on change of eGFR. A chi-square goodness of fit test was used to assess the likelihood of the hypothesized model on our data sample. Our structural equation model did account for repeated measurement: all MMA measurements (1880; 915 metformin, 965 placebo) and all measurements of the neuropathy score (4972 times for 390 individuals in 4.3 years) were included in the analysis.

A.2 RESULTS

A.2.1 Results - Sensitivity analyses

For sensitivity purposes, and to strengthen the validity of our results, three alternative models and selections were tested:

1. T-test: Metformin, as compared to placebo, was associated with a mean increase of MMA of 0.029 $\mu\text{mol/l}$ (95% CI: -0.009 to 0.069, t-test, $p=0.13$).
2. ANCOVA: By adjusting for baseline values, similar results were found: an increase of MMA of 0.036 $\mu\text{mol/l}$ (95% CI: 0.006 to 0.067, ANCOVA, $p=0.02$).
3. Analysis with the full set of all randomized patients without exclusion (including patients with B12 deficiency at baseline and of patients receiving B12 supplementation)

During the 4.3 year of placebo treatment, mean MMA increased from 0.185 (SD 0.811) to 0.200 $\mu\text{mol/l}$ (SD 0.074). During metformin treatment, mean MMA increased from 0.185 (SD 0.073) to 0.222 $\mu\text{mol/l}$ (SD 0.100). Analysis with the mixed model approach showed that the use of metformin was associated with an increase of MMA, both in the analysis with all randomized patients and after exclusion of the patients with B12 deficiency or B12 supplementation: in the analysis with all randomized patients, we detected a significant time-treatment interaction; the difference in MMA at the end of the study, compared with placebo, was 0.020 $\mu\text{mol/l}$ (95%CI: 0.001 to 0.033, $p=0.03$).

A.2.2 Results – Figures

Figure A.1. B12 and MMA at the end of the study
MMA serum levels at the end of the study (geometric means) with standard deviations for patients with a normal B12 (>220 pmol/l), a low B12 (150-220 pmol/l) and B12 deficiency (<150 pmol/l). The number of patients in each treatment group is indicated.

Figure A.2. SEM analysis – Hypothesized structural equation model
The hypothesized structural equation model of the effects of metformin total dose on the neuropathy score. Abbreviations: HbA1c, haemoglobin A1C; eGFR, estimated glomerular filtration rate; Log MMA, neper logarithm of MMA (μmol/l); metformin is provided as gram x years, to examine the effect of treatment dose in time. As metformin dose varies between 0 and 2.55 (0 = placebo), and years vary from 0 to 4.33, metformin gram years vary from 0 to 11.05. *Analyzed baseline variables were age, sex, duration of diabetes and body mass index.

Figure A.3. SEM analysis – Full final parameterized structural equation model
The full final parameterized structural equation model of the effects of metformin total dose on the neuropathy score. Each variable is represented within a rectangle, and existing relationships or paths between variables are represented by a straight line with a direction. Each number nearby a path between two variables is a regression coefficient. For each variable (rectangle) considered as dependent (thus reached by at least one arrow), the first number is the intercept of the regression. For instance for log MMA the number -1.16 is the intercept of the regression by the two variables metformin (gram years) and eGFR, each of them with a coefficient of regression 0.04 and -0.01, so that the regression is $\log \text{MMA} = -1.16 + 0.04 * \text{metformin (gram years)} - 0.01 * \text{eGFR}$. For variables not considered as dependent (metformin and eGFR), the mean and variance are provided. Circles are associated with residuals of regression, their mean is always zero, and the residual variance is provided. For instance, the residual variance associated with the regression of Neuropathy Score by log MMA and HbA1c has a variance of 28.92.
As an example how to interpret the model: patients with the maximum dose of metformin will have had 11.05 gram years at the end of the study. 11.05 gram years of metformin will improve HbA1c by $11.05 * -0.04$, which is associated with a lower neuropathy score of $11.05 * -0.04 * 0.5 = -0.221$, compared to placebo. However, 11 gram years of metformin will increase logMMA by $11.05 * 0.04$, which is associated with an increase of the neuropathy score of $11.05 * 0.04 * 1.06 = 0.469$, compared to placebo. Combined, 11.05 gram years of metformin will increase the neuropathy score with $0.469 - 0.221 = 0.248$.
Abbreviations: HbA1c, haemoglobin A1C; eGFR, estimated glomerular filtration rate; Log MMA, neper logarithm of MMA (μmol/l); metformin is provided as gram x years, to examine the effect of treatment dose in time. As metformin dose varies between 0 and 2.55 (0 = placebo), and years vary from 0 to 4.33, metformin gram years vary from 0 to 11.05.

REFERENCES APPENDIX

1. Valk GD, Nauta JJ, Strijers RL, Bertelsmann FW. Clinical examination versus neurophysiological examination in the diagnosis of diabetic polyneuropathy. *Diabet Med.* 1992;9(8):716-21.
2. Chakraborty H, Gu H. A Mixed Model Approach for Intent-to-Treat Analysis in Longitudinal Clinical Trials with Missing Values. 2009;RTI Press publication No. MR-0009-0903.
3. Akaike H. Information theory as an extension of the maximum likelihood principle. In: Petrov BN, Csaki F, editors. *Second International Symposium on Information Theory.* Budapest: Akademiai Kiado; 1973. p.267-81.
4. Bollen KA. *Structural Equations with Latent Variables.* New York: John Wiley & Sons; 1989.
5. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. *Behav Res Methods.* 2008;40:879-91.

Chapter 3.

Long-term treatment with metformin in type 2 diabetes and vitamin D levels

LONG-TERM TREATMENT WITH METFORMIN IN TYPE 2 DIABETES AND VITAMIN D LEVELS: A POST HOC ANALYSIS OF A RANDOMISED PLACEBO-CONTROLLED TRIAL

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ABSTRACT

Aims: Metformin prevents weight gain, but it also leads to malabsorption of B12 and decreased ileal reabsorption of bile acids. Little is known whether metformin affects absorption of fat-soluble vitamins like vitamin D. Therefore, we studied the effects of metformin, as compared to placebo, on serum levels of vitamin D (25-hydroxyvitamin D [25(OH)D]) in patients with advanced type 2 diabetes.

Materials and Methods: In the HOME trial, a randomised placebo-controlled trial, 390 insulin-treated patients with type 2 diabetes were treated with 850 mg metformin or placebo three times daily for 52 months. In a post-hoc analysis, we analysed changes in the combined levels of 25(OH)D₂ and 25(OH)D₃ at 4 and 16 months during the study.

Results: Mean combined 25(OH)D at baseline was 68.2 nmol/l (95% CI 65.5 to 71.1). In mixed model analysis, metformin, as compared to placebo, had no effect on 25(OH)D levels during 16 months (coefficient: 1.002 per month, multiplicative model; 95%CI: 0.998 to 1.006, p=0.30). Metformin was associated with a small increase of 25(OH)D₂ (coefficient: 1.012 per month; 95% CI 1.003 to 1.021, p=0.008). However, 25(OH)D₂ is only a very small fraction (3%) of 25(OH)D. Seasonal variation had the biggest impact on 25(OH)D levels. Vitamin B12 levels were not associated with the levels of 25(OH)D.

Conclusions: Metformin had no effect on serum 25(OH)D during 16 months in the setting of a clinical randomised controlled trial in patients with type 2 diabetes. Our results support that metformin doesn't lead to vitamin D deficiency.

INTRODUCTION

Metformin is the preferred initial pharmacological agent for type 2 diabetes.¹ It has favourable effects on glycemic control, insulin requirements, weight development and cardiovascular outcomes.^{2,3} Nevertheless, metformin also has side effects, in particular gastrointestinal side effects. Although multiple hypotheses have been proposed, the underlying mechanism remains poorly understood.⁴ In addition, metformin reduces ileal absorption of bile acid,⁵ which may play a role in the B12 deficiency induced by metformin.⁶ Malabsorption may also play a role in the prevention of weight gain by metformin, as a recent study showed that metformin does not affect energy intake.⁷ However, whether the decreased reabsorption of bile acids also causes deficiency of fat-soluble vitamins like vitamin D is unknown.

Vitamin D deficiency is widespread.⁸ Vitamin D is critical for skeletal mineralization and deficiency is linked to fractures.⁹ Although the efficacy of calcium and vitamin D treatment for the prevention of osteoporotic fractures has become controversial during the last years,¹⁰ maintenance of vitamin D stores by supplementation in the elderly combined with sufficient dietary calcium intake (800–1200 mg per day) appears an effective approach for prevention of osteoporotic fractures.^{11,12} In addition, vitamin D deficiency is also associated with increased insulin resistance, decreased insulin production, increased incidence of diabetes and even mortality.^{13,14} However, whether these associations are causal is not clear. Several intervention trials with vitamin D supplementation failed to show significant protection against the onset of diabetes^{15–17} or improvement of cardiovascular risk, insulin sensitivity and glucose regulation.^{18–22} As only two of these trials had a vitamin D deficiency (a 25-hydroxyvitamin D [25(OH)D] below 50 nmol/liter) as inclusion criterion,^{19,20} more research is needed to draw conclusions about the association between vitamin D deficiency and type 2 diabetes. Studies on the effects of metformin on vitamin D levels are sparse. Some data suggest a synergic effect of metformin and vitamin D.^{23,24} Only one cross-sectional study found a negative association between metformin and vitamin D.²⁵ In contrast, two other studies found that metformin did not affect treatment of vitamin D deficiency with supplementation of vitamin D.^{26,27}

Therefore, we investigated whether treatment with metformin vs. placebo during 52 months affects vitamin D levels in patients in the *Hyperinsulinaemia: the Outcome of its Metabolic Effects (HOME) trial*. The HOME trial is the largest known randomised controlled trial (RCT) to evaluate the effects of metformin vs. placebo in patients with type 2 diabetes treated with insulin. The study showed that metformin when added to insulin in patients

	Placebo (n=194)	Metformin (n=196)
Demographic Characteristics, mean±SD		
Men/ women, No.	97/97	81/115
Age (years)	59 (11)	64 (10)
Duration of diabetes (years)	12 (8)	14 (9)
Insulin treatment (years)	6 (6)	7 (8)
Concomitant medication (n (%))		
Calcium supplements	2 (1.0)	3 (1.5)
Vitamin D supplements	0 (0)	0 (0)
Metabolic variables, mean (SD)		
Weight (kg)	87 (15)	85 (16)
Body mass index (kg/m2)	30 (5)	30 (5)
eGFR (ml/min)*	72 (15)	69 (14)
Vitamin D (geometric mean, 95%CI)		
Vitamin 25(OH)D (nmol/l)†	66.1 (62.5 – 69.9)	70.4 (66.2 – 74.8)
Vitamin 25(OH)D ₂ (nmol/l)	2.0 (1.8 – 2.2)	1.9 (1.8 – 2.1)
Vitamin 25(OH)D ₃ (nmol/l)	63.6 (60.0 – 67.4)	68.0 (63.9 – 72.4)
Season at inclusion‡ (n (%))		
Spring	62 (31.9)	59 (30.1)
Summer	69 (35.5)	79 (40.4)
Autumn	31 (15.9)	30 (15.3)
Winter	32 (16.5)	28 (14.2)

Table 1. Baseline Values.

*According to CKD-EPI equation; †Combined levels of serum 25(OH)D₂ and 25(OH)D₃; ‡Spring: March to May; Summer: June to August; September to November; Winter: December to February.

with type 2 diabetes not only improved glycemic control and reduced insulin requirements, but also prevented weight gain.² In the HOME trial, both short and long term outcomes are available. In addition, no other glucose-lowering drugs were used, allowing comparison of metformin vs. placebo in patients receiving insulin. Furthermore, the HOME trial was held in a period in which vitamin D supplementation was uncommon (patients were recruited in 1998 and 1999; follow-up ended in 2003).

MATERIALS AND METHODS

Study design, randomization, interventions and follow-up

The HOME trial was a 4.3 year RCT, that included 390 Caucasian patients aged 30-80 years with type 2 diabetes treated with insulin. Patients were randomly allocated to either metformin 850 mg or placebo (one to three times daily, depending on tolerance and renal function), added to insulin therapy. Patient selection, study design, data collection and power analysis have been described previously.^{2,28} The HOME trial was conducted in the outpatient clinics of three nonacademic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, The Netherlands). All participants provided written informed consent before inclusion. The medical ethical committees of the three participating hospitals approved the trial protocol. The study was conducted in accordance with Good Clinical Practice (CPMP/ICH/135/95; 1996) and with the Declaration of Helsinki (revised version, 2000).

Visits and data collection

Patients visited the clinics at the start of the pre-randomization phase (three months before randomization), at baseline (for randomization to metformin or placebo), one month after baseline (to check tolerance of drug titration), and subsequently every three months until the end of the trial. At every visit, patients were asked whether their medication had changed and whether they used over-the-counter drugs or supplements.

Laboratory investigations

Blood samples were drawn at baseline and after 4, 16, 28, 40, and 52 months (4.3 years), and stored at -80°C until analysis. Concentrations of 25(OH)D₂ and 25(OH)D₃ were determined with the use of ultra-performance liquid chromatography tandem mass spectrometry.²⁹ Based on the inclusion of control samples during the different runs in this study (n=28, as measured in triplicate) we calculated a combined coefficient of variation (inter- and intra assay) of 23.1% and 5.6% for 25(OH)D₂ and 25(OH)D₃, respectively. Validation experiments showed an inter-assay variation (n=23) of 17.1% and 6.8% for 25(OH)D₂ and 25(OH)D₃, respectively. Intra-assay variation (n=6) was 14.8% and 1.8% for 25(OH)D₂ and 25(OH)D₃, respectively. Details of the validation experiments are described in Appendix section S1.1.

STATISTICAL ANALYSIS

Objective

Our primary analysis was to assess the effect of metformin on vitamin D, consisting of the combined levels of 25(OH)D₂ and 25(OH)D₃, the two major metabolites of 25(OH)D.¹⁴ Our secondary analysis was the effect of metformin on the separate levels of 25(OH)D₂ and 25(OH)D₃. In addition, to analyze whether patients with metformin-induced B12 deficiency were more prone to become vitamin D deficient, we repeated the main analysis with B12 added to the model as a covariate. Our main selection was the set of all randomised patients with exclusion of patients on vitamin D supplementation from the time-point at which they received it. On grounds of costs, we decided to limit the initial measurements of 25(OH)D to three time points. If analysis would show significant or nearly significant associations, measurements would be expanded with analysis of all available blood samples. We prespecified that measurements at 0, 4 and 16 months would be the best option for a first evaluation, with a follow-up period long enough to show potential associations, with a minimum of missing values, and with measurements per patient in the same season after 4 and 16 months.

Analysis

Our analysis was conducted through a mixed linear model assessing time effects, treatment effects and their interactions, with the logarithmic value of 25(OH)D at each visit as the dependent variable. Covariates were age, sex, body mass index (BMI) and seasonal variation. Expected seasonal variation was calculated as a sinusoidal curve with the highest vitamin D values at the end of summer and the lowest values at the end of the winter.³⁰ We log transformed vitamin D values before analysis, because their distribution was skewed. Data are given as geometric means with their standard variation, unless stated otherwise. Sensitivity analysis was conducted by separately assessing the constant and change effect of treatment. We analysed the occurrence of missing data with a pattern-mixture approach.³¹ For sensitivity purposes, multiple imputation was used. In case of missing completely at random (MCAR), we added an analysis of the effect of metformin on 25(OH)D levels on completers only (details of handling of missing data are described in Appendix section S1.2). Two-sided P-values < 0.05 are considered statistically significant. Statistical analysis was conducted with R software (release 3.4.0), running on Windows 10.

Figure 1. Recruitment plus retention of patients

Sample size and Power

Original sample size calculations for this trial were based on expected differences in the occurrence of disease-related endpoints, as described previously.²⁸ Post-hoc power calculations for 25(OH)D analysis with two post-baseline measures showed that with our sample size, assuming that the correlation (baseline-final value) is at least R=0.3, a power of at least 0.85 is expected when the variance coefficient is ≤ 40%. If the analysis would be expanded

with measurement of five instead of two post-baseline samples, the power would increase to 0.96 (details of the power calculation are described in Appendix section S1.3).

RESULTS

General trial results

Patients in the metformin group were slightly older than those receiving placebo, had a slightly longer duration of diabetes, and were less often smokers. All other characteristics were comparable between the treatment groups (Table 1). The main outcomes of this trial have been reported previously.² Mean values of duration of diabetes, previous occurrence of cardiovascular disease and age were similar among the three hospitals and between those who did and did not complete the study. Figure 1 shows the recruitment plus retention of patients. At 16 months, laboratory samples were available for 327 patients (84%; 157 metformin, 170 placebo). 1072 laboratory samples were available for measurement of 25(OH)D (530 metformin, 542 placebo). No patients used vitamin D supplementation during the trial.

Analysis

During 16 months of placebo treatment, mean 25(OH)D increased from 66.1±21.4 to 70.7±25.0 nmol/l. During metformin treatment, 25(OH)D increased from 70.4±24.9 to 77.8±22.7 nmol/l (table 2). Mixed model analysis showed that metformin, as compared to placebo, had no effect on 25(OH)D levels during 16 months (coefficient: 1.002 per month, multiplicative model; 95%CI: 0.998 to 1.006, p=0.30; table 3). Analysis of data of completers only showed similar results (Appendix table S1). Seasonal variation had the greatest impact: coefficients for seasonal variation varied from 0.744 in March to 1.256 in September. In addition, 25(OH)D levels were negatively associated with age, BMI and female sex. All patients experienced a slight decrease in time. Sensitivity analyses assessing only constant effects or change in time effects confirmed the absence of effect of metformin on 25(OH)D. Because 25(OH)D consisted for approximately 97% of 25(OH)D₃, the results of the analysis of 25(OH)D₃ alone were very similar to results of the 25(OH)D analysis (table 3). Metformin was associated with a small increase of 25(OH)D₂ (coefficient: 1.012 per month; 95% CI 1.003 to 1.021, p=0.008). No seasonal effect was observed on 25(OH)D₂ levels, as 25(OH)D₂ is only derived from food, but not from sunlight. Finally, the model remained unchanged after addition of B12 as a covariate (the full model is shown in Appendix table S2).

	Placebo		Metformin (n=196)	
	n		n	
25(OH)D*				
Baseline	191	66.1 (62.5 – 69.9)	195	70.4 (66.2 – 74.8)
4 months	181	72.0 (68.1 – 76.1)	178	77.2 (73.0 – 81.7)
16 months	170	70.7 (66.2 – 75.4)	157	77.8 (73.7 – 82.2)
25(OH)D₂				
Baseline	191	2.0 (1.8 – 2.2)	195	1.9 (1.8 – 2.1)
4 months	181	2.0 (1.8 – 2.2)	178	2.1 (2.0 – 2.3)
16 months	170	2.1 (1.9 – 2.3)	157	2.5 (2.3 – 2.7)
25(OH)D₃				
Baseline	191	63.6 (60.0 – 67.4)	195	68.0 (63.9 – 72.4)
4 months	181	69.5 (65.7 – 73.6)	178	74.7 (70.5 – 79.1)
16 months	170	68.2 (63.8 – 72.9)	157	74.6 (70.5 – 79.0)

Table 2. Geometric means of vitamin D values with 95% confidence intervals per treatment group during the study.
*Combined levels of serum 25(OH)D₂ and 25(OH)D₃

	25(OH)D*				25(OH)D ₂			25(OH)D ₃			
	beta	95% CI	p		beta	95% CI	p	beta	95% CI	p	
Intercept (nmol/l)	74.701	60.644 92.018	<0.0001		2.175	1.533 3.084	<0.0001	71.881	58.046 89.014	<0.0001	
Metformin†	1.002	0.998 1.006	0.30		1.012	1.003 1.021	0.008	1.001	0.997 1.005	0.59	
Age‡	0.994	0.991 0.997	0.0001		0.996	0.991 1.001	0.13	0.994	0.991 0.997	<0.0001	
Sex§	0.930	0.869 0.995	0.036		0.850	0.762 0.947	0.004	0.933	0.870 1.000	0.05	
BMI (kg/m2)	0.986	0.979 0.992	<0.0001		0.995	0.984 1.006	0.39	0.986	0.979 0.992	<0.0001	
Season¶	1.256	1.233 1.280	<0.0001		0.973	0.934 1.013	0.19	1.270	1.246 1.295	<0.0001	
Time#	0.998	0.996 1.000	0.044		1.000	0.995 1.005	0.99	0.998	0.995 1.000	0.044	

Table 3. Results of mixed model analysis on mean vitamin D levels.
As logarithmic values have been used for 25(OH)D, the model is multiplicative. *Combined levels of serum 25(OH)D₂ and 25(OH)D₃; †Effect per month of metformin; ‡age minus 60; §female sex; || BMI minus 30; ¶ the seasonal component was calculated as a sinusoidal function with mean 25(OH)D values 25,6% higher or lesser in August or March compared with mid-season (June or November); #per month, independent of treatment.

DISCUSSION

In this post-hoc analysis of the HOME trial, we showed that metformin did not affect 25(OH)D levels, consisting of the combined 25(OH)D₂ and 25(OH)D₃ levels. Metformin was associated with a small increase of 25(OH)D₂ with a negligible clinical impact, as 25(OH)D consisted of less than 3% of 25(OH)D₂. However 25(OH)D₂ is a more specific marker of oral intake because it is not endogenously synthesized in the skin. Therefore our observations further emphasize that metformin treatment does not lead to vitamin D malabsorption. Our study is the first report of the effect of metformin on vitamin D levels in the setting of a randomised placebo controlled trial.

The prevalence of vitamin D deficiency varies widely depending on which definition is used and which population is studied.³² In our study vitamin D deficiency, defined as a 25(OH)D level < 50 nmol/l,³³ was present in 80 patients (21%). Compared to other populations, this is a rather moderate to low prevalence. Previously, analysis of a subsample from the Dutch population based Hoorn study showed a prevalence of vitamin D deficiency of 38% with a mean 25(OH)D level of 57 nmol/l in patients with type 2 diabetes.³⁴

Our present finding that metformin does not affect vitamin D levels in advanced type 2 diabetes is in contrast with our earlier finding that metformin significantly reduces B12 levels with increased methylmalonic acid (MMA) levels. This supports the theory that metformin might induce a more specific malabsorption of B12. This is biologically plausible because vitamin D is absorbed in the duodenum and proximal intestine and vitamin B12 is absorbed in the terminal ileum. Fluorodeoxyglucose positron emission tomography (FDG-PET) studies show a more distant uptake of metformin in the digestive system with sparing of the duodenum but increased uptake in ileum and colon.³⁵

Our findings are in accordance with previous studies, which found that metformin does not negatively affect treatment of vitamin D deficiency in patients with diabetes.^{26,27} One cross sectional study showed an inverse correlation between metformin and vitamin D levels.²⁵ Because of the study design, no causality can be inferred from that observation.

Although randomised placebo-controlled studies are lacking, several epidemiological trials show favourable associations between the use of metformin and bone fracture risk.³⁶ In comparison with other glucose lowering therapies, metformin might have either an overall beneficial or neutral effect on bone.³⁷ Our study shows that metformin treatment does not influence vitamin D levels and that changes in fracture risk cannot be explained by changes in vitamin D levels.

Strengths and limitations of the study

Strengths of our study include the randomised, placebo-controlled, double-blind design, relatively long term period of follow-up. Furthermore, the HOME trial was held in a period in which vitamin D supplementation was uncommon. Our study has also some limitations. First, we limited the measurements of D to three time points (0, 4 and 16 months). However, power calculations showed that this did not invalidate the current findings. Second, we did not assess other D-related potential outcomes, notably calcium, phosphate and parathyroid hormone. Finally, we studied middle-aged, Caucasian, insulin-treated individuals with type 2 diabetes who received regular dietary advice. The risk of D deficiency may be higher in older individuals and those with poor dietary habits.

In conclusion, we showed that metformin had no effect on D levels during 16 months in the setting of a clinical randomised controlled trial in patients with type 2 diabetes.

ARTICLE INFORMATION

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Conflict of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

AK and CDS designed the study. MO and WT did literature searches. MO and PL researched the data; MO and PL performed the statistical analyses; MO and WT wrote the article. AK and CDS contributed to the discussion and reviewed and edited the article. CAS performed the analysis of serum levels of vitamin D. MO, WT, AK and PL are guarantors and, as such,

had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

Trial Registration

Clinicaltrials.gov NCT00375388.

REFERENCES

- American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment. *Diabetes Care*. 2017;40(Suppl 1):S64-74.
- Kooy A, de Jager J, Leher P, et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch Intern Med*. 2009;169(6):616-25.
- Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-Year Follow-Up of Intensive Glucose Control in Type 2 Diabetes. *N Engl J Med*. 2008;359(15):1577-89.
- McCreight LJ, Stage TB, Connelly P, et al. Pharmacokinetics of metformin in patients with gastrointestinal intolerance. *Diabetes Obes Metab*. 2018;.
- Scarpello JH, Hodgson E, Howlett HC. Effect of metformin on bile salt circulation and intestinal motility in type 2 diabetes mellitus. *Diabet Med*. 1998;15(8):651-6.
- Out M, Kooy A, Leher P, Schalkwijk CA, Stehouwer CDA. Long-term treatment with metformin in type 2 diabetes and methylmalonic acid: Post hoc analysis of a randomized controlled 4.3year trial. *J Diabetes Complications*. 2017;.
- Out M, Miedema I, Jager-Wittenaar H, et al. Metformin-associated prevention of weight gain in insulin-treated type 2 diabetic patients cannot be explained by decreased energy intake: A post hoc analysis of a randomized placebo-controlled 4.3-year trial. *Diabetes Obes Metab*. 2017;.
- van Schoor NM, Lips P. Worldwide vitamin D status. *Best Pract Res Clin Endocrinol Metab*. 2011;25(4):671-80.
- Cauley JA, Lacroix AZ, Wu L, et al. Serum 25-hydroxyvitamin D concentrations and risk for hip fractures. *Ann Intern Med*. 2008;149(4):242-50.
- Black DM, Rosen CJ. Clinical Practice. Postmenopausal Osteoporosis. *N Engl J Med*. 2016;374(3):254-62.
- Rosen CJ. Vitamin D supplementation: bones of contention. *Lancet*. 2014;383(9912):108-10.
- Avenell A, Mak JC, O'Connell D. Vitamin D and vitamin D analogues for preventing fractures in post-menopausal women and older men. *Cochrane Database Syst Rev*. 2014;(4):CD000227. doi(4):CD000227.
- Rosen CJ, Adams JS, Bikle DD, et al. The nonskeletal effects of vitamin D: an Endocrine Society scientific statement. *Endocr Rev*. 2012;33(3):456-92.
- Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266-81.
- Davidson MB, Duran P, Lee ML, Friedman TC. High-dose vitamin D supplementation in people with prediabetes and hypovitaminosis D. *Diabetes Care*. 2013;36(2):260-6.
- Jorde R, Sollid ST, Svartberg J, et al. Vitamin D 20,000 IU per Week for Five Years Does Not Prevent Progression From Prediabetes to Diabetes. *J Clin Endocrinol Metab*. 2016;101(4):1647-55.
- Moreira-Lucas TS, Duncan AM, Rabasa-Lhoret R, et al. Effect of vitamin D supplementation on oral glucose tolerance in individuals with low vitamin D status and increased risk for developing type 2 diabetes (EVIDENCE): A double-blind, randomized, placebo-controlled clinical trial. *Diabetes Obes Metab*. 2017;19(1):133-41.
- Wagner H, Alvarsson M, Mannheimer B, Degerblad M, Ostenson CG. No Effect of High-Dose Vitamin D Treatment on beta-Cell Function, Insulin Sensitivity, or Glucose Homeostasis in Subjects With Abnormal Glucose Tolerance: A Randomized Clinical Trial. *Diabetes Care*. 2016;39(3):345-52.
- Mousa A, Naderpoor N, de Courten MP, et al. Vitamin D supplementation has no effect on insulin sensitivity or secretion in vitamin D-deficient, overweight or obese adults: a randomized placebo-controlled trial. *Am J Clin Nutr*. 2017;105(6):1372-81.
- Gulseth HL, Wium C, Angel K, Eriksen EF, Birkeland KI. Effects of Vitamin D Supplementation on Insulin Sensitivity and Insulin Secretion in Subjects With Type 2 Diabetes and Vitamin D Deficiency: A Randomized Controlled Trial. *Diabetes Care*. 2017;40(7):872-8.
- Krul-Poel YH, Westra S, ten Boekel E, et al. Effect of Vitamin D Supplementation on Glycemic Control in Patients With Type 2 Diabetes (SUNNY Trial): A Randomized Placebo-Controlled Trial. *Diabetes Care*. 2015;38(8):1420-6.
- Manousopoulou A, Al-Daghri NM, Garbis SD, Chrousos GP. Vitamin D and cardiovascular risk among adults with obesity: a systematic review and meta-analysis. *Eur J Clin Invest*. 2015;45(10):1113-26.
- Amin SN, Hussein UK, Yassa HD, Hassan SS, Rashed LA. Synergistic actions of vitamin D and metformin on skeletal muscles and insulin resistance of type 2 diabetic rats. *J Cell Physiol*. 2017;.
- Figurova J, Dravecka I, Petrikova J, Javorsky M, Lazurova I. The effect of alfalcidol and metformin on metabolic disturbances in women with polycystic ovary syndrome. *Horm Mol Biol Clin Investig*. 2017;29(3):85-91.
- van Orten-Luiten AC, Janse A, Dhonukshe-Rutten RA, Witkamp RF. Vitamin D deficiency as

- adverse drug reaction? A cross-sectional study in Dutch geriatric outpatients. *Eur J Clin Pharmacol.* 2016;72(5):605-14.
26. Kos E, Liszek MJ, Emanuele MA, Durazo-Arvizu R, Camacho P. Effect of metformin therapy on vitamin D and vitamin B(1)(2) levels in patients with type 2 diabetes mellitus. *Endocr Pract.* 2012;18(2):179-84.
 27. Alkharfy KM, Al-Daghri NM, Sabico SB, et al. Vitamin D supplementation in patients with diabetes mellitus type 2 on different therapeutic regimens: a one-year prospective study. *Cardiovasc Diabetol.* 2013;12:113,2840-12-113.
 28. Wulfele MG, Kooy A, Leher P, et al. Combination of insulin and metformin in the treatment of type 2 diabetes. *Diabetes Care.* 2002;25(12):2133-40.
 29. Engelen L, Schalkwijk CG, Eussen SJ, et al. Low 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 levels are independently associated with macroalbuminuria, but not with retinopathy and macrovascular disease in type 1 diabetes: the EURODIAB prospective complications study. *Cardiovasc Diabetol.* 2015;14:67,015-0231-2.
 30. Lips P, van Schoor NM, de Jongh RT. Diet, sun, and lifestyle as determinants of vitamin D status. *Ann N Y Acad Sci.* 2014;1317:92-8.
 31. Little RJ, Wang Y. Pattern-mixture models for multivariate incomplete data with covariates. *Biometrics.* 1996;52(1):98-111.
 32. LeFevre ML, U.S. Preventive Services Task Force. Screening for vitamin D deficiency in adults: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med.* 2015;162(2):133-40.
 33. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2011;96(7):1911-30.
 34. Pilz S, van den Hurk K, Nijpels G, et al. Vitamin D status, incident diabetes and prospective changes in glucose metabolism in older subjects: the Hoorn study. *Nutr Metab Cardiovasc Dis.* 2012;22(10):883-9.
 35. Gontier E, Fourme E, Wartski M, et al. High and typical 18F-FDG bowel uptake in patients treated with metformin. *Eur J Nucl Med Mol Imaging.* 2008;35(1):95-9.
 36. McCarthy AD, Cortizo AM, Sedlinsky C. Metformin revisited: Does this regulator of AMP-activated protein kinase secondarily affect bone metabolism and prevent diabetic osteopathy. *World J Diabetes.* 2016;7(6):122-33.
 37. Shanbhogue VV, Mitchell DM, Rosen CJ, Bouxsein ML. Type 2 diabetes and the skeleton: new insights into sweet bones. *Lancet Diabetes Endocrinol.* 2016;4(2):159-73.

APPENDIX

S1 METHODS

S1.1 Laboratory analyses - validation experiments

Validation experiments were performed to test reproducibility, recovery and linearity for both metabolites. Calibration curves for 25-hydroxyvitamin D₂ (25(OH)D₂) were tested in water and in four different plasma samples. Mean response factor of these calibration curves was 0.763 (8.9 %CV). Inter-assay variation of 25(OH)D₂ was tested in two different plasma samples with a mean concentration of 5.7 nmol/L (15.9 %CV) and 15.7 (12.9 %CV), respectively. We have tested the effect of thawing and freezing the samples for four cycles. We did not find any change in concentration, as this was stable during four cycles of freeze-thawing for both metabolites. Samples were not tested in the same batch; however two control samples were used to test inter-assay variation during several days of analysis, and appeared to be between 7.4 and 15.9 %CV for both metabolites. These validation experiments show the reliability of our method and make any findings by chance due to lab technique variability unlikely. All the measurements were performed in the Laboratory of Metabolism and Vascular Medicine, Maastricht University Medical Center, The Netherlands.

S1.2 Missing Data Allocation

Missing completely at random (MCAR) assumption: When assuming that the occurrence of missing data is completely at random, the significance of the tested drug will be assessed on completers in testing the significance of the interaction term quantifying the average difference in trend lines between the two treatments

Missing not at random (MNAR) assumption: The level of extent to which missing at random (MAR) assumption constitutes a viable hypothesis is unknown, thus sensitivity analysis is needed to assess departure from this assumption. We base our analysis on a Pattern Mixture approach (PM), the principle of which is to identify a pattern variable affecting the probability and the appropriate imputation of missingness. Little introduced pattern mixture models for longitudinal data that are used to identify whether missing data patterns are informative and to include the missing data patterns as covariates in the model to control for the effect of missing data patterns on the outcome.^{1,2} Subjects are classified with the number of missing data.³ To examine changes in an outcome over time, the interaction

between the missing value pattern and time will allow evaluation of whether trends over time differ according to the presence of a missing value pattern. For sensitivity purposes, multiple imputation was used.

S1.3 SAMPLE SIZE AND POWER

S1.3.1 Preliminary Note

This study is a post-hoc analysis based on a fixed sample size. Thus, the sample size cannot be calculated but the corresponding power should be demonstrated as sufficient related with a clinically relevant difference.

S1.3.2 Assumptions

We assume simplifying hypotheses: (1) Vitamin D absorption is constant during the whole Post-baseline period, (2) Linearity of Baseline effect (25(OH)D)b, (3) known correlation of the partial predicting value (25(OH)D)b+cos(kt.t) such that the Pearson Linear correlation R is 0.30 <R<0.60 . (3) Admitted two-tailed alpha risk reduced to p<0.05. (4) 25(OH)D at base-line was measured only at D0 (once). (5) A minimum clinically relevant of 10% difference between the two groups, (6) using the relative difference over baseline as the assumed true difference, we assume the Coefficient of variation varying from 20% until 50% , noticing that 20% corresponds to a minimum clinical relevant Cohen’Rule of thumb (Standardized Mean difference ≤0.5) .

S1.3.3 Calculation

Based on the previous assumed values, we calculate the power in varying V from 0.20 until 0.50, and the number of post-baseline measures from 1 to 5 (There are 6 samples but one baseline, thus 5 post-baseline possible measures).

Assuming that the correlation (Baseline-Final) is at least R=0.30, a power of at least 0.85 is expected when the variance coefficient is ≤0.40 (40%), when at least 2 post-baseline measures are available. Besides, in using all the samples (n=5), this power increase to 0.96 thus roughly 10% more.

However, assuming R=0.70, even with V=0.50, the power remains 0.85 with 2 post-baseline values and we only gain 10% more in using the 5 values. We finally note that a Variation coefficient V=0.50 should be unusually large and unexpected.

	25(OH)D*				25(OH)D ₂				25(OH)D ₃			
	beta	95% CI		p	beta	95% CI		p	beta	95% CI		p
Intercept (nmol/l)	78.199	69.805	87.603	<0.0001	2.576	2.152	3.083	<0.0001	75.071	66.807	84.357	<0.0001
Metformin†	1.002	0.998	1.006	0.35	1.011	1.003	1.020	0.01	1.001	0.997	1.005	0.59
Age‡	0.995	0.992	0.999	0.005	0.997	0.992	1.002	0.28	0.995	0.992	0.999	0.007
Sex§	0.929	0.866	0.997	0.042	0.847	0.758	0.946	0.004	0.932	0.867	1.002	0.06
BMI (kg/m2)	0.987	0.980	0.995	0.001	0.995	0.983	1.007	0.39	0.987	0.980	0.995	0.001
Season¶	1.255	1.230	1.279	<0.0001	0.964	0.926	1.003	0.07	1.268	1.243	1.294	<0.0001
Time#	0.998	0.995	1.000	0.35	1.000	0.995	1.005	0.97	0.998	0.995	1.000	0.036

Supplemental table S1. Results of mixed model analysis on mean vitamin D levels in completers. As logarithmic values have been used for 25(OH)D, the model is multiplicative. *Combined serum levels of 25(OH)D₂ and 25(OH)D₃; †Effect per month of metformin; ‡age minus 60; §female sex; || BMI minus 30; ¶ the seasonal component was calculated as a sinusoidal function with mean 25(OH)D values in August or March compared with mid-season (June or November); #per month, independent of treatment..

	25(OH)D*			
	beta	95% CI		p
Intercept (nmol/l)	78.168	62.953	97.059	<0.0001
Metformin†	1.003	0.999	1.008	0.13
Age‡	0.994	0.991	0.997	<0.0001
Sex§	0.932	0.871	0.997	0.04
BMI (kg/m2)	0.986	0.979	0.993	0.001
Season¶	1.252	1.227	1.277	<0.0001
Time#	0.997	0.994	0.999	0.01
B12 (pmol/l)	1.000	1.000	1.000	0.17

Supplemental table S2. Results of mixed model analysis on mean vitamin D levels with addition of B12 as a covariate. As logarithmic values have been used for 25(OH)D, the model is multiplicative. *Combined serum levels of 25(OH)D₂ and 25(OH)D₃; †Effect per month of metformin; ‡age minus 60; §female sex; || BMI minus 30; ¶ the seasonal component was calculated as a sinusoidal function with mean 25(OH)D values in August or March compared with mid-season (June or November); #per month, independent of treatment.

REFERENCES APPENDIX

1. Little RJ, Wang Y. Pattern-mixture models for multivariate incomplete data with covariates. *Biometrics* 1996;52:98-111
2. Raghunathan TE. What do we do with missing data? some options for analysis of incomplete data. *Annu Rev Public Health* 2004;25:99-117
3. Little RJ, Long Q, Lin X. A comparison of methods for estimating the causal effect of a treatment in randomized clinical trials subject to noncompliance. *Biometrics* 2009;65:640-649

Chapter 4.

Metformin-associated prevention of weight gain in insulin-treated type 2 diabetic patients cannot be explained by decreased energy intake

$$E = MC^2$$

METFORMIN-ASSOCIATED PREVENTION OF WEIGHT GAIN IN INSULIN-TREATED TYPE 2 DIABETIC PATIENTS CANNOT BE EXPLAINED BY DECREASED ENERGY INTAKE: A POST HOC ANALYSIS OF A RANDOMIZED PLACEBO-CONTROLLED 4.3 YEAR TRIAL.

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ABSTRACT

Metformin prevents weight gain in patients with type 2 diabetes (T2D). However, the mechanisms involved are still unknown. In this post-hoc analysis of the HOME trial, we aimed to determine whether metformin affects energy intake. Patients with T2D were treated with 850 mg metformin or placebo (1-3 times daily) added to insulin for 4.3 years. Dietary intake was assessed at baseline, after 1 year and after 4.3 years, according to the dietary history method. Of the 310 included participants, 179 completed (93 placebo, 86 metformin) all three dietary assessments. We found no significant difference in energy intake after 4.3 years between the groups (metformin vs placebo: -31.0 kcal/day; 95% CI -107.4 to +45.4; F-value 1.3, df=415, p=0.27). Body weight in placebo-users increased significantly more than in metformin-users during 4.3 years (4.9 ± 4.9 vs 1.1 ± 5.2 kg; t-test: $p \leq 0.001$). Linear mixed models did not show a significant effect of energy intake as explanation for the difference in weight gain between the groups (F-value 0.1, df=1, p=0.82). In conclusion, the prevention from weight gain by metformin cannot be explained by reduced energy intake.

INTRODUCTION

A major side-effect of insulin therapy in type 2 diabetes (T2D) is weight gain, which is associated with increasing insulin resistance and cardiovascular risk.¹ Metformin prevents weight gain in T2D patients.² However, the mechanisms involved are still unknown. Less energy intake, malabsorption and metabolic effects may contribute. In animal experiments, metformin reduced food intake.³ Studies of the effects of metformin on energy intake in human are sparse and inconclusive,⁴⁻⁹ although a majority of the studies found associations between the use of metformin and lower energy intake.⁴⁻⁷ However, sample sizes of

these studies were small and long-term placebo-controlled trials are lacking. Therefore, we investigated whether treatment with metformin vs placebo during 4.3 years affects energy intake in patients in the *Hyperinsulinemia: the Outcome of its Metabolic Effects (HOME) trial* (ClinicalTrials.gov identifier NCT00375388). The HOME trial is the largest known randomized controlled trial (RCT) to evaluate the effects of metformin vs placebo in patients with T2D treated with insulin. The study showed that metformin when added to insulin in patients with T2D not only improved glycemic control and reduced insulin requirements, but also prevented weight gain.² In the HOME trial, both short- and long-term outcomes are available. Moreover, no other glucose-lowering drugs were used, allowing comparison of metformin vs placebo in patients receiving insulin, but no other antidiabetic agents. The main objective of the current study was to investigate whether treatment with metformin affects energy intake.

MATERIALS AND METHODS

Study design, randomization, interventions and follow-up

The HOME trial is a 4.3 year RCT, that included 390 Caucasian patients aged 30-80 years with T2D treated with insulin. Patient selection, study design, data collection and power analysis have been described previously.² The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, The Netherlands). All participants provided written informed consent before inclusion. The medical ethical committees of the three participating hospitals approved the trial protocol. The study was conducted in accordance with Good Clinical Practice (CPMP/ICH/135/95; 1996) and with the Declaration of Helsinki (revised version, 2000).

Figure 1 shows the trial design and recruitment plus retention of patients.² Patients were randomly allocated to either metformin 850 mg or placebo (one to three times daily, depending on tolerance and renal function), in addition to insulin therapy.

Visits and data collection

Patients visited the clinics at the start of the pre-randomization phase (three months before randomization), at baseline (for randomization to metformin or placebo), one month after baseline (to check tolerance of drug titration), and subsequently every three months until the end of the trial. All participants received dietary counseling. Dietary intake was

Figure 1. Trial design (A) and flow diagram of recruitment and retention of patients in both groups (B).

independently assessed by four trained registered dietitians at baseline, after 1 year, and after 4.3 years. For dietary assessment, patients were asked about their intake during the preceding week according to the validated dietary history method.¹⁰ Mean energy intake per day (kcal) was calculated using food calculation software (Becel Institute Nutrition Software; Vodisys Medical Software, Groningen, The Netherlands). The dietitians were blinded for treatment allocation. Body weight was measured every three months.

STATISTICAL ANALYSES

In the current analysis, our main objective was to test for the effect of metformin vs placebo on energy intake. For our main analysis, we used a linear mixed model assessing time effects (baseline, one year, 4.3 years), treatment effects and their interactions, based on intention to treat. In the model, we statistically controlled for the fixed effects of sex, age, duration of diabetes and smoking habits. Missing measurements on dietary intake and body weight were not part of the analysis.

In addition, we performed three secondary analyses: 1) a per protocol analysis including only those patients who completed the whole study according to the research protocol; 2) an analysis to investigate the degree to which possible differences in energy intake between treatment groups were associated with weight gain during the study. For this secondary analysis, treatment dose, time, smoking habits and sex were taken as fixed factors, energy intake, age and duration of diabetic disease as covariates, and patients as random effects; 3) a mediation analysis to study whether reduction of the daily dose of insulin is a significant contributing factor to the reduced weight gain. F-tests were used to for the significance of effects. P-values < 0.05 were considered statistically significant; “±” denotes standard deviation. The analysis of mixed models was carried out with R release 3.03.

RESULTS

General trial results

Dietary assessments were available from two hospitals, providing data of 334 out of 390 patients randomized in the original HOME study. 24 patients were excluded, as their baseline dietary assessment was performed after having started with metformin or placebo treatment, resulting in inclusion of 310 patients. Of the 310 participants, 179 (93 placebo and 86 metformin) completed all three dietary assessments. Patients in the metformin

	Placebo (n=151)	Metformin (n=159)
Demographic Characteristics, mean±SD		
Men/ women, No.	72/79	72/87
Age (years)	59±11	63±9*
Duration of diabetes (years)	12±8	14±9*
Insulin treatment (years)	6±6	7±8
Current smoking, No. (%)	39 (26)	33 (21)*
Use of alcohol (U/day)	0.7±1.2	0.7±1.2
Metabolic variables, mean±SD		
Weight (kg)	87±16	86±16
Body mass index (kg/m ²)	30±5	30±5
Plasma HbA1c (%)	7.9±1.2	7.9±1.2
Daily dose of insulin (IU/day)	64±25	62±29
Energy intake (kcal)	1649±419	1613±426

Table 1. Baseline characteristics

group were slightly older than those receiving placebo, had a slightly longer duration of diabetes, and were less often smokers. All other characteristics were comparable between the treatment groups (Table 1). The main outcomes of this trial have been reported previously.² Mean values of duration of diabetes, previous occurrence of cardiovascular disease, age and weight were similar between the three hospitals and between those who did and did not complete the study.

Main analysis

Mean daily energy intake after 4.3 years decreased from 1649±419 to 1632±453 kcal in the placebo group and from 1613±426 to 1567±419 kcal in the metformin group (mean difference of 71.0 kcal/day in metformin vs placebo; 95% CI -199.4 to +54.5, p=0.28) (figure 2).

Mixed model analyses did not indicate a significant difference in energy intake after 4.3 years: -31.0 kcal/day in the metformin compared to the placebo group (95% CI -107.4 to +45.4; F-value 1.3, df=415, p = 0.27). In addition, a per protocol analysis of the data of patients with the intended maximum dose of metformin of 850 mg three times daily showed similar results (-36.8 kcal/day, 95% CI -144.7 to +71.1; F-value 1.3, df=238, p = 0.28).

Body weight of the patients in the placebo group increased by 4.9±4.9 kg in 4.3 years, significantly more than in the metformin group (+1.1±5.2kg; t-test: p<0.001). However, linear

Figure 2. Mean energy intake at baseline, after 1 year and 4.3 years for the placebo and metformin group

mixed models taking random patient effects into account did not indicate a significant effect of energy intake on weight gain between treatment and control groups (F-value 0.1, df=1, p = 0.82), after controlling for several factors. A mediation analysis showed that at the end of the study, a patient on the actual mean dose of metformin (2050mg), had 3.2kg±0.5 less weight gain (p=0.0001), as compared to placebo, of which 1.2kg±0.0 (p<0.0001; 37.5%) could be explained by a reduction of insulin intake by the use of metformin. (p=0.0001) (See Appendix S1 for further details).

DISCUSSION

This long-term RCT has previously shown that metformin prevents weight gain in T2D patients with insulin therapy.² The present analysis shows that metformin did not reduce energy intake. Therefore, in this study, the prevention of weight gain by metformin cannot be explained by a reduction of energy intake.

Our present findings are not consistent with the majority of previous studies. Previously, metformin was associated with reduced food intake in two very small short term placebo-controlled trials^{4,5} a small open label trial⁶ and a post-hoc trial with matched controls.⁷ On the

other hand, two RCTs in T2D patients showed no change in energy intake by metformin, as compared to placebo.^{8,9} In our study, we analysed the effect of metformin on energy intake after 1 and 4.3 years. Therefore, we cannot entirely exclude that metformin reduces energy intake at the very short term. However, the current evidence suggests that other mechanisms must be involved in the prevention of weight gain by metformin during insulin therapy.

Reduction in insulin requirements. Our mediation analysis showed that reduction in insulin requirements by the use of metformin² prevents weight gain. Several effects of insulin may cause weight gain.¹¹ First, in the months after initiation of insulin therapy, weight gain is attributed to the improvement in glycemic control and correction of fluid balance. Second, insulin has anabolic effects and stimulates lipogenesis. Third, genetic factors may contribute to the sensitivity to gain weight due to insulin therapy. Fourth, insulin acts independently of glycaemia and may induce hypoglycemia and lead to enhanced hunger, inducing reactive eating behavior and inhibiting an active lifestyle.¹¹

Malabsorption by metformin. Metformin may cause malabsorption of nutrients possibly through reducing ileal bile salt reabsorption.¹² To what extent this may affect weight development remains to be established.

Effects of metformin through the incretin system. Metformin may affect the incretin system at different levels by acting as an incretin mimetic, as an incretin enhancer, and as an incretin sensitizer.¹³ Such a composite effect increases the bioavailability and efficacy of glucagon-like peptide-1 (GLP-1), the most relevant incretin. GLP-1 is known to slow gastric emptying, to improve glycemic control, to prevent hypoglycemia (and reactive eating behavior), and to prevent weight gain.

Potential other effects of metformin affecting regulation of body weight. An important effect of metformin is the activation of AMP-activated protein kinase (AMPK), the cell's principle energy sensor.¹⁴ AMPK affects cell energy metabolism and might influence body weight regulation. In obese mice, metformin improves the balance of white adipose tissue and brown adipose tissue, which also might affect body weight control.¹⁵ Next, metformin alters the microbiome in mice and humans, which may cause an increase of intestinal gluconeogenesis.¹² In rodents, increased intestinal gluconeogenesis decreases body weight, despite comparable food intake.¹⁶ Such an effect may contribute to the prevention of weight gain in metformin use. Finally, metformin might affect energy expenditure or physical activity. However, several studies found no influence of metformin on energy expenditure or physical activity.⁴

Strengths and limitations of the study

Strengths of our study include the randomized, placebo-controlled, double-blind design and its relatively long period of follow-up, as well as the relatively large sample size. A limitation is the assessment of dietary intake by the dietary history method, which usually underestimates intake.¹⁰ Intake may have been further underestimated, as we did not assess snacking in case of hypoglycemia. However, the frequency of hypoglycemic events was similar in both groups.² Thus, the occurrence of hypoglycemia and related snacking probably will not have influenced the results. In addition, we did not register physical activity during the present trial. Finally, it is difficult to unravel 'cause-and-effect relationships' between insulin requirement and weight development, even in an RCT like the HOME trial since they might be bi-directional.

In conclusion, in this long-term RCT in patients with T2D treated with insulin, metformin treatment did not affect energy intake as compared to placebo. Therefore, reduced energy intake cannot serve as an explanatory factor for the prevention of weight gain caused by metformin during insulin therapy in the long term that was reported earlier. However, approximately one third of difference in weight gain between metformin and placebo users can be explained by the reduction of insulin requirements by metformin. Further research is needed to determine the degree of involvement of potential other mechanisms discussed.

ARTICLE INFORMATION

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Conflict of interest

All authors declare that there is no duality of interest associated with their contribution to this paper.

Author contributions

AK and CS designed the study. MO and IM did literature searches. MO, IM, WK and PL researched the data; MO, IM and WK performed the statistical analyses; MO, IM and AK wrote the article. HJW, CS, WK, PL and CS contributed to the discussion and reviewed and edited the article. MO, IM, WK and AK are guarantors. All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

Trial Registration

Clinicaltrials.gov NCT00375388.

REFERENCES

- DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care*. 1991;14(3):173-94.
- Kooy A, de Jager J, Lehert P, et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch Intern Med*. 2009;169(6):616-25.
- Lv WS, Wen JP, Li L, et al. The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. *Brain Res*. 2012;1444:11-9.
- Lee A, Morley JE. Metformin decreases food consumption and induces weight loss in subjects with obesity with type II non-insulin-dependent diabetes. *Obes Res*. 1998;6(1):47-53.
- Paolisso G, Amato L, Eccellente R, et al. Effect of metformin on food intake in obese subjects. *Eur J Clin Invest*. 1998;28(6):441-6.
- Makimattila S, Nikkila K, Yki-Jarvinen H. Causes of weight gain during insulin therapy with and without metformin in patients with Type II diabetes mellitus. *Diabetologia*. 1999;42(4):406-12.
- Yki-Jarvinen H, Nikkila K, Makimattila S. Metformin prevents weight gain by reducing dietary intake during insulin therapy in patients with type 2 diabetes mellitus. *Drugs*. 1999;58 Suppl 1:53,4; discussion 75-82.
- Aviles-Santa L, Sinding J, Raskin P. Effects of metformin in patients with poorly controlled, insulin-treated type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med*. 1999;131(3):182-8.
- Jacob AN, Salinas K, Adams-Huet B, Raskin P. Weight gain in type 2 diabetes mellitus. *Diabetes Obes Metab*. 2007;9(3):386-93.
- Jain M, Howe GR, Johnson KC, Miller AB. Evaluation of a diet history questionnaire for epidemiologic studies. *Am J Epidemiol*. 1980;111(2):212-9.
- McFarlane SI. Insulin therapy and type 2 diabetes: management of weight gain. *J Clin Hypertens (Greenwich)*. 2009;11(10):601-7.
- McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. *Diabetologia*. 2016;59(3):426-35.
- Cho YM, Kieffer TJ. New aspects of an old drug: metformin as a glucagon-like peptide 1 (GLP-1) enhancer and sensitiser. *Diabetologia*. 2011;54(2):219-22.
- Pryor R, Cabreiro F. Repurposing metformin: an old drug with new tricks in its binding pockets. *Biochem J*. 2015;471(3):307-22.
- Kim EK, Lee SH, Jhun JY, et al. Metformin Prevents Fatty Liver and Improves Balance of White/Brown Adipose in an Obesity Mouse Model by Inducing FGF21. *Mediators Inflamm*. 2016;2016:5813030.
- De Vadder F, Kovatcheva-Datchary P, Goncalves D, et al. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell*. 2014;156(1-2):84-96.

APPENDIX**S1 RESULTS**

Mediation analysis to study whether reduction of the daily dose of (DDI) is a significant contributing factor to the reduced weight gain. As compared to placebo, one gram year of metformin (the use of one gram of metformin during one year) was associated with a lower DDI of $-1.68 \text{ IU/d} \pm 0.35$ ($P < 0.0001$), which was in turn associated with a difference in weight gain of 0.13 kg per gram year of metformin ($p < 0.0001$). Independently of insulin reduction, one gram year of metformin was associated with a difference in weight gain of $0.23 \text{ kg} \pm 0.06$ ($p = 0.0001$). As an example: at the end of the study, a patient on the actual mean dose of metformin (2050 mg) had a lower DDI of $-14.9 \text{ IU/d} \pm 3.1$ ($P < 0.0001$) as compared to placebo, which was in turn associated with a difference in weight gain of $1.2 \text{ kg} \pm 0.0$ ($p < 0.0001$). Independently of insulin reduction, the use of metformin was associated with a difference in weight gain of $2.0 \text{ kg} \pm 0.5$ ($p = 0.0001$). As a result, the difference in weight gain in the study between metformin and placebo users can be explained for approximately one third ($1.2 / (1.2 + 2.0) = 37.5\%$) by a reduction of insulin intake by the use of metformin."

Chapter 5.

A gene variant near ATM affects the response to metformin and metformin plasma levels

A GENE VARIANT NEAR ATM AFFECTS THE RESPONSE TO METFORMIN AND METFORMIN PLASMA LEVELS - A POST HOC ANALYSIS OF AN RCT

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ABSTRACT

Aim: To determine the influence of polymorphisms on the effects of metformin on HbA1c, daily dose of insulin (DDI) and metformin plasma concentration

Methods: In a post hoc analysis of a 4.3 year placebo controlled RCT with 390 patients with T2D already on insulin, we analyzed the influence of polymorphisms in genes coding for ATM and the transporters OCT1 and MATE1. Outcome measures were a combined HbA1c + DDI Z score and metformin plasma concentrations.

Results: rs11212617 (ATM) was associated with an improved Z score and a lower metformin plasma concentration. In addition, the major allele of rs2289669 (MATE1) was also associated with an improved Z score.

Conclusion: The ATM SNP rs11212617 significantly affected the effect of metformin and MPC. Further research is needed to determine the clinical importance of these findings, in particular the effects on metformin plasma concentration.

INTRODUCTION

Metformin is the preferred initial pharmacological agent for type 2 diabetes (T2D).¹ Several intervention studies have shown favorable effects of metformin on glycemic control, insulin requirements, weight development and cardiovascular outcomes.^{2,3} The response to metformin is highly variable, even after adjusting for adherence to medication.⁴ Despite the progress in the understanding of the pharmacokinetics and pharmacodynamics of metformin, the variability in response remains only partly understood.⁵ Metformin requires drug transporters for absorption, distribution, and elimination, as it poorly diffuses passively over membranes.^{6,7} The drug is transported from the intestine into the bloodstream by plasma membrane monoamine transporter (PMAT) and organic cation transporter 1

(OCT1),^{8,9} taken up into the liver by OCT1 and OCT3, into the kidneys by OCT2, and passed from proximal tubule cells into the urine via multidrug and toxin extrusion transporter 1 (MATE1) and MATE2.⁵ Variation in the expression of genes involved in the transport and action of metformin may influence the response of metformin.¹⁰

Over the past few years, several studies showed associations between genetic variation of metformin transporters and the effect of metformin.¹¹⁻¹⁸ In addition, variability in the ataxia telangiectasia mutated (ATM) gene, which acts upstream of AMP-activated protein kinase, may play a role.¹⁹⁻²¹ However, the results of most studies on polymorphisms are still inconclusive, as several studies failed to replicate the associations.²²⁻²⁴ Small sample sizes, study designs without control groups, the low doses of metformin used and/or variety in ethnicity, all together, limit definite conclusions to be drawn.²⁵ In addition, studies about the effect of polymorphisms on metformin plasma concentration are sparse and contradictory.^{16,17,26,27} More studies are required to unravel the possible genetic factors that underlie the complex variation in the transport of and the response to metformin.

Therefore, we analyzed the association between genetic variations, metformin plasma concentrations and the effect of metformin in a post hoc analysis of the *Hyperinsulinemia: the Outcome of its Metabolic Effects* (HOME) trial. The HOME trial is the largest RCT to evaluate the effects of metformin vs placebo in patients with T2D treated with insulin. The study showed that metformin vs placebo, when added to insulin in patients with T2D, improved glycemic control, reduced insulin requirements and prevented weight gain.^{3,28} In the HOME trial, both short term and long term outcomes are available. Moreover, patients in the HOME trial used no other glucose-lowering medication than metformin (or placebo) and insulin. Dose dependency can be studied between metformin and the single-nucleotide polymorphisms (SNPs), with the option to use the placebo group as a control group with zero gram of metformin. In addition, the setting of a RCT ensures the consistency of data, with many measurements per patient during a long period of follow up. Furthermore, metformin plasma levels are available. Therefore, the design of the HOME trial allows analyzing the possible effects of polymorphisms on the key long-term effect of metformin: reduction in DDI related to glycemic control, as an aggregate result of metformin's multiple actions: decrease of hepatic glucose output, reduction of insulin resistance and increase of GLP-1 release.²⁹

Figure 1. Trial design (A) and flow diagram of recruitment and retention of patients in both groups (B).

MATERIALS AND METHODS

The HOME trial is a 4.3 year randomized placebo controlled trial that included 390 Caucasian patients aged 30-80 years with T2D treated with insulin. Patient selection, study design, data collection and power analysis have been described previously.^{3,28} Figure 1 shows the trial design and the recruitment plus retention of patients for the current study.^{3,28,30} Patients were randomly allocated to either metformin 850 mg or placebo (one to three times daily, depending on tolerance and renal function), in addition to insulin therapy. No other glucose-lowering drugs were used, allowing comparison of metformin with placebo in patients receiving insulin, but no other antidiabetic agents. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial has been conducted in accordance with the Committee for Medicinal Products for Human Use note for guidance on good clinical practice (CPMP/ICH/135/95), dated 17 July 1996, and in accordance with the Declaration of Helsinki (revised version of Hong Kong in 1989 and Edinburgh in 2000). All patients gave written informed consent.

Laboratory investigations

Measurement of HbA1c was described previously.²⁸ For measurement of trough metformin plasma concentrations, we used blood samples taken before morning metformin dose. Blood samples were stored at -80°C until analysis. The plasma concentrations were measured with high-performance liquid chromatography with UV Detector (HPLC-UV).³¹ The mobile phase contained 30% acetonitrile and 70% aqueous phosphate buffer at pH 7. The column used was a reversed-phase phenyl column (Spherisorb 5 Phenyl G100x3.0µm), kept at a temperature of 40 degrees Celsius. The wavelength of the detector was set at 236 nm.

Genotyping

We selected the SNPs that were previously associated with metformin response or pharmacokinetic parameters in at least two independent populations and with a minor allele frequency of at least 5% in Caucasian populations. On this basis we analyzed the polymorphisms in the genes coding for OCT1 (*SLC22A1*; rs12208357^{11,17,18} and rs622342^{14,32,33}), MATE1 (*SLC47A1*; rs2289669^{12,15,34}) and their interaction,^{14,35} and for the ATM gene (rs11212617¹⁹⁻²¹). DNA samples were collected at baseline on IsoCode Stix and stored at basement climate, according to the manufacturer's instruction (Schleicher & Schuell, Keene, N.H., USA). Genotyping was done using Taqman allelic discrimination assays. The staff performing genotyping was masked to the outcome.

STATISTICAL ANALYSIS

We analyzed the previously reported associations between polymorphisms and the treatment effect of metformin on hyperglycemia (HbA1c) and DDI. Our main endpoint was the assessment of the polymorphisms’ effect on two important treatment effects of metformin: the reduction of HbA1c and DDI. These bivariate simultaneous effects were assessed through an aggregated Z score of baseline (b) and final (f) values of HbA1c and DDI, as follows:³⁶

$$HbA1c+DDI\ Z\ score = \frac{HbA1c\ (f) - \text{mean}\ HbA1c(b)}{SD(\text{mean}\ HbA1c(b))} + \frac{DDI(f) - \text{mean}\ DDI(b)}{SD(\text{mean}\ DDI(b))}$$

A better metformin response will thus result in a lower Z score. Our main selection was the set of all randomized patients. As secondary analysis, we tested the influence of the polymorphisms on the effect of metformin on HbA1c and DDI. For our main analysis, we used a linear model with a full factorial design (see the supplements section 1 for details). For each polymorphism, the associations between the number of minor variant alleles and treatment response were assessed (wild type = 0, heterozygous = 1, homozygous = 2), for short term (16 weeks of treatment) and long term (4.3 years). The analyses were adjusted for baseline Z score, age, sex and duration of diabetes. Intention to treat sample was considered as the main population. Final values for dropouts were imputed through last observation carried forward (LOCF). In addition, we tested for interactions between the OCT1 SNP rs622342 and the MATE1 SNP rs2289669.

Next, we studied the effect of the plasma metformin concentration on HbA1c and DDI to find out whether an association of the SNPs with the Z score (if any) can be explained by a difference in metformin plasma concentration, with adjustment for age, sex, duration of diabetes, daily dose of metformin and the estimated glomerular filtration rate (according to the CKD-EPI equation)³⁷. Since this analysis was necessarily restricted to metformin users, the sample size was reduced accordingly for this evaluation.

As we performed an analysis of four different polymorphisms, we set the significance level to 0.0125 for two-sided P-values (0.05 divided by the number of tests, according to the Bonferroni method).³⁸ For this report, we used the current guidelines and recent recommendations for reporting of genetic association studies.^{25,39}

	Placebo (n=194)	Metformin (n=196)	p value
Demographic Characteristics			
<i>Sex, no. (%)</i>			
Men	97 (50)	81 (41)	0.08
Women	97 (50)	115 (59)	<0.001*
Age (years)	59±11	64±10	
<i>Race, no. (%)</i>			
White	194 (100)	196 (100)	N/A
Duration of diabetes (years)	12±8	14±9	0.01*
Insulin treatment (years)	6±6	7±8	0.15
Current smoking, no. (%)	59 (30)	38 (19)	0.01*
Metabolic variables			
Weight (kg)	87±15	85±16	0.25
Body mass index (kg/m2)	30±5	30±5	0.77
HbA1c (%)	7.9±1.2	7.9±1.2	0.90
Daily dose of insulin (IU/day)	64±25	62±29	0.57
eGFR (ml/min)	72.1±14.9	68.9±13.8	0.03*

Table 1. Baseline data
Data are shown as mean ± SD or as indicated. There were no significant differences in baseline characteristics between the study groups, except that patients in the metformin group were slightly older than those receiving placebo (p<0.001), had a more extensive cardiovascular disease history (p=0.03) and were less often smokers (p=0.01). *P<0.05

RESULTS

General trial results

A total of 390 subjects provided written informed consent and enrolled into the trial; 196 subjects were randomly assigned to receive metformin and 194 to receive placebo (Table 1). Of the 390 included patients, 277 (71%) completed the 4.3 year HOME trial. The main outcomes of this trial have been reported previously.³ The mean dose in the metformin group was 2050 mg during the long term trial. Those who did and did not complete the study did not differ with respect to duration of diabetes, previous occurrence or severity of cardiovascular disease, age or weight.

Gene	SNP		MAF	pHWE
OCT1	rs12208357	C>T	6%	0.52
	rs622342	A>C	40%	0.42
MATE1	rs2289669	G>A	40%	0.34
ATM-Locus	rs11212617	A>C	46%	0.91

Table 2. Analyzed polymorphisms
MAF: minor allele frequency; pHWE: p value for Hardy-Weinberg equilibrium

		Short term (n=321)*	Long term (n=331)†		
Gene	SNP	Beta (95%CI)	p	Beta (95%CI)	p
A. HbA1c+DDI Z score					
OCT1	rs12208357	-0.01 (-0.26 – 0.25)	1.00	0.40 (-0.20 – 1.01)	0.31
	rs622342	-0.01 (-0.11 – 0.10)	0.99	0.24 (0.00 – 0.49)	0.09
MATE1	rs2289669	0.03 (-0.07 – 0.14)	0.44	0.35 (0.09 – 0.60)	0.006
ATM-Locus	rs11212617	-0.11 (-0.20 – -0.01)	0.04	-0.19 (-0.42 – 0.05)	0.12
B. HbA1c					
OCT1	rs12208357	-0.08 (-0.35 – 0.19)	0.67	0.12 (-0.27 – 0.51)	0.57
	rs622342	-0.04 (-0.15 – 0.07)	0.63	0.02 (-0.14 – 0.17)	0.77
MATE1	rs2289669	-0.02 (-0.13 – 0.09)	0.80	0.03 (-0.13 – 0.19)	0.71
ATM-Locus	rs11212617	-0.09 (-0.19 – 0.02)	0.10	-0.07 (-0.22 – 0.08)	0.39
C. Daily dose of insulin					
OCT1	rs12208357	2.09 (-1.94 – 6.12)	0.30	7.19 (-6.77 – 21.14)	0.48
	rs622342	0.05 (-1.61 – 1.72)	0.93	5.43 (-0.23 – 11.09)	0.10
MATE1	rs2289669	0.35 (-1.31 – 2.02)	0.64	7.58 (1.79 – 13.38)	0.01
ATM-Locus	rs11212617	-0.56 (-2.12 – 1.01)	0.49	-3.42 (-8.81 – 1.96)	0.21

Table 3. Polymorphisms and treatment effect
Estimates of the association between polymorphisms and the effect of treatment (daily dose of metformin, gram per day) on the aggregated HbA1c + DDI Z score, HbA1c and DDI. Covariates included in the model were baseline Z score (or either HbA1 or DDI), age, sex and duration of diabetes. Full models are shown in Supplemental Table 4 and 5. *After 16 weeks; †At the end of the study (LOCF)

Genotyping

DNA samples were available for 164 patients in the metformin group and 172 patients in the placebo group (Table 2). Genotyping was successful for all DNA samples. All variants were in Hardy-Weinberg equilibrium ($p > 0.05$). There were no significant differences in baseline characteristics between genotyped and non-genotyped patients (see Supplemental Table S1 for baseline characteristics per genotype).

Polymorphisms and treatment effect

During the study, the aggregated HbA1c + DDI Z score decreased in the placebo group from 0.019 ± 1.6 to 0.016 ± 1.66 at short term (16 weeks), and increased to 1.31 ± 2.46 at long term (LOCF). In the metformin group, the HbA1c + DDI Z score decreased from -0.05 ± 1.7 at baseline to -0.79 ± 1.7 at short term, and increased to 0.22 ± 2.2 at long term. Results of the unadjusted analysis are shown in supplemental Tables 2 and 3). Figure 2 shows the mean values of the Z score per polymorphism during the trial.

Figure 2. Mean values of the HbA1c + DDI Z score during the HOME trial per polymorphism. For patients on metformin, mean values are shown per number of minor alleles. For patients on placebo, mean values are shown for the whole group together, as analyses showed that the polymorphisms had no effect on the Z score in placebo users (see supplemental Table 3 for full results of the unadjusted analysis).

In the main analysis, the minor allele of the ATM polymorphism rs11212617 showed a trend to an association with improved effect of treatment on the aggregated HbA1c + DDI Z score at short term, and to a lesser extent at LOCF. However, this association was not significant with the application of the Bonferroni method (Table 3; see Supplemental Tables 4 and 5 for the full factorial models).

We found no association between the separate OCT1 polymorphisms and treatment. The MATE1 SNP had a significant association with treatment: for all patients, the minor allele was associated with an increase of both the Z score and DDI. The estimates for the association between polymorphisms and the effect of treatment on both HbA1c and DDI were similar to the estimates of the primary endpoint (Table 3). The association between MATE1 and treatment effect appeared to be influenced by an interaction between the OCT1 SNP rs622342 and the MATE1 SNP rs2289669 ($p=0.007$ at short term, $p=0.02$ at LOCF; Table 4).

For patients with the OCT1 genotype AA or genotype AC, the minor allele of the MATE1 polymorphism was associated with decreased treatment effect. In contrast, for patients with the OCT1 genotype CC, the minor allele of MATE1 was associated with a positive treatment effect: a decrease of the Z score per daily gram of metformin at short term of -0.37 (95% CI -0.63 to -0.11) and at long term of -0.44 (95% CI -0.94 to -0.06). We also found a significant interaction between rs622342 and rs2289669 for HbA1c, but not for DDI (Supplemental Table 6).

Based on the Z score, it is possible to get an estimation of the change of HbA1c or DDI, by multiplying the coefficients by the standard deviations of the mean baseline values of HbA1c (1.2%) or DDI (27.1 IU). For example, at short term, the minor allele of the ATM SNP was associated with a decrease of 0.11 of the aggregated HbA1c + DDI Z score per gram of

OCT1 & MATE1	Short term*			Long term†		
	Beta (95%CI)	p value		Beta (95%CI)	p value	
rs622342 & rs2289669	n=321 -0.20 (-0.35 – -0.06)	0.007		n=331 -0.41 (-0.77 – -0.06)	0.024	
rs622342 (AA) & rs2289669	n=118 0.15 (-0.02 – 0.32)	N/A		n=123 0.61 (0.16 – 1.06)	N/A	
rs622342 (AC) & rs2289669	n=149 0.07 (-0.09 – 0.23)	N/A		n=153 0.35 (-0.02 – 0.71)	N/A	
rs622342 (CC) & rs2289669	n=54 -0.37 (-0.63 – -0.11)	N/A		n=55 -0.44 (-0.94 – 0.06)	N/A	

Table 4. Metformin, interaction rs622342, rs2289669 and the aggregated HbA1c + DDI Z score
Estimates of the interaction between the MATE1 SNP rs2289669 (estimate per minor allele) and treatment (daily dose of metformin, gram per day) per genotype of the OCT1 SNP rs622342 on the aggregated HbA1c + DDI Z score. Covariates included in the model were baseline Z score, age, sex and duration of diabetes. *After 16 weeks; †At the end of the study (LOCF)

Figure 3. Mean metformin plasma concentration per polymorphism

metformin per day, corresponding with an estimated decrease of HbA1c of $0.11 \times 1.2 = 0.13\%$ or a decrease of DDI of $0.11 \times 27.1 = 3.0$ IU (per gram of metformin per day per minor allele). Although not significant, the association at long term was comparable: a decrease of the Z score of 0.19 per minor allele at LOCF (per gram of metformin, HbA1c: -0.23%; DDI: -5.0 IU).

Polymorphisms and metformin plasma concentration

Mean metformin plasma concentration was 1.79 ± 0.39 mg/ml ($n=158$). The metformin plasma concentration was associated with a reduction of the Z score (adjusted for baseline values): an increase of the metformin plasma concentration with 1mg/ml was associated with a decrease of the Z score after 16 weeks with 0.05 (95% CI: -0.11 to 0.02; $p=0.02$) and at LOCF with a decrease of 0.09 (95% CI: -0.23 to 0.04; $p=0.03$). Figure 3 shows the mean plasma concentration per polymorphism.

The minor allele of the ATM SNP rs11212617 was associated with a lower metformin plasma concentration, closely to significance with application of the Bonferroni method (-0.42 mg/ml per minor allele, 95% CI: -0.81 to -0.03; $p=0.0128$; Table 5).

There were no associations between the OCT1 polymorphisms or the MATE1 polymorphism and metformin plasma concentration. Furthermore, there was no significant interaction between OCT1, MATE1 and metformin plasma concentration in all patients.

Gene	SNP	Metformin Plasma Concentration (n=155)	
		Beta (95%CI)	p
OCT1	rs12208357	0.39 (-0.37 – 1.15)	0.09
	rs622342	0.27 (-0.30 – 0.84)	0.29
MATE1	rs2289669	0.12 (-0.49 – 0.73)	0.74
ATM-Locus	rs11212617	-0.42 (-0.81 – -0.03)	0.0128
OCT1 & MATE1	rs622342 & rs2889669	-0.31 (-0.88 – 0.25)	0.62

Table 5. Polymorphisms and Metformin Plasma Concentration

Adjusted for age, sex, estimated glomerular filtration rate, daily dose of metformin and duration of diabetes

In addition, the daily dose of metformin predicted the metformin plasma concentration. An increase in the daily dose of metformin with 1 gram/day was associated with an increase of the metformin plasma concentration of 0.82 mg/ml (95% CI 0.48 to 1.15; $p < 0.001$). Furthermore, of all variables studied, the daily dose of metformin and age had the strongest association with metformin response: an increase of the daily dose of metformin with 1 gram/day was associated with a mean decrease of the HbA1c + DDI Z Score of 0.62 (95% CI: -0.96 to -0.29; $p < 0.001$), age was also associated with a decrease (-0.05 per year of age, 95% CI: -0.08 to -0.02; $p < 0.001$).

DISCUSSION

In this post hoc analysis of a long-term randomised placebo-controlled trial in T2D patients treated with insulin, we could not confirm previously described associations between the effect of metformin and polymorphisms of genes involved in the transport of metformin. However, we did find interesting results between the ATM SNP rs11212617, the effect of metformin and metformin plasma concentrations. Metformin plasma concentrations were associated with the treatment outcome. However, of all variables studied, the daily dose of metformin and age had the strongest association with metformin response.

Metformin & SNPs

The rs11212617 variant near the ATM gene was associated with the treatment response and with the metformin plasma concentrations. Furthermore, we replicated an interaction between the OCT1 polymorphism rs622342 and the MATE1 polymorphism rs2289669.

Rs11212617 was associated with treatment outcome, albeit not significant after application of the Bonferroni method. At short term, the minor allele was associated with a decrease

of 0.11 of the aggregated HbA1c + DDI Z score per gram of metformin and, the association at long term was comparable: a decrease of the Z score of 0.19 per minor allele at LOCF. Several studies have reported the association between the ATM-SNP rs11212617 and treatment response.^{19,20,40} Furthermore, a recent study found an association between the polymorphism and coronary artery disease in men.⁴¹

Our study is the first to report the association between rs11212617 and metformin plasma concentrations. Interestingly, the minor allele of the SNP was not only associated with improved treatment outcome, but also with lower metformin plasma concentrations. Previously, a study of OCT1 polymorphisms in healthy volunteers has shown comparable results: the OCT1 reference allele was associated with a lower metformin plasma concentration and a stronger glucose-lowering effect of metformin, compared to OCT1 variant alleles.¹³ An explanation could be that the SNP rs11212617 or related genes might lead to greater intracellular uptake of metformin to exert its effect more effectively, leading to lower insulin requirements. The target gene of rs11212617 is unknown yet. The ATM gene is suggested as the most likely candidate, as one of the upstream targets of ATM is known to be involved in the actions of metformin.¹⁹ Furthermore, patients with loss of function ATM mutations, resulting in the autosomal recessive condition ataxia telangiectasia, have marked insulin resistance.⁴² However, the assumption that ATM is the most likely gene being responsible for the effect has been a matter of debate.^{43–45} Recently, a study in participants with ataxia telangiectasia in the absence of a diagnosis of diabetes showed that mutations in ATM are associated with elevated glycaemia and low peripheral insulin sensitivity, but not with a reduction in hepatic insulin sensitivity.⁴⁶ As metformin predominantly acts in the liver, this observation is at odds with an association between ATM and metformin response. Our finding that the minor allele was associated with lower plasma concentrations makes it less likely that ATM is the target gene, as ATM is not known to be involved in the pharmacokinetics of metformin. Therefore, our findings might support the theory that the SNP rs11212617 influences the expression of transporters involved in the cellular uptake of metformin in the liver or the gut.

In the primary analysis at LOCF, the minor allele of MATE1 SNP rs2289669 was associated with decreased metformin response (an increase of the Z score with 0.35 per gram of metformin per minor allele). Studies of the association between MATE1 and the effect of metformin are inconclusive, with reports linking the minor allele of rs2289669 to both increased^{12,15,27} and reduced³⁴ glucose-lowering effect of metformin. In addition, studies about MATE1 and

metformin plasma concentrations and renal clearance found contradictory results.^{17,27} In this study, we show that a possible cause of these seemingly contradicting results might be due to an interaction between the OCT1 SNP rs622342 and the MATE1 SNP rs2289669. For patients with one or two major alleles of the OCT1 polymorphism, the minor allele of the MATE1 polymorphism was associated with decreased treatment effect. At the opposite, for patients with two minor alleles of the OCT1 polymorphism, the minor allele of MATE1 was associated with a positive treatment effect. So far, two studies found a similar interaction between the OCT1 polymorphism rs622342 and the MATE1 polymorphism rs2289669.^{14,35} However, a recent meta-analysis by the Metformin Genetics Consortium (MetGen) could not confirm the interaction between both SNPs.⁴⁷ In addition, our findings that both SNPs do not affect metformin plasma concentrations make it difficult to explain a possible interaction between the two transporter genes.

Metformin and the gut

Our results confirmed that metformin plasma concentration is associated with treatment effect.^{29,48} However, the daily dose of metformin was a better predictor of the effect of metformin. The essential question is at which site metformin exerts its main effect. Many data support an important action of metformin in the liver, and less pronounced in muscle and fat tissue, by different mechanism.²⁹ However, novel evidence shows that a significant part of the effect of metformin is caused by action in the intestines.⁴⁹ Therefore, it might be speculated upon that for its main effect, metformin may not need transport from the intestinal cells to the portal circulation, into the liver or into other organs by transport through the systemic circulation. The intestinal absorption of metformin is not only mediated by OCT1, but also by other transporters: the plasma membrane monoamine transporter, the serotonin reuptake transporter (SERT) and possibly the choline high-affinity transporter (CHT).⁹ So far, two studies found no association between polymorphisms of PMAT and the effect of metformin or plasma concentrations.^{16,50}

Strengths and limitations of study

Strengths of our study include the randomized, placebo-controlled, double-blind design and its relatively long period of follow-up (4.3 years), with both short term and long term outcomes available. Patients in the HOME trial used no other glucose-lowering medication than metformin and insulin. Therefore, it was possible not only to analyze the effects

of metformin on glycaemic control, but also on another important effect: the reduction in insulin requirements. In addition, the study design allowed using a full factorial model to analyze the main effects of both metformin and polymorphisms independently and their interaction with the placebo group as a control group. Since the HOME trial is not a genome-wide association study (GWAS), but a placebo controlled long term trial with a large number of measurements over time, the study design offers a unique opportunity to study the relations between well-established SNPs from exploratory studies or GWAS and relevant clinical and pharmacokinetic endpoints in the setting of a long term RCT, like HbA1C, DDI and metformin plasma concentrations. 390 patients using different daily doses of metformin (0 mg (placebo), 850 mg, 1.700 mg and 2.550 mg) were included, and followed during a period of 4.3 years with frequent blood samplings.

Our study has also some limitations. First, the power analysis of the HOME trial was primarily based on the aggregation of mortality and macrovascular and microvascular endpoints. For a genetic study, our sample size was relatively small. Therefore, our analysis should be seen as exploratory, and not confirmative. Second, we studied middle-aged, Caucasian, insulin-treated individuals with a long duration of diabetes at baseline. It is not clear whether our findings can be generalized to non-Caucasians, to individuals not treated with insulin or with pre-diabetes or recently diagnosed diabetes. Third, our selection of SNPs may only explain part of the variation. However, analyzing all the variations would ultimately lead to sequencing the whole gene without knowing the relevance of the new SNPs.

In conclusion, we found interesting associations between the ATM SNP and treatment: the minor allele of rs11212617 was not only associated with better treatment outcome, but also with a lower metformin plasma concentration. A lower metformin plasma concentration might reflect a better cellular uptake and action of metformin. The polymorphisms involved in the transportation or action of metformin need further research to draw definite conclusions about the clinical importance. The meta-analysis by the Metformin Genetics Consortium (MetGen) did not find any significant associations.⁴⁷ However, the association with metformin plasma concentration wasn't studied in this meta-analysis. Therefore, further research is needed to determine the clinical importance of these findings, in particular the effects of rs11212617 on metformin plasma concentration.

ARTICLE INFORMATION

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Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Trial Registration

Clinicaltrials.gov NCT00375388.

REFERENCES

- American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment. *Diabetes Care*. 2017;40(Suppl 1):S64-74.
- Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-Year Follow-Up of Intensive Glucose Control in Type 2 Diabetes. *N Engl J Med*. 2008;359(15):1577-89.
- Kooy A, de Jager J, Lehert P, et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch Intern Med*. 2009;169(6):616-25.
- Rena G, Pearson ER, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? *Diabetologia*. 2013;56(9):1898-906.
- Gong L, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenet Genomics*. 2012;22(11):820-7.
- Graham GG, Punt J, Arora M, et al. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet*. 2011;50(2):81-98.
- Becker ML, Pearson ER, Tkac I. Pharmacogenetics of oral antidiabetic drugs. *Int J Endocrinol*. 2013;2013:686315.
- Muller J, Lips KS, Metzner L, Neubert RH, Koepsell H, Brandsch M. Drug specificity and intestinal membrane localization of human organic cation transporters (OCT). *Biochem Pharmacol*. 2005;70(12):1851-60.
- Han TK, Proctor WR, Costales CL, Cai H, Everett RS, Thakker DR. Four cation-selective transporters contribute to apical uptake and accumulation of metformin in Caco-2 cell monolayers. *J Pharmacol Exp Ther*. 2015;352(3):519-28.
- Zhou K, Donnelly L, Yang J, et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. *Lancet Diabetes Endocrinol*. 2014;2(6):481-7.
- Shu Y, Sheardown SA, Brown C, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest*. 2007;117(5):1422-31.
- Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH. Genetic variation in the multidrug and toxin extrusion 1 transporter protein influences the glucose-lowering effect of metformin in patients with diabetes: a preliminary study. *Diabetes*. 2009;58(3):745-9.
- Shu Y, Brown C, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther*. 2008;83(2):273-80.
- Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH. Interaction between polymorphisms in the OCT1 and MATE1 transporter and metformin response. *Pharmacogenet Genomics*. 2010;20(1):38-44.
- Jablonski KA, McAteer JB, de Bakker PI, et al. Common variants in 40 genes assessed for diabetes incidence and response to metformin and lifestyle intervention in the diabetes prevention program. *Diabetes*. 2010;59(10):2672-81.
- Christensen MM, Brasch-Andersen C, Green H, et al. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. *Pharmacogenet Genomics*. 2011;21(12):837-50.
- Tzvetkov MV, Vormfelde SV, Balen D, et al. The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin. *Clin*

- Pharmacol Ther. 2009;86(3):299-306.
18. Nies AT, Koepsell H, Winter S, et al. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology*. 2009;50(4):1227-40.
 19. GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group, Wellcome Trust Case Control Consortium 2, Zhou K, et al. Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. *Nat Genet*. 2011;43(2):117-20.
 20. van Leeuwen N, Nijpels G, Becker ML, et al. A gene variant near ATM is significantly associated with metformin treatment response in type 2 diabetes: a replication and meta-analysis of five cohorts. *Diabetologia*. 2012;55(7):1971-7.
 21. Tkac I. Replication of the association of gene variant near ATM and response to metformin. *Pharmacogenomics*. 2012;13(12):1331-2.
 22. Zhou K, Donnelly LA, Kimber CH, et al. Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes*. 2009;58(6):1434-9.
 23. Tkac I, Klimcakova L, Javorsky M, et al. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in type 2 diabetes. *Diabetes Obes Metab*. 2013;15(2):189-91.
 24. Florez JC, Jablonski KA, McAteer JB, et al. Effects of genetic variants previously associated with fasting glucose and insulin in the Diabetes Prevention Program. *PLoS One*. 2012;7(9):e44424.
 25. Maruthur NM, Gribble MO, Bennett WL, et al. The pharmacogenetics of type 2 diabetes: a systematic review. *Diabetes Care*. 2014;37(3):876-86.
 26. Christensen MM, Hojlund K, Hother-Nielsen O, et al. Steady-state pharmacokinetics of metformin is independent of the OCT1 genotype in healthy volunteers. *Eur J Clin Pharmacol*. 2015;71(6):691-7.
 27. He R, Zhang D, Lu W, et al. SLC47A1 gene rs2289669 G>A variants enhance the glucose-lowering effect of metformin via delaying its excretion in Chinese type 2 diabetes patients. *Diabetes Res Clin Pract*. 2015;109(1):57-63.
 28. Wulfele MG, Kooy A, Lehert P, et al. Combination of insulin and metformin in the treatment of type 2 diabetes. *Diabetes Care*. 2002;25(12):2133-40.
 29. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. *Cell Metab*. 2014;20(6):953-66.
 30. Wulfele MG, Kooy A, Lehert P, Bets D, Donker AJ, Stehouwer CD. Does metformin decrease blood pressure in patients with Type 2 diabetes intensively treated with insulin? *Diabet Med*. 2005;22(7):907-13.
 31. Porta V, Schramm SG, Kano EK, et al. HPLC-UV determination of metformin in human plasma for application in pharmacokinetics and bioequivalence studies. *J Pharm Biomed Anal*. 2008;46(1):143-7.
 32. Umamaheswaran G, Praveen RG, Damodaran SE, Das AK, Adithan C. Influence of SLC22A1 rs622342 genetic polymorphism on metformin response in South Indian type 2 diabetes mellitus patients. *Clin Exp Med*. 2014;.
 33. Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH. Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. *Pharmacogenomics J*. 2009;9(4):242-7.
 34. Davis RL, Giacomini KM, Yee SW, Jenkins G, McCarthy CA, Wilke RA. Response to Metformin and Genetic Variants of Organic Cation and Multidrug and Toxin Extrusion Transporters (abstract HMORN 2010). *Clin Med Res*. 2010;8(3-4):191.
 35. Joerger M, van Schaik RH, Becker ML, et al. Multidrug and toxin extrusion 1 and human organic cation transporter 1 polymorphisms in patients with castration-resistant prostate cancer receiving metformin (SAKK 08/09). *Prostate Cancer Prostatic Dis*. 2015;.
 36. O'Brien PC. The appropriateness of analysis of variance and multiple-comparison procedures. *Biometrics*. 1983;39(3):787-94.
 37. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150(9):604-12.
 38. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *BMJ*. 1995;310(6973):170.
 39. Little J, Higgins JP, Ioannidis JP, et al. Strengthening the REporting of Genetic Association studies (STREGA)--an extension of the STROBE statement. *Eur J Clin Invest*. 2009;39(4):247-66.
 40. Zhou Y, Guo Y, Ye W, et al. RS11212617 is associated with metformin treatment response in type 2 diabetes in Shanghai local Chinese population. *Int J Clin Pract*. 2014;68(12):1462-6.
 41. Schiekofer S, Bobak I, Kleber ME, et al. Association between a gene variant near ataxia telangiectasia mutated and coronary artery disease in men. *Diab Vasc Dis Res*. 2014;11(1):60-3.
 42. Bar RS, Levis WR, Rechler MM, et al. Extreme insulin resistance in ataxia telangiectasia: defect in affinity of insulin receptors. *N Engl J Med*. 1978;298(21):1164-71.
 43. Yee SW, Chen L, Giacomini KM. The role of ATM in response to metformin treatment and activation of AMPK. *Nat Genet*. 2012;44(4):359-60.
 44. Woods A, Leiper JM, Carling D. The role of ATM in response to metformin treatment and activation of AMPK. *Nat Genet*. 2012;44(4):360-1.
 45. Zhou K, Bellenguez C, Sutherland C, et al. The role of ATM in response to metformin treatment and activation of AMPK. *Nat Genet*. 2012;44(4):361-2.
 46. Connelly PJ, Smith N, Chadwick R, Exley AR, Shneerson JM, Pearson ER. Recessive mutations

- in the cancer gene Ataxia Telangiectasia Mutated (ATM), at a locus previously associated with metformin response, cause dysglycaemia and insulin resistance. *Diabet Med.* 2016;33(3):371-5.
47. Dujic T, Zhou K, Yee SW, et al. Variants in pharmacokinetic transporters and glycemic response to metformin: A metagen meta-analysis. *Clin Pharmacol Ther.* 2016;.
 48. He L, Wondisford FE. Metformin action: concentrations matter. *Cell Metab.* 2015;21(2):159-62.
 49. Buse JB, DeFronzo RA, Rosenstock J, et al. The Primary Glucose-Lowering Effect of Metformin Resides in the Gut, Not the Circulation: Results From Short-term Pharmacokinetic and 12-Week Dose-Ranging Studies. *Diabetes Care.* 2016;39(2):198-205.
 50. Duong JK, Kumar SS, Kirkpatrick CM, et al. Population pharmacokinetics of metformin in healthy subjects and patients with type 2 diabetes mellitus: simulation of doses according to renal function. *Clin Pharmacokinet.* 2013;52(5):373-84.

APPENDIX

1. Statistical Analysis - full factorial model

For our main analysis, we used a linear model in testing a full factorial design including metformin daily dose, the polymorphisms' separate main effects and their first order interaction. A full factorial design allows testing three effects simultaneously:

1. the overall effect of the daily dose of metformin on the Z score (independently of any SNP, with the daily dose varying from zero gram in the placebo group to 2.55 gram in the metformin group);
2. the overall effect of the polymorphisms (independently of the use of metformin) and
3. the interaction between metformin and polymorphisms: in addition to the possible main effects of metformin and polymorphisms, is there an effect of metformin, that varies per genotype.

rs12208357				rs622342				
	Wildtype homozygous (n=296)	Heterozygous (n=38)	Variant homozygous (n=2)	p value	Wildtype homozygous (n=125)	Heterozygous (n=154)	Variant homozygous (n=57)	p value
Demographic Characteristics								
Sex, no. (%)								
Men	138 (47)	19 (50)	2 (100)		57 (46)	77 (50)	25 (44)	
Women	158 (53)	19 (50)	0 (0)	0.13	68 (54)	77 (50)	32 (56)	0.69
Age (years)	60.9±10.7	63.2±11.1	65.6±1.2	0.76	62.0±11.1	60.8±10.3	60.8±11.1	0.68
Duration of diabetes (years)	13.6±8.3*	10.1±6.9*	2.6±2.5*	0.07	12.6±8.8	13.3±7.8	14.0±8.4	0.51
Insulin treatment (years)	6.4±7.0*	3.9±4.6*	2.6±2.5*	0.63	5.7±6.7	6.2±6.9	6.9±6.8	0.51
Metabolic variables								
Weight (kg)	86.1±15.0	82.5±13.2	79.0±12.7	0.73	84.5±14.0	87.0±15.7	84.5±14.0	0.41
Body mass index (kg/m ²)	29.7±4.8	29.3±4.7	28.8±3.9	0.95	29.3±4.4	30.0±5.1	29.5±4.5	0.55
HbA1c (%)	7.9±1.2*	7.4±1.1*	7.5±0.2*	0.75	7.8±1.1	7.9±1.2	7.8±1.2	0.73
Daily dose of insulin (IU/day)	63.8±28.1	56.6±20.5	60.2±9.6	0.91	61.1±24.8	65.3±29.0	60.4±27.8	0.42
eGFR (ml/min)	70.6±14.4	70.7±14.4	85.1±1.9	0.15	69.8±15.3	71.3±14.1	71.3±13.2	0.74

rs2289669													rs11212617															
Wildtype homozygous (n=125)													Heterozygous (n=153)		Variant homozygous (n=58)		p value		Wildtype homozygous (n=97)		Heterozygous (n=168)		Variant homozygous (n=71)		p value			
Demographic Characteristics																												
Sex, no. (%)																												
Men													62		68		29				51		78		30			
Women													63		85		29		0.70		46		90		41		0.34	
Age (years)													61.5±10.2		61.4±10.9		60.1±11.4		0.62		60.2±11.1		62.4±10.8		59.9±9.8		0.20	
Duration of diabetes (years)													13.7±8.6		12.8±8.2		12.9±7.6		0.74		11.6±8.5		14.2±8.6		12.7±6.9		0.07	
Insulin treatment (years)													6.0±6.3		6.4±7.5		5.6±5.8		0.72		5.6±7.1		6.9±7.2		5±4.8		0.11	
Metabolic variables																												
Weight (kg)													84.5±14.1		86.8±16.0		85.1±12.9		0.56		86.4±14.4		84.8±14.6		86.6±16.0		0.68	
Body mass index (kg/m²)													29.0±4.6		30.2±5.1		29.8±4.0		0.20		29.6±4.8		29.7±4.7		29.7±4.9		0.98	
HbA1c (%)													7.9±1.2		7.8±1.2		7.8±1.2		0.53		7.9±1.3		7.8±1.1		7.8±1.1		0.86	
Daily dose of insulin (IU/day)													62.0±28.0		62.5±26.7		66.1±27.6		0.54		61.6±28.0		63.3±26.6		63.7±28.4		0.85	
eGFR (ml/min)													69.9±13.8		70.2±14.5		74.0±15.0		0.09		73.4±14.8		69.5±14.2		70.1±13.9		0.12	

Supplemental Table 1. Baseline data per genotype
Data are shown as mean ± SD or as indicated. There were no significant differences in baseline characteristics between the genotypes (p<0.05; t-test)

Gene	SNP	Short term (n=321)*		Long term (n=331)†	
		Beta (95%CI)	p	Beta (95%CI)	p
OCT1	rs12208357	-0.19 (-0.79 – 0.42)	0.60	0.11 (-0.80 – 1.01)	1.00
	rs622342	-0.05 (-0.29 – 0.20)	0.96	0.21 (-0.15 – 0.56)	0.19
MATE1	rs2289669	-0.08 (-0.32 – 0.17)	0.66	0.19 (-0.17 – 0.56)	0.25
ATM-Locus	rs11212617	-0.27 (-0.50 – -0.04)	0.02	-0.45 (-0.79 – -0.11)	0.01

Supplemental Table 2. Metformin, Polymorphisms and the HbA1c+DDI Z score – Unadjusted Analysis
Unadjusted estimates of the interaction between polymorphisms and treatment (daily dose of metformin, gram per day) on the aggregated HbA1c + DDI Z score (after 16 weeks and at LOCF). Full models are shown in Supplemental Table 3. *After 16 weeks; †At the end of the study (LOCF)

	Short Term (n=325)		Long Term (n=335)	
	Beta (95%CI)	p value	Beta (95%CI)	p value
Treatment & rs12208357*	-0.19 (-0.79 – 0.42)	0.60	0.11 (-0.80 – 1.01)	1.00
Treatment & rs622342*	-0.05 (-0.29 – 0.20)	0.96	0.21 (-0.15 – 0.56)	0.19
Treatment & rs2289669*	-0.08 (-0.32 – 0.17)	0.66	0.19 (-0.17 – 0.56)	0.25
Treatment & rs11212617*	-0.27 (-0.50 – -0.04)	0.02	-0.45 (-0.79 – -0.11)	0.01
Treatment & rs622342 & rs2889669*	-0.20 (-0.53 – 0.13)	0.24	-0.66 (-1.15 – -0.16)	0.01
Treatment †	-0.04 (-0.48 – 0.39)	<0.001	-0.58 (-1.20 – 0.05)	<0.001
rs12208357 [§]	-0.48 (-1.29 – 0.32)	0.02	-0.72 (-1.80 – 0.35)	0.15
rs622342 [§]	0.01 (-0.33 – 0.35)	0.83	-0.41 (-0.87 – 0.06)	0.22
rs2289669 [§]	0.05 (-0.28 – 0.37)	0.93	-0.34 (-0.78 – 0.10)	0.28
rs11212617 [§]	0.35 (0.00 – 0.69)	0.50	0.45 (-0.02 – 0.91)	0.78
rs622342 & rs2889669 [§]	0.39 (0.08 – 0.85)	0.24	0.55 (-0.07 – 1.18)	0.94
Intercept	-0.32 (-0.92 – 0.28)	0.30	1.58 (0.77 – 2.39)	<0.001

Supplemental Table 3. Metformin, Polymorphisms and the HbA1c+DDI Z score – Unadjusted Analysis
Unadjusted analysis: full factorial models of the effects of metformin and polymorphisms on the DDI+HbA1c Z score (after 16 weeks and at LOCF):

* The first order interaction of the daily dose of metformin and polymorphisms

† The effect of metformin daily dose (gram per day)

[§] The effects of the polymorphisms, independently of treatment

	Short term (n=321)		Long term (n=331)	
	Beta (95%CI)	p	Beta (95%CI)	p
Treatment & rs12208357*	-0.01 (-0.26 – 0.25)	1.00	0.40 (-0.20 – 1.01)	0.31
Treatment & rs622342*	-0.01 (-0.11 – 0.10)	0.99	0.24 (0.00 – 0.49)	0.09
Treatment & rs2289669*	0.03 (-0.07 – 0.14)	0.44	0.35 (0.09 – 0.60)	0.01
Treatment & rs11212617*	-0.11 (-0.20 – -0.01)	0.04	-0.19 (-0.42 – 0.05)	0.12
Treatment & rs622342 & rs2889669*	-0.20 (-0.35 – -0.06)	0.007	-0.41 (-0.77 – -0.06)	0.02
Treatment†	-0.33 (-0.52 – -0.15)	<.001	-0.92 (-1.35 – -0.50)	<.001
rs12208357 [§]	-0.08 (-0.42 – 0.27)	0.50	-0.40 (-1.13 – 0.32)	0.90
rs622342 [§]	0.02 (-0.13 – 0.17)	0.88	-0.42 (-0.74 – -0.11)	0.06
rs2289669 [§]	-0.07 (-0.21 – 0.07)	0.46	-0.43 (-0.73 – -0.13)	0.11
rs11212617 [§]	0.19 (0.04 – 0.33)	0.14	0.26 (-0.06 – 0.57)	0.39
rs622342 & rs2889669 [§]	0.27 (0.08 – 0.47)	0.20	0.38 (-0.04 – 0.80)	0.87
Z Score (baseline)	0.93 (0.88 – 0.98)	<.001	1.04 (0.93 – 1.15)	<.001
Age	-0.01 (-0.02 – -0.01)	<.001	-0.03 (-0.05 – -0.01)	<.001
Sex	-0.16 (-0.32 – 0.00)	0.03	-0.31 (-0.65 – 0.03)	0.05
Duration of diabetes	0.00 (-0.01 – 0.01)	0.96	-0.02 (-0.05 – 0.00)	0.01
Intercept	0.77 (0.24 – 1.30)	<.001	3.99 (2.83 – 5.14)	<.001

Supplemental Table 4. Metformin, Polymorphisms and the HbA1c+DDI Z score

Main analysis: full factorial model of the effects of metformin and polymorphisms on the DDI+HbA1c Z score, including the covariates baseline Z score, age, sex and duration of diabetes (after 16 weeks and at LOCF). The MATE1 SNP had a significant interaction with treatment: for all patients. The rs11212617 polymorphism had a significant effect influence on treatment effect at short term and a near significant treatment interaction at LOCF. As expected, metformin had a beneficial effect on the Z score, independent of the SNPs. On the other hand, no independent associations were found between the SNPs and the change in Z score,

* The first order interaction of the daily dose of metformin and polymorphisms

† The effect of metformin daily dose (gram per day)

[§] The effects of the polymorphisms, independently of treatment

Gene	SNP	Short term (n=335)		Long term (n=335)	
		Beta (95%CI)	p	Beta (95%CI)	p
Treatment & rs12208357*		-0.08 (-0.35 – 0.19)	0.67	0.12 (-0.27 – 0.51)	0.57
Treatment & rs622342*		-0.04 (-0.15 – 0.07)	0.63	0.02 (-0.14 – 0.17)	0.77
Treatment & rs2289669*		-0.02 (-0.13 – 0.09)	0.80	0.03 (-0.13 – 0.19)	0.71
Treatment & rs11212617*		-0.09 (-0.19 – 0.02)	0.10	-0.07 (-0.22 – 0.08)	0.39
Treatment & rs622342 & rs2889669*		-0.21 (-0.36 – -0.07)	0.004	-0.25 (-0.46 – -0.03)	0.03
Treatment†		-0.18 (-0.37 – 0.01)	<.001	-0.13 (-0.40 – 0.15)	0.02
rs12208357§		-0.03 (-0.38 – 0.33)	0.37	-0.23 (-0.70 – 0.24)	0.48
rs622342§		0.07 (-0.08 – 0.23)	0.54	-0.03 (-0.23 – 0.18)	0.81
rs2289669§		-0.02 (-0.17 – 0.13)	0.53	-0.07 (-0.26 – 0.12)	0.49
rs11212617§		0.14 (-0.01 – 0.30)	0.33	0.14 (-0.06 – 0.35)	0.31
rs622342 & rs2889669§		0.23 (0.03 – 0.44)	0.61	0.14 (-0.13 – 0.41)	0.63
HbA1c (baseline)		0.63 (0.56 – 0.71)	<.001	0.41 (0.31 – 0.50)	<.001
Age		-0.01 (-0.02 – 0.00)	<.001	-0.02 (-0.03 – -0.01)	<.001
Sex		-0.20 (-0.37 – -0.04)	0.01	-0.19 (-0.41 – 0.03)	0.08
Duration of diabetes		0.01 (0.00 – 0.02)	0.00	0.01 (-0.01 – 0.02)	0.23
Intercept		2.95 (2.17 – 3.72)	<.001	5.98 (4.95 – 7.02)	<.001

Supplemental Table 5a. SNPs and HbA1c

Gene	SNP	Short term (n=321)		Long term (n=332)	
		Beta (95%CI)	p	Beta (95%CI)	p
Treatment & rs12208357*		2.09 (-1.94 – 6.12)	0.30	7.19 (-6.77 – 21.14)	0.48
Treatment & rs622342*		0.05 (-1.61 – 1.72)	0.93	5.43 (-0.23 – 11.09)	0.10
Treatment & rs2289669*		0.35 (-1.31 – 2.02)	0.64	7.58 (1.79 – 13.38)	0.01
Treatment & rs11212617*		-0.56 (-2.12 – 1.01)	0.49	-3.42 (-8.81 – 1.96)	0.21
Treatment & rs622342 & rs2889669*		1.05 (-1.26 – 3.35)	0.38	-3.11 (-11.43 – 5.20)	0.46
Treatment†		-3.99 (-6.89 – -1.09)	<.001	-20.00 (-29.77 – -10.23)	<.001
rs12208357§		-2.58 (-7.99 – 2.83)	0.86	-6.69 (-23.49 – 10.10)	0.96
rs622342§		-0.79 (-3.07 – 1.50)	0.40	-10.53 (-17.78 – -3.27)	0.02
rs2289669§		-1.25 (-3.44 – 0.95)	0.26	-11.43 (-18.32 – -4.55)	0.07
rs11212617§		0.91 (-1.41 – 3.24)	0.74	2.64 (-4.63 – 9.92)	0.63
rs622342 & rs2889669§		-0.11 (-3.20 – 2.99)	0.60	2.57 (-7.21 – 12.35)	0.78
DDI (baseline)		1.17 (1.13 – 1.22)	<.001	1.41 (1.27 – 1.56)	<.001
Age		-0.11 (-0.24 – 0.01)	<.001	-0.35 (-0.74 – 0.04)	0.001
Sex		0.35 (-2.13 – 2.84)	0.98	-2.58 (-10.47 – 5.32)	0.36
Duration of diabetes		-0.27 (-0.43 – -0.11)	0.00	-0.49 (-0.99 – 0.02)	0.02
Intercept		7.28 (-1.41 – 15.98)	<.001	52.00 (24.27 – 79.74)	<.001

Supplemental Table 5b. SNPs and DDI

Full factorial models of the effects of metformin and polymorphisms on a) HbA1c and b) DDI, including the covariates baseline values of HbA1c and DDI, age, sex and duration of diabetes (after 16 weeks and at LOCF). The estimates for the interaction between treatment, the polymorphisms and both HbA1c and DDI were similar to the estimates of the primary endpoint. However, only the MATE1 SNP showed a significant treatment interaction at LOCF for DDI: the minor allele was associated with an increase of DDI. As expected, the beneficial effect of metformin was confirmed in all analyses, independent of the SNPs. On the other hand, no independent associations were found between the SNPs and the change in HbA1c or DDI, except for one (between rs622342 and DDI at LOCF).

* The first order interaction of the daily dose of metformin and polymorphisms

† The effect of metformin daily dose (gram per day)

§ The effects of the polymorphisms, independently of treatment

OCT1 & MATE1	Short term			Long term		
		Beta (95%CI)	p		Beta (95%CI)	p
A. HbA1c						
rs622342 & rs2289669	n=335	-0.21 (-0.36 – -0.07)	0.004	n=335	-0.25 (-0.46 – -0.03)	0.03
rs622342 (AA) & rs2289669	n=125	0.13 (-0.05 – 0.32)	N/A	n=125	0.17 (-0.11 – 0.45)	N/A
rs622342 (AC) & rs2289669	n=153	-0.04 (-0.21 – 0.14)	N/A	n=153	0.04 (-0.20 – 0.27)	N/A
rs622342 (CC) & rs2289669	n=57	-0.34 (-0.54 – -0.15)	N/A	n=57	-0.48 (-0.82 – -0.15)	N/A
B. DDI						
rs622342 & rs2289669	n=321	1.05 (-1.26 – 3.35)	0.38	n=332	-3.11 (-11.43 – 5.20)	0.46
rs622342 (AA) & rs2289669	n=118	-0.09 (-2.96 – 2.78)	N/A	n=123	11.72 (1.13 – 22.30)	N/A
rs622342 (AC) & rs2289669	n=149	1.07 (-1.46 – 3.61)	N/A	n=154	5.86 (-2.51 – 14.22)	N/A
rs622342 (CC) & rs2289669	n=54	0.89 (-2.43 – 4.22)	N/A	n=55	3.65 (-8.80 – 16.10)	N/A

Supplemental Table 6. Metformin, interaction rs622342 & rs2289669 Estimates of the interaction between the MATE1 SNP rs2289669 (estimate per minor allele) and treatment (daily dose of metformin, gram per day) per genotype of the OCT1 SNP rs622342 on a) HbA1c and b) DDI. Covariates included in the model were baseline values of HbA1c and DDI, age, sex and duration of diabetes.

Chapter 6.

Variation in the glucose transporter gene SLC2A2 is associated with glycemic response to metformin

VARIATION IN THE GLUCOSE TRANSPORTER GENE SLC2A2 IS ASSOCIATED WITH GLYCEMIC RESPONSE TO METFORMIN

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Metformin is the first-line antidiabetic drug with over 100 million users worldwide, yet its mechanism of action remains unclear¹. Here the Metformin Genetics (MetGen) Consortium reports a three-stage genome-wide association study (GWAS), consisting of 13,123 participants of different ancestries. The C allele of rs8192675 in the intron of *SLC2A2*, which encodes the facilitated glucose transporter GLUT2, was associated with a 0.17% ($P = 6.6 \times 10^{-14}$) greater metformin-induced reduction in hemoglobin A1c (HbA1c) in 10,577 participants of European ancestry. rs8192675 was the top cis expression quantitative trait locus (*cis*-eQTL) for *SLC2A2* in 1,226 human liver samples, suggesting a key role for hepatic GLUT2 in regulation of metformin action. Among obese individuals, C-allele homozygotes at rs8192675 had a 0.33% (3.6 mmol/mol) greater absolute HbA1c reduction than T-allele homozygotes. This was about half the effect seen with the addition of a DPP-4 inhibitor, and equated to a dose difference of 550 mg of metformin, suggesting rs8192675 as a potential biomarker for stratified medicine.

INTRODUCTION

Metformin was commercialized before the modern era of target-based drug discovery. It typically reduces HbA1c by 1–1.5% (11–16 mmol/mol) and has an excellent safety record,

Figure 1. Pharmacogenetic impact of rs8192675 on metformin response in participants of European ancestry. The forest plot shows meta-analyses of association test results for metformin induced change in HbA1c in a total number of 10,557 participants from 10 MetGen cohorts. Results from linear regression models with (left) and without (right) adjustment for baseline HbA1c are presented. The x axis represents the impact on metformin-induced HbA1c reduction of each copy of the C allele. HbA1c was measured in percentage.

but considerable variation exists in how well patients respond to metformin^{2,3}. We have recently established that genetic factors influence glycemic response to metformin, with many common variants across the genome together explaining a substantial proportion of the variation, ranging from 21% to 34%, depending on how glycemic response had been measured⁴. Hypothesis-driven studies of pharmacokinetic variants have shown no consistent results^{5–10}. The only GWAS published to date showed an association with rs11212617 near the *ATM* locus, which has been replicated^{11,12}.

Here we extended the previous GWAS by an additional 345 samples to a screening set of 1,373 participants. As in our previous report¹², rs11212617 remained the top signal with no other genome-wide significant hits (Supplementary Fig. 1). We undertook a systematic three-stage replication, with the workflow shown in Supplementary Figure 2. Only rs8192675 in the intron of *SLC2A2* was replicated through the first two stages with a combined $P = 1 \times 10^{-7}$ derived from a linear regression meta-analysis of 3,456 participants (Supplementary Data and Supplementary Table 1).

The MetGen Consortium performed the final replication of rs8192675 as a meta-analysis. Measures of glycemic response to metformin were aligned across the cohorts as the absolute HbA1c reduction (expressed as reduction in percentage of HbA1c). Within each

Figure 2. HbA1c reduction by BMI group and rs8192675 genotype. HbA1c reduction by BMI group and rs8192675 genotype. Participants were stratified into obese (BMI ≥ 30 kg/m²) and nonobese (BMI < 30 kg/m²) groups. The number of obese and nonobese individuals in each genotype group is noted along the x axis. Error bars, s.e.m.

cohort, we tested associations with rs8192675 using two multiple linear models with or without the adjustment of baseline HbA1c, in addition to other available clinical covariates (Supplementary Table 2). In the meta-analysis of 10,557 participants of European ancestry (Fig. 1), each copy of the C allele was associated with a greater HbA1c reduction of 0.07% ($P = 2 \times 10^{-8}$, and Cochran’s heterogeneity statistic’s P value ($P_{\text{het}} = 0.35$) when adjusting for baseline HbA1c; without adjustment the allelic effect of the C allele was 0.17% ($P = 6.6 \times 10^{-14}$, $P_{\text{het}} = 0.52$). There was no effect of rs8192675 on the efficacy of metformin in delaying progression to diabetes, or on metformin efficacy in a small insulin-treated cohort (Table 1).

We tested the pharmacogenetic effect of rs8192675 in 2,566 participants of non-European ancestries (Supplementary Table 4). The meta-analysis showed that the C allele was associated with a 0.08% greater HbA1c reduction ($P = 0.006$, $P_{\text{het}} = 0.63$) when adjusting for baseline HbA1c, and without the baseline adjustment the allelic effect of the C allele was 0.15% ($P = 0.005$, $P_{\text{het}} = 0.95$). In the meta-analysis of 13,123 participants of any ancestry (data not shown), we observed no genetic heterogeneity ($P_{\text{het}} > 0.29$) between different ethnic groups despite the frequency of the C allele ranging from 24% in Latino populations to around 70% in African-American populations.

Study	Allele	N	Effect Size	95% CI	P
HOME	C	163	0.08	[-0.02,0.18]	0.286
DPP	C	511	0.97	[0.72,1.31]	0.832

Table 1. Association between rs8192675 and alternative measures of metformin efficacy in MetGen. In HOME study of patients using metformin as add on treatment to insulin, the outcome of the change in daily dose of insulin (unit) was modelled in linear regression. In the DPP study of metformin prevention in pre-diabetes patients, the outcome of diabetes incidence was modelled with proportional hazard Cox regression.

We examined whether rs8192675 had an impact on baseline HbA1c, because the effect sizes of its association with glycemic response to metformin differed depending on whether there was adjustment for the baseline HbA1c. In the 10,557 participants of European ancestry, the C allele was associated with a 0.13% ($P = 2.6 \times 10^{-8}$) higher baseline HbA1c but a 0.04% ($P = 0.007$) lower on-treatment HbA1c, which together contributed to the observed 0.17% ($P = 6.6 \times 10^{-14}$) pharmacogenetic impact on HbA1c reduction in the model without baseline adjustment (Supplementary Fig. 3).

Given the association of rs8192675 with HbA1c before treatment with metformin, we assessed whether this variant was marking a general ability to respond to any antihyperglycemic treatment. Therefore we studied the pharmacogenetic impact of rs8192675 in 2,654 participants treated with sulfonylureas (Supplementary Table 5), another commonly used class of antidiabetic drug^{13,14}. As in metformin users, the C allele was also associated with a higher baseline HbA1c in these users of sulfonylureas (beta = 0.15%, $P = 3.1 \times 10^{-4}$). However, in contrast to the case for users of metformin, the C allele remained associated with a higher on-treatment HbA1c (beta = 0.09%, $P = 0.006$) in the users of sulfonylureas, which resulted in no net pharmacogenetic impact (beta = 0.04%, $P = 0.44$) on sulfonylurea-induced HbA1c reduction. These data suggest that rs8192675 is marking a genetic defect in glucose metabolism in type 2 diabetes that is ameliorated by metformin treatment but not by sulfonylurea treatment. The fact that rs8192675 is not associated with sulfonylurea response strongly supports a specific role for this variant on glycemic response to metformin, rather than simply reflecting the higher pretreatment (baseline) HbA1c seen in carriers of this C allele. In addition, the association with metformin-induced HbA1c reduction remained significant ($P = 2 \times 10^{-8}$; Fig. 1) after adjustment for baseline HbA1c, corroborating a specific effect on response beyond its effect on baseline glycemia.

Metformin is particularly recommended for the treatment of diabetes in obese individuals owing to its beneficial effect on body weight^{15–17}. Therefore, we explored whether the

Figure 3. Regional plots of the SLC2A2 locus. SNPs are plotted by position on chromosome 3 against association with meta-analysis of HbA1c reduction without baseline adjustment ($-\log_{10} P$) in 7,223 participants (left) and meta-analysis of SLC2A2 expression ($-\log_{10} P$) in 1,226 liver samples (right). In both plots rs8192675 (purple circle) and its proxies are the top signals. The nonsynonymous SNP rs5400 (arrow) was also nominally associated with HbA1c reduction. Estimated recombination rates (cM/Mb) are plotted in blue to reflect the local LD structure. The SNPs surrounding the most significant SNP, rs8192675, are color coded to reflect their LD with this SNP. This LD was taken from pairwise r^2 values from the HapMap CEU data. Genes, the position of exons and the direction of transcription from the UCSC Genome Browser are noted.

pharmacogenetic impact of rs8192675 varied by body mass index (BMI) in the MetGen cohorts ($n = 7,581$ participants). BMI was associated with HbA1c reduction ($\beta = -0.01\%$; $P = 1.7 \times 10^{-4}$) but not rs8192675 genotype ($P = 0.52$). Adjusting for BMI did not attenuate the observed pharmacogenetic effect of rs8192675 (Supplementary Table 6). When we stratified participants into nonobese ($\text{BMI} < 30 \text{ kg/m}^2$) and obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) groups, there was a significant ($P = 0.02$) gene by BMI group interaction (Fig. 2). The pharmacogenetic effect size of the C allele was 0.13% (s.e.m. = 0.04% , $P = 0.001$) in the nonobese participants as compared to that of 0.24% (s.e.m. = 0.04% , $P = 5.0 \times 10^{-11}$) in the obese participants.

We performed a locus-wise meta-analysis to narrow down the candidate causal gene and variant list. Variant rs8192675 and its proxies showed the strongest association with HbA1c reduction (Fig. 3). The linkage disequilibrium (LD) block covered three genes, of which SLC2A2 encodes the facilitated glucose transporter GLUT2, and EIF5A2 and RPL22L1 have little known functionality. Previous GWAS showed that the nonsynonymous rs5400 in SLC2A2 is the main variant associated with glycemic traits such as fasting glucose and HbA1c^{18,19}. Because rs8192675 and rs5400 are in partial LD ($D' = 1$; $r^2 = 0.35$), here rs5400 was also associated with metformin response ($\beta = 0.13\%$, $P = 5.2 \times 10^{-4}$). However, when conditioning on rs5400, rs8192675 remained strongly associated with metformin response

Figure 4. Genetic impact of GLUT2 variants on glucose homeostasis in different physiological and pharmacologic states. In patients with the monogenic Fanconi–Bickel syndrome (FBS), the loss-of-function variants led to lower fasting glucose but higher post-prandial glucose; the expression-reducing C allele at rs8192675 was associated with lower HbA1c in normal glycemia state but higher HbA1c in hyperglycemia state (before pharmacological treatment was indicated in patients with type 2 diabetes (T2D)); metformin but not sulfonylurea treatment reversed the genetic impact on HbA1c.

($\beta = 0.21\%$, s.e.m. = 0.04% , $P = 2.3 \times 10^{-3}$); when conditioning on rs8192675, rs5400 was not significant ($P = 0.29$). These results suggest that the pharmacogenetic impact of rs8192675 is unlikely to be via the amino acid change of GLUT2 at rs5400.

Given that liver is the most established site of metformin action, we examined whether rs8192675 is an eQTL in 1,226 liver samples of European ancestry. In Figure 3 we show rs8192675 as the top cis-eQTL for SLC2A2, with the C allele associated with decreased ($P = 4.2 \times 10^{-12}$) expression. In the 48 tissues examined by the Genotype–Tissue Expression (GTEx) Project, SLC2A2 was sufficiently expressed in seven tissues (Supplementary Table 7). rs8192675 showed a significant ($P = 5.7 \times 10^{-4}$) impact on SLC2A2 expression in the 271 samples of transformed fibroblasts, but no other significant associations²⁰. Beyond GTEx, we sought additional eQTL evidence for other tissues that have been implicated in metformin action or glucose homeostasis. We found directionally consistent and supportive evidence of rs8192675 or its proxies being SLC2A2 cis-eQTLs in 118 islets (rs8192675, $P = 0.0025$)²¹,

173 intestinal samples (rs5398, $P = 0.007$)²² and 44 kidney samples (rs1905505, $P = 0.04$) (Supplementary Table 7).

Patients with Fanconi–Bickel syndrome (Online Mendelian Inheritance in Man (OMIM), 227810), who carry rare loss-of-function variants of GLUT2, can provide useful insight into the role of GLUT2 in glucose homeostasis and into the differing impact of common GLUT2 variants in various physiological states (Fig. 4). Patients with Fanconi–Bickel syndrome exhibit low fasting glucose but high postprandial glucose^{23,24}. In parallel, the C allele of rs8192675 that is associated with reduced *SLC2A2* expression is associated with lower fasting glucose and HbA1c among individuals of normal glycemia^{18,19}. We found that, in patients with type 2 diabetes, the expression-decreasing C allele of rs8192675 was associated with higher HbA1c before treatment with either metformin or sulfonylureas. This deleterious genetic effect of rs8192675 on HbA1c was reversed with metformin treatment (C allele associated with lower on-treatment HbA1c and therefore better response to metformin), but not by sulfonylurea treatment.

In humans, GLUT2 is a facilitative glucose transporter that is highly produced in the liver, kidney, small intestine and islets, and to a lesser extent in certain brain regions and other tissues. Defects in the GLUT2-encoding gene could potentially alter glucose homeostasis at any or all of these sites⁴. Metformin's main site of action is widely believed to be the liver, where it primarily acts to suppress hepatic glucose production^{1,25–27}. In mice with *Glut2* inactivation, glucose and glucose-6-phosphate accumulated in the cytoplasm owing to reduced glucose efflux, resulting in increased expression of nuclear ChREBP, l-pyruvate kinase and lipogenic genes²⁸. Our eQTL data in liver samples (Fig. 3) and corresponding reporter assays (Supplementary Fig. 4) showed that the C allele at rs8192675 was associated with lower expression of *SLC2A2*. This suggests that the variant may lead to similar effects on hepatic gene expression in humans, which will be potentially modulated by metformin's well-described effect on hepatic glucose production and lipogenesis^{29,30}. An alternative explanation could be that reduced *SLC2A2* expression owing to rs8192675 is associated with reduced glucose-mediated glucose clearance (glucose effectiveness) owing to a decreased ability for glucose to enter the liver. This is seen in mice lacking *Glut2* in the liver, and is an effect that is improved by metformin treatment³¹, although the mechanism for this is not understood.

Metformin is also increasingly believed to exert some of its beneficial effects by acting on the intestines to increase gut glucose uptake and non-oxidative glucose disposal, as well as

increasing bile acid reabsorption, GLP-1 secretion and altering the microbiome³². In leptin-deficient (ob/ob) mice, metformin has been shown to increase translocation of Glut2 to the apical surface resulting in improved glucose homeostasis³³. In light of the interaction we observed between rs8192675 and BMI on metformin response, obese humans are reported to have altered GLUT2 localization in the fasting state compared to nonobese humans³³, suggestive of dysregulation of glucose sensing and transport in obese individuals. If reduced *SLC2A2* expression owing to rs819265 were to result in reduced apical GLUT2, metformin could potentially overcome this by restoring GLUT2 transport in enterocytes and improving glucose homeostasis.

Finally, given that metformin is transported into different tissues by several organic cation transporters, including OCTs, MATEs and THTR2³⁴, we examined whether *GLUT2* can transport metformin in *Xenopus laevis* oocytes. Our results suggested that metformin was not a substrate or an inhibitor of *GLUT2* (Supplementary Fig. 5). Detailed human physiological studies, as well as functional exploration in animal and cellular model systems, are required to fully elucidate the role of *GLUT2* in metformin response, and whether this is mediated via a hepatic, an intestinal or another mechanism.

We examined the potential clinical impact of rs8192675. An unbiased (from the nondiscovery cohorts) estimate of its allelic effect was a 0.15% absolute reduction in percentage of HbA1c. This is equivalent to the pharmacological impact of taking 250 mg extra metformin per day, which is 26% of the average daily dose. We saw more clinical potential in obese patients as the homozygote carriers of the C allele had a 0.33% (s.e.m. = 0.09%, $P = 6.6 \times 10^{-4}$) greater reduction in percentage of HbA1c than those homozygous for the T allele; this equates to 24% of the average glycemic reduction seen with metformin treatment in the MetGen cohorts and is equivalent to the impact of 550 mg extra metformin. Given that newer agents such as DPP-4 inhibitors only reduce HbA1c by 0.6–0.8% on average³⁵, this genetic effect is large and has potential to be of clinical utility. C-allele homozygotes could be treated with lower doses, and be exposed to fewer side effects; conversely T-allele carriers could be treated with doses higher than normally recommended to achieve a response. This may be of particular importance in African Americans where 49% of the population is homozygous for the C allele, in contrast to only 9% in European Americans. Stratified clinical trials, in different ethnic groups, are required to evaluate the potential for this pharmacogenetic variant to influence clinical care.

In conclusion, we established a robust association between rs8192675 and metformin-induced HbA1c reduction with a large multiethnic cohort. rs8192675 was the top *cis*-eQTL for *SLC2A2* in the liver and potentially islets, kidney and intestine. Reduced *SLC2A2* expression resulted in a defect in glucose homeostasis in type 2 diabetes before initiation of therapy, which could be ameliorated by metformin treatment. The clinically appreciable impact in obese patients suggests that rs8192675 has the potential to be a biomarker for stratified medicine.

METHODS

Data access

The three liver eQTL data sets published previously are available with Gene Expression Omnibus (GEO) accession numbers: GSE39036, GSE25935 and GSE9588.

Studies and samples

Both GWAS screening and the first-stage replication analyzed participants with type 2 diabetes of European ancestry from the GoDARTS cohort. The current GWAS screening used 1,373 participants, which included data from 345 samples released after our initial GWAS report on 1,028 participants¹². The first-stage replication included up to 1,473 samples from the remaining GoDARTS participants depending on the call rate and genotyping assay. The second-stage replication consisted of 1,223 participants of European ancestry from the UKPDS study. The final replication and meta-analysis was conducted within the MetGen Consortium which included an extra 6,488 participants of European ancestry and 2,566 participants of non-European ancestry. Detailed information on the MetGen participants is provided in Supplementary Table 2. Of note, about 50% of the MetGen cohort is from PMT, which represents ethnically diverse US populations. These cohorts were used extensively in our multiethnic analysis for replication purposes. Participants from the largest PMT cohort, PMT2, were selected from the Genetic Epidemiology Research on Adult Health and Aging (GERA) cohort, a subsample of the Kaiser Permanente Research Program on Genes, Environment, and Health (RPEGH)³⁶. Three MetGen cohorts, GoDARTS, UKPDS and DCS, also provided data on response to sulfonylureas. All human research was approved by the relevant institutional review boards, and all participants provided written informed consent.

Genotyping and quality control

Genotyping for the GWAS screening and the first-stage CardioMetaboChip replication in GoDARTS cohort has been described before by WTCCC2 and DIAGRAM^{12,37}. Standard quality-control procedures were applied to both data sets to filter SNPs with minor allele frequency (MAF) < 1% or call rate < 98% or Hardy–Weinberg equilibrium deviation ($P < 10^{-4}$). Samples with call rate < 98% or extra heterozygosity (more than 3 s.d. away from the mean) or correlated with another sample (identity by descent (IBD) > 0.125) were filtered out. In-house genotyping of the GoDARTS samples in the first-stage replication was performed with Sequenom MassArray for 66 SNPs and TaqMan-based Allelic Discrimination assays for nine SNPs. Details of the SNP selection procedure are described in Supplementary Data. All 75 SNPs had call rate > 90% and no deviation from Hardy–Weinberg equilibrium ($P > 0.005$). The second-stage genotyping of the UKPDS sample was carried out in duplicate runs using standard TaqMan assays. All the SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$), and only samples with concordant genotypes from both runs were analyzed. The third-stage replication used high-quality genotypes from either TaqMan assay or GWAS imputed data on rs8192675 (Supplementary Table 2).

Assessment of glycemic response to metformin and sulfonylureas

As with our previous GWAS¹², two correlated measures of glycemic response to metformin were used in the current GWAS screening and the first-stage replication. A quantitative measure of HbA1c reduction (baseline minus on-treatment HbA1c) and a categorical measure of whether achieving a target of treatment HbA1c ≤ 7% were used for genetic association tests. Therefore only participants with type 2 diabetes and a baseline HbA1c > 7% were included. Baseline HbA1c was measured within 6 months before metformin start, and on-treatment HbA1c was taken as the minimum achieved within 18 months after metformin start.

In the second-stage replication and the meta-analysis in the third-stage replication, we opted to maximize the sample size by synchronizing the measurement of metformin efficacy in a wider spectrum of participants with type 2 diabetes (including those with baseline HbA1c < 7%) across the MetGen. Therefore only the quantitative outcome of HbA1c reduction was used to assess the glycemic response to metformin. To maintain relative clinical homogeneity, only participants with type 2 diabetes on metformin monotherapy or using metformin as an add-on therapy to another oral agent were included.

Data from two MetGen cohorts, which used alternative measures of glycemic response, were not included in the current meta-analyses, but the results are shown in Supplementary Table 4. In the DPP cohort of prediabetes participants, Cox proportional hazards regression was used to evaluate the genetic impact on the time to diabetes incidence⁸. In the HOME cohort, a multiple linear regression was used to test the genetic association with the difference in daily dose of insulin because metformin was used in conjunction with insulin in these participants³⁸.

Assessment of glycemic response to sulfonylureas adopted a similar approach as the quantitative outcome of metformin response in the MetGen. Baseline HbA1c and on-treatment HbA1c were captured in a similar manner as those in defining metformin response. Only participants with type 2 diabetes who were on sulfonylurea monotherapy or using sulfonylurea as an add-on therapy to metformin were included. All participants had a baseline HbA1c > 7%.

STATISTICAL ANALYSIS

In the GWAS screening and first-stage replication, each SNP was tested for association with the continuous measure and categorical measure of glycemic response to metformin separately with PLINK software using linear and logistic regression respectively³⁹. Baseline HbA1c, adherence, metformin dose, creatinine clearance and treatment scheme (whether on metformin monotherapy or dual therapy of metformin add-on to sulfonylureas) and the first 10 principal components from EIGENSTRAT were used as covariates⁴⁰. Statistical evidence of the two associations at each SNP was averaged by taking the geometric mean of the two *P* values in cases in which the direction of effect was consistent (for example more HbA1c reduction and more likely to achieve the treatment target both indicate better response).

In the second- and third-stage replications, association with HbA1c reduction was tested with multiple linear regression. Within each cohort, two linear models were fitted either with or without adjustment for baseline HbA1c. Baseline HbA1c has been shown to be the strongest predictor of metformin-induced HbA1c reduction in pharmaco-epidemiological studies⁴¹. Adjusting for baseline HbA1c could reduce the confounding of measurement error in baseline HbA1c and increase the statistical power for pharmacogenetic studies⁴². However, if a variant is associated with baseline HbA1c, adjusting for baseline HbA1c would lead to a reduced estimate of its pharmacogenetic effect compared to a model that did not adjust for the baseline HbA1c. Therefore we presented both models in the current study. Other

clinical factors such as creatinine clearance (or other measurement of kidney function) and treatment scheme were included as covariates where available (Supplementary Table 2). Association results from individual cohorts were combined by a fixed-effect inverse-variance-weighted meta-analysis as applied in GWAMA⁴³. Cochran's heterogeneity statistic's *P* value was reported as *P*_{het}.

For the genetic association tests with response to sulfonylureas, multiple linear regression was used to assess the association between rs8192675 and baseline HbA1c, on-treatment HbA1c, HbA1c reduction and baseline-adjusted HbA1c reduction. Treatment scheme (whether on sulfonylurea monotherapy or using sulfonylurea as add-on treatment to metformin) was included as a covariate when modeling sulfonylurea-induced HbA1c reduction. Association test results from the three cohorts were combined with fixed-effect inverse-variance-weighted meta-analysis in GWAMA.

Locus-wise association was performed with GWAS imputed data of 7,223 participants available in the GoDARTS and PMT2-EU. Software IMPUTE2 was used to impute the post-quality-control GWAS data for the 1-Mb region flanking rs8192675 against the 1000 Genomes reference panel⁴⁴. Only SNPs with high imputation quality (info > 0.9 and MAF > 0.02) in both cohorts were tested for association with SNPTTEST⁴⁵. Summary statistics from GoDARTS and PMT2-EU were combined with fixed-effect inverse-variance-weighted meta-analysis in GWAMA.

To evaluate the translational potential of rs8192675, we derived an unbiased estimate of its allelic effect by excluding the discovery cohort in the meta-analysis. This effect size was aligned to the clinical impact observed in the PMT2-EU, which was the biggest replication cohort and used the median average daily dose in the MetGen. The average daily dose and dosing impact in PMT2-EU were 962 mg/d and an extra 0.6% HbA1c reduction per gram metformin, respectively. The evaluation of rs8192675 genotype by BMI group interaction was performed with linear regression by adjusting for treatment group, sex and study cohort.

Expression quantitative trait locus analyses

We used four liver eQTL data sets comprising a total of 1,226 liver samples from individuals of European ancestry (Supplementary Table 8). Tissue procurement, gene expression analysis, genotyping and eQTL analyses have been described previously for three of the data sets^{46–48}. The fourth data set was contributed by E. Schadt (E. Schadt, C. Molony, E. Chudin, K. Hao, X. Yang et al., personal communication). Genotypes were imputed to the

1000 Genome reference panel with IMPUTE2. Expression probe sequences were mapped to ENSEMBL genes and only the common genes across all data sets were included for subsequent analyses. Within each data set, the genome-wide eQTL analysis was run with an additive genetic model including data-set-specific covariates to examine *cis*-associations within a 100-kb flanking window. Results from the four data sets were then combined with a modified meta test statistic which was calculated using the following approach: $t_{\text{meta}} = (\sum w_i t_i) / \sqrt{(\sum w_i^2)}$, $w = \sqrt{(n - (\# \text{ covariates}) - 1)}$, where i = data sets 1–4 and n = sample size⁴⁹. P values were generated by assuming the meta test statistics were normally distributed; a Benjamini–Hochberg multiple-testing correction was applied to the P values. For the current study, we extended the *cis*-association tests to all SNPs within a 1-Mb window of SLC2A2 and report the locus-wise P values of the meta test statistic.

We investigated whether rs8192675 is a *cis*-eQTL in other tissues in the GTEx data release V6. Because of the sample size limitation, rs8192675 is not a genome-wide significant *cis*-eQTL for SLC2A2 in any of the tissues examined. However, given the strong evidence of the variant being a *cis*-eQTL in the large liver samples reported in this study, we considered a directionally consistent association with $P < 0.05$ as supportive evidence. The eQTL data for islet and intestine were acquired through contacting the authors of the original publications^{21,22}. The eQTL data for kidney were obtained by quantitative real-time PCR of 44 kidney samples genotyped with the Affymetrix Axiom array. Sample acquirement and tissue preparation was described previously⁵⁰. The transcript levels of *SLC2A2* were determined using TaqMan probe (ID Hs01096908_m1). The relative expression level of *SLC2A2* transcript was calculated by the comparative method ($\Delta\Delta C_t$) normalized to the housekeeping gene *GAPDH*, as described previously⁵¹.

Accession codes

Part of the unpublished eQTL data set 4 (Supplementary Table 8), which covers the SLC2A2 locus, has been deposited in the Figshare: <http://dx.doi.org/10.6084/m9.figshare.3438362>. Phenotype and genotype data used in the first-stage GWAS screening have been deposited at the European Genome-phenome Archive: EGAS00001001875 and EGAD00010000282, respectively.

ARTICLE INFORMATION

Any Supplementary Information including author affiliations and Source Data files are available in the online version of the paper. This chapter has been modified from its original version: Table 1 has been moved from the supplements to the main article.

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Conflict of Interest

The authors declare no competing financial interests.

Author contributions

Conception and design of the study: E.R.P. and K.M.G.; data analysis: K.Z., S.W.Y., E.L.S., N.v.L., A.A.v.d.H., J.W.B., C.E.d.K., L.Z., D.M.R., M.O., K.A.J., L.C., M.J., A.M.L., L.K.W., T.D. and A.A.M.-R.; data collection and genotyping: S.W.Y., C.S., R.T., A.J.B., C.J.G., R.L.C., L.L., L.K.W., T.D., S.S., M.K., M.M.H., H.-C.C., F.I., S.M., J.S.W., L.W., J.Ž., I.T., A.K., R.H.N.v.S., C.D.A.S., J.K., V.P., A.H., B.H.S., M.J.W., L.M.H., J.C.F., R.R.H., M.I.M. and C.N.A.P.; manuscript writing: E.R.P., K.Z., S.W.Y. and K.M.G. with contributions from all authors on the final version.

REFERENCES

1. Madiraju, A.K. et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* 510, 542–546 (2014).
2. DeFronzo, R.A. & Goodman, A.M. Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 333, 541–549 (1995).
3. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 352, 854–865 (1998).
4. Zhou, K. et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. *Lancet Diabetes Endocrinol.* 2, 481–487 (2014).
5. Pawlyk, A.C., Giacomini, K.M., McKeon, C., Shuldiner, A.R. & Florez, J.C. Metformin pharmacogenomics: current status and future directions. *Diabetes* 63, 2590–2599 (2014).

6. Tkáč, I. et al. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in type 2 diabetes. *Diabetes Obes. Metab.* 15, 189–191 (2013).
7. Stocker, S.L. et al. The effect of novel promoter variants in MATE1 and MATE2 on the pharmacokinetics and pharmacodynamics of metformin. *Clin. Pharmacol. Ther.* 93, 186–194 (2013).
8. Jablonski, K.A. et al. Common variants in 40 genes assessed for diabetes incidence and response to metformin and lifestyle intervention in the diabetes prevention program. *Diabetes* 59, 2672–2681 (2010).
9. Shu, Y. et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin. Pharmacol. Ther.* 83, 273–280 (2008).
10. Zhou, K. et al. Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes* 58, 1434–1439 (2009).
11. van Leeuwen, N. et al. A gene variant near ATM is significantly associated with metformin treatment response in type 2 diabetes: a replication and meta-analysis of five cohorts. *Diabetologia* 55, 1971–1977 (2012).
12. GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group, Wellcome Trust Case Control Consortium 2 & MAGIC investigators. Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. *Nat. Genet.* 43, 117–120 (2011).
13. Zhou, K. et al. Loss-of-function CYP2C9 variants improve therapeutic response to sulfonylureas in type 2 diabetes: a Go-DARTS study. *Clin. Pharmacol. Ther.* 87, 52–56 (2010).
14. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352, 837–853 (1998).
15. Diabetes Prevention Program Research Group. Long-term safety, tolerability, and weight loss associated with metformin in the Diabetes Prevention Program Outcomes Study. *Diabetes Care* 35, 731–737 (2012).
16. Diabetes Prevention Program Research Group. 10-year follow-up of diabetes incidence and weight loss in the Diabetes Prevention Program Outcomes Study. *Lancet* 374, 1677–1686 (2009).
17. Kahn, S.E. et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N. Engl. J. Med.* 355, 2427–2443 (2006).
18. Scott, R.A. et al. Large-scale association analyses identify new loci influencing glycemic traits and provide insight into the underlying biological pathways. *Nat. Genet.* 44, 991–1005 (2012).
19. Soranzo, N. et al. Common variants at 10 genomic loci influence hemoglobin A1c levels via glycemic and nonglycemic pathways. *Diabetes* 59, 3229–3239 (2010).
20. GTEx Consortium. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science* 348, 648–660 (2015).
21. van de Bunt, M. et al. Transcript expression data from human islets links regulatory signals from genome-wide association studies for type 2 diabetes and glycemic traits to their downstream effectors. *PLoS Genet.* 11, e1005694 (2015).
22. Kabachiev, B. & Silverberg, M.S. Expression quantitative trait loci analysis identifies associations between genotype and gene expression in human intestine. *Gastroenterology* 144, 1488–496 (2013).
23. Manz, F. et al. Fanconi–Bickel syndrome. *Pediatr. Nephrol.* 1, 509–518 (1987).
24. Fanconi, G. & Bickel, H. Chronic aminoaciduria (amino acid diabetes or nephrotic-glucosuric dwarfism) in glycogen storage and cystine disease. *Helv. Paediatr. Acta* 4, 359–396 (1949).
25. Miller, R.A. et al. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* 494, 256–260 (2013).
26. Hosokawa, M. & Thorens, B. Glucose release from GLUT2-null hepatocytes: characterization of a major and a minor pathway. *Am. J. Physiol. Endocrinol. Metab.* 282, E794–E801 (2002).
27. Burcelin, R., Dolci, W. & Thorens, B. Glucose sensing by the hepatoportal sensor is GLUT2-dependent: in vivo analysis in GLUT2-null mice. *Diabetes* 49, 1643–1648 (2000).
28. Seyer, P. et al. Hepatic glucose sensing is required to preserve β cell glucose competence. *J. Clin. Invest.* 123, 1662–1676 (2013).
29. Hundal, R.S. et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* 49, 2063–2069 (2000).
30. Fullerton, M.D. et al. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nat. Med.* 19, 1649–1654 (2013).
31. Pau, C.T., Keefe, C., Duran, J. & Welt, C.K. Metformin improves glucose effectiveness, not insulin sensitivity: predicting treatment response in women with polycystic ovary syndrome in an open-label, interventional study. *J. Clin. Endocrinol. Metab.* 99, 1870–1878 (2014).
32. McCreight, L.J., Bailey, C.J. & Pearson, E.R. Metformin and the gastrointestinal tract. *Diabetologia* 59, 426–435 (2016).
33. Ait-Omar, A. et al. GLUT2 accumulation in enterocyte apical and intracellular membranes: a study in morbidly obese human subjects and ob/ob and high fat-fed mice. *Diabetes* 60, 2598–2607 (2011).
34. Gong, L., Goswami, S., Giacomini, K.M., Altman, R.B. & Klein, T.E. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenet. Genomics* 22, 820–827 (2012).
35. Bailey, C.J. The current drug treatment landscape for diabetes and perspectives for the future. *Clin. Pharmacol. Ther.* 98, 170–184 (2015).

36. Banda, Y. et al. Characterizing race/ethnicity and genetic ancestry for 100,000 subjects in the Genetic Epidemiology Research on Adult Health and Aging (GERA) cohort. *Genetics* 200, 1285–1295 (2015).
37. Morris, A.P. et al. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. *Nat. Genet.* 44, 981–990 (2012).
38. Kooy, A. et al. Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus. *Arch. Intern. Med.* 169, 616–625 (2009).
39. Purcell, S. et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* 81, 559–575 (2007).
40. Price, A.L. et al. Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* 38, 904–909 (2006).
41. Sherifali, D., Nerenberg, K., Pullenayegum, E., Cheng, J.E. & Gerstein, H.C. The effect of oral antidiabetic agents on A1C levels: a systematic review and meta-analysis. *Diabetes Care* 33, 1859–1864 (2010).
42. Postmus, I. et al. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. *Nat. Commun.* 5, 5068 (2014).
43. Mägi, R. & Morris, A.P. GWAMA: software for genome-wide association meta-analysis. *BMC Bioinformatics* 11, 288 (2010).
44. Howie, B.N., Donnelly, P. & Marchini, J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet.* 5, e1000529 (2009).
45. Marchini, J., Howie, B., Myers, S., McVean, G. & Donnelly, P. A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat. Genet.* 39, 906–913 (2007).
46. Innocenti, F. et al. Identification, replication, and functional fine-mapping of expression quantitative trait loci in primary human liver tissue. *PLoS Genet.* 7, e1002078 (2011).
47. Schröder, A. et al. Genomics of ADME gene expression: mapping expression quantitative trait loci relevant for absorption, distribution, metabolism and excretion of drugs in human liver. *Pharmacogenomics J.* 13, 12–20 (2013).
48. Schadt, E.E. et al. Mapping the genetic architecture of gene expression in human liver. *PLoS Biol.* 6, e107 (2008).
49. Xia, K. et al. seeQTL: a searchable database for human eQTLs. *Bioinformatics* 28, 451–452 (2012).
50. Dahlin, A. et al. Gene expression profiling of transporters in the solute carrier and ATP-binding cassette superfamilies in human eye substructures. *Mol. Pharm.* 10, 650–663 (2013).
51. Liang, X. et al. Metformin is a substrate and inhibitor of the human thiamine transporter, THTR-2 (SLC19A3). *Mol. Pharm.* 12, 4301–4310 (2015).

Chapter 7.

Variants in Pharmacokinetic Transporters and Glycemic Response to Metformin

VARIANTS IN PHARMACOKINETIC TRANSPORTERS AND GLYCEMIC RESPONSE TO METFORMIN: A METGEN META-ANALYSIS

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ABSTRACT

Therapeutic response to metformin, a first-line drug for type 2 diabetes (T2D), is highly variable, in part likely due to genetic factors. To date, metformin pharmacogenetic studies have mainly focused on the impact of variants in metformin transporter genes, with inconsistent results. To clarify the significance of these variants in glycemic response to metformin in T2D, we performed a large-scale meta-analysis across the cohorts of the Metformin Genetics Consortium (MetGen). Nine candidate polymorphisms in five transporter genes (organic cation transporter [OCT]1, OCT2, multidrug and toxin extrusion transporter [MATE]1, MATE2-K, and OCTN1) were analyzed in up to 7,968 individuals. None of the variants showed a significant effect on metformin response in the primary analysis, or in the exploratory secondary analyses, when patients were stratified according to possible confounding genotypes or prescribed a daily dose of metformin. Our results suggest that candidate transporter gene variants have little contribution to variability in glycemic response to metformin in T2D.

INTRODUCTION

Metformin is the first-line pharmacological therapy for type 2 diabetes (T2D) and the most widely prescribed antidiabetic drug. The glycemic response to metformin is, however, highly variable. In patients receiving metformin as an initial treatment for T2D, less than two-thirds achieve acceptable glycemic control or a target HbA_{1c} < 7% (53 mmol/mol).^{1,2} Genetic factors play an important role in the variable glycemic response to metformin, with up to 34% of variance in HbA_{1c} reduction explained by common genetic variants each conferring a

small to moderate impact.³ As a result a large sample size is required for pharmacogenetic studies aiming to discover these common metformin response variants.

Previously published studies of metformin pharmacogenetics have mostly focused on the candidate genes involved in drug pharmacokinetics, with the expectation that these might have a large clinical effect. For example, polymorphisms in the transporters, organic anion-transporting polypeptide 1B1 and breast cancer resistance protein, have been associated with large effect sizes with the pharmacokinetics and pharmacodynamics of several drugs including statins.⁴ Metformin is not metabolized and is excreted unchanged by the kidneys. Its primary mode of action appears to be an increase of hepatic insulin sensitivity through inhibition of gluconeogenesis,⁵ although there is increasing recognition of its role in the gut.⁶ As metformin is an organic cation at physiologic pH, cation-selective carrier proteins mediate its transport across cell membranes in the intestine, liver and kidneys. Organic cation transporter 1 (OCT1; encoded by *SLC22A1*) is expressed on the sinusoidal

Gene	dbSNP ID	Nucleotide change	Amino acid change	MAF ^a	Reference
OCT1 (<i>SLC22A1</i>)	rs12208357	c.181C> T	R61C	0.06	Shu et al., 2007 ¹⁵ ; Shu et al., 2008 ¹⁶ ; Tzvetkov et al., 2009 ²⁸ ; Zhou et al., 2009 ¹⁹ ; Christensen et al., 2011 ²³
	rs72552763	c.1260GAT> del	M420del	0.19 ^{15,45}	Shu et al., 2007 ¹⁵ ; Shu et al., 2008 ¹⁶ ; Tzvetkov et al., 2009 ²⁸ ; Zhou et al., 2009 ¹⁹ ; Christensen et al., 2011 ²³
	rs622342	Intron A> C		0.38	Becker et al., 2009 ²⁰ ; Christensen et al., 2011 ²³
OCT2 (<i>SLC22A2</i>)	rs316019	c.808G> T	A270S	0.11	Song et al., 2008 ²⁹ ; Wang et al., 2008 ³⁰ ; Chen et al., 2009 ¹⁷ ; Christensen et al., 2011 ²³ ; Tkac [~] et al., 2013 ²⁵
MATE1 (<i>SLC47A1</i>)	rs2289669	Intron G> A		0.42	Becker et al., 2009 ²¹ ; Tzvetkov et al., 2009 ²⁸ ; Jablonski et al., 2010 ³¹ ; Christensen et al., 2011 ²³ ; Tkac [~] et al., 2013 ²⁵
	rs2252281	g.-66T> C		0.41	Stocker et al., 2013 ¹⁸
MATE2-K (<i>SLC47A2</i>)	rs12943590	g.-130G> A		0.27	Choi et al., 2011 ²⁴ ; Stocker et al., 2013 ¹⁸
OCTN1 (<i>SLC22A4</i>)	rs272893	c.917C> T	T306I	0.41	Yoon et al., 2013 ¹⁴
	rs1050152	c.1507C> T	L503F	0.39	Tzvetkov et al., 2009 ²⁸

Table 1. Single-nucleotide polymorphisms explored in the meta-analysis
Minor alleles are shown in bold.
dbSNP, single nucleotide polymorphism database; ID, identification; MAF, minor allele frequency; MATE1, multidrug and toxin extrusion transporter; OCT1, organic cation transporter.
^aMinor allele frequencies from 1000 Genome Project Phase 3 EUR population (www.ncbi.nlm.nih.gov/projects/SNP).

membrane of hepatocytes and is the main transporter of metformin into the liver.⁷ Organic cation transporter 2 (OCT2; encoded by *SLC22A2*) is expressed primarily at the basolateral membrane in the kidney tubular cells and facilitates the uptake of metformin from the blood into kidney.⁸ The multidrug and toxin extrusion transporter 1 (MATE1; encoded by *SLC47A1*) and MATE2-K (encoded by *SLC47A2*), are H⁺/drug antiporters located in the apical membrane of the renal tubular cells, and facilitate metformin excretion from tubular cells into urine.⁹⁻¹¹ A recent study showed that metformin is also a substrate of carnitine/cation transporter 1 (OCTN1; encoded by *SLC22A4*).¹² OCTN1 is highly expressed at the apical membranes in renal proximal tubules and could also contribute to metformin elimination.^{13,14} To date, several polymorphisms in these transporter genes have been associated with the pharmacokinetics and pharmacodynamics of metformin in healthy volunteers¹⁵⁻¹⁸ and with metformin response in T2D.¹⁸⁻²⁵ In addition, a few studies have reported gene-gene interactions between polymorphisms in transporter genes.^{18,22,26} However, the results of these studies have been inconsistent and the impact of the established pharmacokinetic variants on metformin clinical response in T2D is uncertain.²⁷ Apart from the different measures of glycemic response used in these studies, the small sample size and reporting bias is another potential explanation for the observed inconsistency.

To clarify the role of genetic variants in these transporters on metformin clinical response, we performed a large-scale meta-analysis of the impact of known candidate variants on metformin efficacy in T2D, across the cohorts of recently established Metformin Genetics Consortium (MetGen). This resource has now in excess of 10,000 individuals where metformin response can be defined, and offers a unique opportunity for a highly powered pharmacogenetic meta-analysis of glycemic response to metformin.

RESULTS

We studied the effect of nine candidate variants in transporter genes OCT1,^{15,16,19,20,23,28} OCT2,^{17,23,25,29,30} MATE1,^{18,21,23,25,28,31} MATE2-K^{18,24} and OCTN1^{14,28} (Table 1) on metformin glycemic response in 7,968 MetGen participants of European ancestry. Of these, definition of metformin response could be aligned for a meta-analysis in 7,656 participants, of whom 5,836 were initiated on metformin monotherapy and 1,820 were initiated on metformin as add-on treatment for sulfonylureas (dual therapy; Table 2). Forest plots for the meta-analyses of the individual variants in the monotherapy group are presented in Figure 1. There was no significant heterogeneity between the studies for any polymorphism. None

Characteristic	DCS	GoDARTS	Kosice	PMT1-EU	PMT2-EU	Riga	Rotterdam	Sarajevo
No. of participants	1,380	3,170	148	125	2,358	64	323	87
Age, years	62.1 ± 10.6	61.9 ± 11.1	57.5 ± 10.4	57.2 ± 12.9	66.2 ± 9.8	59.7 ± 10.7	69.7 ± 10.6	58.2 ± 9.0
Sex, male, %	757 (55)	1,813 (57)	72 (49)	61 (49)	1,283 (54)	19 (30)	147 (46)	37 (43)
BMI, kg/m ²	30.9 ± 5.2	31.7 ± 5.9	31.5 ± 4.6	37.7 ± 9.1	32.3 ± 6.8	34.4 ± 5.2	29.5 ± 4.7	31.2 ± 4.3
Pretreatment HbA _{1c} , %	7.3 ± 1.3	8.9 ± 1.4	7.6 ± 1.1	7.8 ± 1.3	7.8 ± 1.5	7.1 ± 1.1	7.8 ± 1.3	7.8 ± 1.4
On-treatment HbA _{1c}	6.6 ± 0.8	7.2 ± 1.1	7.0 ± 0.8	6.7 ± 0.8	6.5 ± 0.8	6.4 ± 0.5	6.7 ± 0.7	6.7 ± 0.7
Creatinine clearance, ml/min ^a	105 ± 38	93.2 ± 35.4	104 ± 36	80.2 ± 20.1	100.0 ± 38.1	122.4 ± 44.5	85.2 ± 32.6	109.5 ± 30.4
Metformin daily dose, mg	1,089 ± 597	1,321 ± 515	1,400 ± 540	913 ± 326	932 ± 490	1,704 ± 579	800 ± 480	1,200 ± 608
Adherence	-	82.4 ± 16.2	-	>80 ^b	88.9 ± 20.4	-	79.6 ± 34	-
Metformin monotherapy, %	85	69	100	100	75	100	88	100

Table 2. Characteristics of cohort participants included in the meta-analysis

BMI, body mass index.
^aCreatinine clearance was estimated using the Cockcroft-Gault formula, except for the PMT1-EU study in which the MDRD formula was used.
^bPatients needed to have 80% adherence to be included in the study.

Figure 1. Effects of candidate variants in transporters genes on metformin glycemic response assessed as HbA_{1c} reduction in patients on metformin monotherapy. Beta values obtained from individual studies are presented with 95% confidence interval (CI); arrowheads indicate the CI exceeding the limits of the graph.

Overall betas are presented as black diamonds. The organic cation transporter 1 (OCT1) reduced-function (RF) alleles denote combined genotype for R61C and M420del – number of RF alleles. MATE1, multidrug and toxin extrusion transporter 1.

SNP	Effect allele	HOME				SDDS			
		N	Beta	SE	p	N	Beta	SE	p
OCT1 R61C	T	163	0.063	0.201	0.753	149	-0.010	0.186	0.960
OCT1 M420del	del	-	-	-	-	148	0.060	0.133	0.650
OCT1 RF alleles*		-	-	-	-	148	0.040	0.120	0.720
OCT1 rs622342	C	163	-0.019	0.113	0.863	148	-0.180	0.102	0.072
OCT2 A270S	T	-	-	-	-	149	-0.320	0.161	0.050
MATE1 rs2289669	A	163	0.099	0.118	0.402	149	-0.030	0.094	0.760
MATE1 rs2252281	C	-	-	-	-	146	0.070	0.092	0.440

Table 3. Association of individual SNPs with metformin glycaemic response in the HOME and SDDS studies. N, number of patients. *Combined genotype for R61C and M420del - number of reduced-function (RF) alleles. A positive beta is a greater glycaemic response to metformin associated with the effect allele.

of the variants were significantly associated with glycemic response to metformin (Figure 1). Similarly, when patients on monotherapy and dual therapy were analyzed together, no SNP showed significant association with HbA_{1c} reduction (Supplementary Table S1). The results from the HOME and SDDS studies, two MetGen cohorts where metformin was added to insulin therapy, did not show significant impact on metformin response assessed as HbA_{1c} reduction (Table 3).

As transport of metformin could depend on its concentration, we next explored possible gene by dose interactions, using a dose as a proxy of metformin concentration. In the meta-analysis of SNP x dose interaction effects, none of the interactions showed significant effect in the monotherapy group (Table 4) or the total group when patients on dual therapy were added to the analysis (Supplementary Table S3a). Likewise, no significant associations were found in the separate meta-analyses of the effects of variants on metformin response in individuals treated with low (≤ 1000 mg) or high daily doses of metformin (> 1000 mg) (Table 5 and Supplementary Table S3b).

We next explored the potential interactions between these transporters that might affect metformin response. The interactions were tested by examining the impact of one SNP in two separate subgroups of participants, homozygotes for the reference and variant allele of a potential confounding SNP. Tables 6a and 6b show the results from meta-analyses of participants on monotherapy for transporters in the liver and kidney, respectively. Supplementary Tables S4a and S4b show the meta-analyses results for all participants,

SNP	Effect allele	No. of studies	No. of patients	Continuous dose					Dichotomous dose				
				Beta	SE	p	I ²	p(Q)	Beta	SE	p	I ²	p(Q)
OCT1 R61C	T	5	4,376	0.005	0.019	0.816	0.0	0.965	-0.005	0.078	0.946	0.0	0.720
OCT1 M420del	del	4	4,297	0.006	0.014	0.669	0.0	0.502	0.043	0.089	0.631	61.8	0.049
OCT1 RF alleles ^a		4	3,986	0.001	0.012	0.923	0.0	0.659	0.041	0.049	0.394	44.0	0.148
OCT1 rs622342	C	5	5,273	0.028	0.010	0.006	39.8	0.156	0.092	0.037	0.014	0.0	0.413
OCT2 A270S	T	6	5,002	0.008	0.017	0.647	30.8	0.205	-0.003	0.056	0.952	23.0	0.261
MATE1 rs2289669	A	6	4,980	0.014	0.008	0.094	0.2	0.415	0.081	0.036	0.024	0.0	0.485
MATE1 rs2252281	C	4	2,528	0.004	0.015	0.791	0.0	0.505	0.059	0.043	0.177	0.0	0.484
MATE2 rs12943590	A	6	4,273	-0.008	0.011	0.467	0.0	0.663	0.007	0.044	0.875	0.0	0.827
OCTN1 T306I	T	6	4,647	-0.005	0.010	0.624	0.0	0.466	-0.006	0.037	0.879	0.0	0.875
OCTN1 L503F	T	4	4,202	0.025	0.016	0.110	60.4	0.056	0.120	0.085	0.161	76.0	0.006

Table 4. Meta-analysis results of single-nucleotide polymorphism 3 dose interaction effects in participants on metformin monotherapy I², heterogeneity index; MATE, multidrug and toxin extrusion transporter; OCT1, organic cation transporter 1; P(Q), P value for Cochrane's Q statistic; RF, reduced function; SNP, single-nucleotide polymorphism. ^aCombined genotype for R61C and M420del – number of reduced-function alleles. A positive beta is a greater glycemic response to metformin associated with the effect allele.

including both monotherapy and dual therapy. These exploratory subgroup analyses revealed no significant interactions between SNPs that affect metformin glycemic response.

In the supplemental locus-wise meta-analysis of the association of all common SNPs in transporter gene regions with metformin glycemic response, none of the variants showed significant signal after correction for multiple testing ($P > 1.4 \times 10^{-5}$, Supplementary Figure S1).

DISCUSSION

Despite the established role of cation-selective transporters in metformin pharmacokinetics, polymorphisms in these transporters showed no significant impact on glycemic response to metformin in this large study of patients with T2D. This meta-analysis had 80% power to detect an allelic effect of HbA_{1c} reduction $> 0.14\%$ (1.5 mmol/mol) for any of the candidate SNPs at nominal significance level of $\alpha=0.005$. Thus, none of the SNPs reported as being associated with metformin response in previous literature is likely to have an allelic impact on HbA_{1c} reduction of $>0.14\%$. Furthermore, it is unlikely that other SNPs, such as cis-regulatory variants, in these genes could have a significant impact on metformin glycemic response, as shown by a locus-wise meta-analysis of all common SNPs within genes' proximity in 6,964 patients.

Our findings contrast to most of those previously reported in healthy subjects,¹⁵⁻¹⁸ although it is in keeping with a recent study showing no impact of the OCT1 genotype on the glucose production in fasting healthy subjects.³² This may reflect the fact that our study was in patients with type 2 diabetes and as such we were able to assess HbA_{1c} reduction rather than other surrogates of metformin response. Some^{18,20,21,23-25} but not all 19 previous studies have reported an association of a variant altering metformin transport and glycemic response to metformin in patients with type 2 diabetes. These studies have varied in size but have generally been small, and have varied in their definition of glycemic response to metformin and analytical approaches. In this MetGen meta-analysis, we included all studies that have previously explored associations between transporters variants and glycemic response to metformin in T2D patients of European ancestry with both positive and negative findings,¹⁸⁻²⁵ and also other cohorts for which results have not been published. In this way we have reduced chance of reporting bias and maximized the power. In addition, we have reduced heterogeneity by aligning metformin response definition, models and covariates for all studies included in the meta-analysis. Our results suggest that metformin

SNP	Effect allele	No. of studies	No. of patients	Dose ≤ 1,000 mg				Dose >1,000 mg						
				Beta	SE	p	I ²	p(Q)	No. of patients	Beta	SE	p	I ²	p(Q)
OCT1 R61C	T	5	3,015	0.019	0.038	0.619	0.0	0.855	1,361	0.000	0.000	1.000	0.0	0.719
OCT1 M420del	del	4	2,903	-0.001	0.050	0.987	55.6	0.080	1,394	0.013	0.040	0.747	44.9	0.142
OCT1 RF alleles ^a		4	2,690	0.006	0.025	0.812	0.2	0.391	1,296	0.032	0.039	0.412	26.1	0.255
OCT1 rs622342	C	5	3,571	-0.004	0.019	0.837	0.0	0.523	1,702	0.055	0.030	0.064	0.0	0.679
OCT2 A270S	T	6	3,388	0.013	0.029	0.659	0.0	0.749	1,614	0.062	0.082	0.453	50.9	0.070
MATE1 rs2289669	A	6	3,428	-0.014	0.018	0.452	38.1	0.152	1,552	0.060	0.030	0.046	20.0	0.282
MATE1 rs2252281	C	4	1,629	0.004	0.026	0.877	0.0	0.750	899	-0.006	0.031	0.843	32.2	0.219
MATE2 rs12943590	A	6	3,009	-0.012	0.023	0.592	0.0	0.799	1,264	-0.070	0.088	0.431	93.1	0.000
OCTN1 T306I	T	6	3,116	0.017	0.020	0.401	0.0	0.728	1,531	0.038	0.033	0.251	0.0	0.971
OCTN1 L503F	T	4	2,889	-0.044	0.020	0.028	35.8	0.197	1,313	-0.029	0.036	0.434	48.4	0.121

Table 5. Meta-analysis results for the effects of candidate variants in transporter genes on metformin glycemic response in participants on metformin monotherapy, stratified by metformin dose
I², heterogeneity index; MATE, multidrug and toxin extrusion transporter; OCT1, organic cation transporter 1; P(Q), P value for Cochrane's Q statistic; RF, reduced function;
SNP, single-nucleotide polymorphism.
^aCombined genotype for R61C and M420del – number of reduced-function alleles. A positive beta is a greater glycemic response to metformin associated with the effect allele.

transporters do not have a significant role in how patients with type 2 diabetes respond to metformin therapy.

Transporters exhibit asymmetry in their kinetic properties; thus, for facilitated transporters that are bidirectional, the direction of the transport will depend on the substrate concentration.^{33,34} Systemic plasma levels of metformin are dependent on dose; therefore, in our secondary analyses, we analyzed dose x SNP interactions and assessed the effect of the studied variants separately in individuals prescribed low (≤ 1000 mg) or high (> 1000 mg) doses of the drug. We did not find significant impact of the analyzed interactions on metformin glycemic response, and accordingly, significant association between any variant and response in the dose-stratified analysis. However, we used prescribed dose as a proxy of metformin concentration. There were differences in the characteristics of patients between the cohorts, and for instance, older age and lower estimated glomerular filtration rate in the Rotterdam cohort could result in higher metformin serum levels despite being prescribed lower doses. Also, data on drug adherence were available only in four studies, although all studies were adjusted for creatinine clearance and other known clinical covariates which could influence metformin response.

Studies of small cohorts have reported gene-gene interactions affecting glycemic response to metformin previously.^{18,22} Here we explored whether such interactions could explain the lack of association between metformin response and transporter variants. The exploratory analyses of SNP x SNP interactions, assessed as the impact of studied polymorphisms on metformin response in the subgroups of patients homozygous for possibly confounding SNPs, did not show any significant effects. However, these subgroup analyses had substantially less power to detect moderate effects due to smaller sample sizes, especially for the rarer SNPs. Larger studies would be needed to detect these effects and to explore possible, but more complex multiple gene-gene interactions.

This is the largest metformin pharmacogenetic study reported to date, despite a few limitations due to the need to align cohorts. Our finding that there is no significant role for metformin transporter variants in mediating glycemic response to metformin challenges our understanding of metformin action in patients with type 2 diabetes chronically treated with metformin. For example, there is increasing recognition that metformin works pre-systemically in the gut, via a number of mechanisms, to improve glycemia.^{6,35-37} Indeed a recent delayed release metformin achieves low systemic metformin concentrations but is effective at lowering blood glucose in patients with type 2 diabetes.³⁸ It is also possible,

Subgroup	SNP	Effect allele	No. of studies	No. of patients	Beta	SE	P value	I ²	p(Q)
0 OCT1 RF alleles ^a	MATE1 rs2289669	A	5	1,736	0.004	0.029	0.879	0.0	0.468
	MATE1 rs2252281	C	4	891	0.028	0.045	0.542	0.0	0.976
2 OCT1 RF alleles	MATE1 rs2289669	A	5	179	-0.095	0.108	0.380	80.6	0.000
	MATE1 rs2252281	C	4	102	0.167	0.136	0.221	66.5	0.030
OCT1 rs622342 wt/wt	MATE1 rs2289669	A	6	1,899	0.007	0.026	0.789	0.0	0.429
	MATE1 rs2252281	C	4	997	0.046	0.036	0.204	0.0	0.799
OCT1 rs622342 v/v	MATE1 rs2289669	A	6	596	0.084	0.063	0.178	61.7	0.023
	MATE1 rs2252281	C	4	329	0.060	0.051	0.243	35.5	0.200
MATE1 rs2289669 wt/wt	OCT1 RF alleles		5	1,179	0.025	0.036	0.494	31.6	0.211
	OCT1 rs622342	C	6	1,608	0.035	0.049	0.477	53.2	0.058
MATE1 rs2289669 v/v	OCT1 RF alleles		5	292	-0.008	0.080	0.921	0.0	0.779
	OCT1 rs622342	C	6	527	0.024	0.053	0.653	0.0	0.505
MATE1 rs2252281 wt/wt	OCT1 RF alleles		4	606	0.064	0.052	0.213	0.0	0.837
	OCT1 rs622342	C	4	956	0.040	0.035	0.250	0.0	0.870
MATE1 rs2252281 v/v	OCT1 RF alleles		4	256	-0.005	0.092	0.957	49.1	0.117
	OCT1 rs622342	C	4	373	0.019	0.054	0.734	0.0	0.575

Table 6a. Meta-analysis results for the effects of individual single-nucleotide polymorphisms in the subgroups of patients homozygous for the wild-type or variant allele of possible confounding single-nucleotide polymorphisms – interactions between metformin liver transporters – monotherapy group
I², heterogeneity index; MATE, multidrug and toxin extrusion transporter; OCT1, organic cation transporter 1; P(Q), P value for Cochrane’s Q statistic; RF, reduced function;
SNP, single-nucleotide polymorphism; v/v, homozygous variant allele carriers; wt/wt, homozygous wild-type allele carriers.
^aCombined genotype for R61C and M420del - number of RF alleles. A positive beta is a greater glycemic response to metformin associated with the effect allele.

that the influence of the analyzed transporters genes variants is less prominent clinically than expected due to the redundancy of transporters *in vivo*.²⁷ If one or more transporters have reduced function, other transporters may take over their roles and mediate metformin uptake or efflux from the organs. In addition, there might be more membrane transporters of metformin yet to be identified that could have a role in its absorption, distribution and elimination. In the current study, we focused on the candidate variants in the membrane transporters genes that have been associated previously with metformin pharmacokinetics or response. However, in addition to liver and kidney transporters, transporters in the intestine may play a significant role in metformin levels. Recent studies have suggested

Subgroup	SNP	Effect allele	No. of studies	No. of patients	Beta	SE	P value	I ²	p(Q)
OCT2 A270S wt/wt	MATE1 rs2289669	A	7	3,869	0.008	0.018	0.673	41.9	0.112
	MATE1 rs2252281	C	5	1,835	-0.008	0.027	0.756	0.0	0.964
	MATE2 rs12943590	A	7	3,176	-0.011	0.023	0.642	0.0	0.481
	OCTN1 T306I	T	7	3,642	0.030	0.019	0.118	0.0	0.660
	OCTN1 L503F	T	5	3,437	-0.040	0.019	0.034	0.0	0.720
OCT2 A270S v/v	MATE1 rs2289669	A	3	59	0.045	0.210	0.831	0.0	0.950
	MATE1 rs2252281	C	2	35	0.575	0.175	0.001	31.8	0.226
	MATE2 rs12943590	A	3	48	0.170	0.337	0.615	0.0	0.557
	OCTN1 T306I	T	3	51	0.164	0.212	0.440	34.6	0.217
	OCTN1 L503F	T	3	50	-0.017	0.206	0.934	20.3	0.285
MATE1 rs2289669 wt/wt	OCT2 A270S	T	7	1,598	0.021	0.042	0.613	0.0	0.568
	MATE2 rs12943590	A	7	1,288	-0.066	0.071	0.347	58.9	0.024
	OCTN1 T306I	T	7	1,412	0.044	0.054	0.418	63.4	0.012
	OCTN1 L503F	T	5	1,328	-0.069	0.029	0.015	0.0	0.884
	OCTN1 L503F	T	5	1,328	-0.069	0.029	0.015	0.0	0.884
MATE1 rs2289669 v/v	OCT2 A270S	T	6	495	-0.066	0.146	0.655	65.0	0.014
	MATE2 rs12943590	A	7	363	0.065	0.052	0.215	0.0	0.529
	OCTN1 T306I	T	7	410	0.138	0.053	0.009	6.4	0.379
	OCTN1 L503F	T	5	365	-0.156	0.055	0.004	0.0	0.612
	OCTN1 L503F	T	5	365	-0.156	0.055	0.004	0.0	0.612
MATE1 rs2252281 wt/wt	OCT2 A270S	T	5	862	-0.014	0.056	0.802	0.0	0.942
	MATE2 rs12943590	A	5	720	0.006	0.046	0.902	9.2	0.354
	OCTN1 T306I	T	5	726	-0.037	0.042	0.382	47.5	0.107
	OCTN1 L503F	T	4	662	-0.017	0.046	0.711	19.9	0.290
	OCTN1 L503F	T	4	662	-0.017	0.046	0.711	19.9	0.290
MATE1 rs2252281 v/v	OCT2 A270S	T	4	329	0.129	0.076	0.087	0.0	0.592
	MATE2 rs12943590	A	5	276	-0.042	0.061	0.494	0.0	0.829
	OCTN1 T306I	T	5	283	0.013	0.067	0.853	22.1	0.274
	OCTN1 L503F	T	4	262	-0.011	0.074	0.884	0.0	0.650
	OCTN1 L503F	T	4	262	-0.011	0.074	0.884	0.0	0.650
MATE2 rs12943590 wt/wt	OCT2 A270S	T	7	1,964	0.001	0.036	0.978	22.4	0.259
	MATE1 rs2289669	A	7	1,980	-0.010	0.023	0.673	0.0	0.454
	MATE1 rs2252281	C	5	1,014	0.051	0.034	0.138	0.0	0.661
	OCTN1 T306I	T	7	1,828	0.043	0.026	0.095	39.7	0.127
	OCTN1 L503F	T	5	1,636	-0.050	0.027	0.062	26.4	0.246
MATE2 rs12943590 v/v	OCT2 A270S	T	7	298	-0.017	0.088	0.849	0.0	0.830
	MATE1 rs2289669	A	7	300	0.183	0.115	0.112	51.3	0.055
	MATE1 rs2252281	C	5	139	0.089	0.097	0.357	0.0	0.534
	OCTN1 T306I	T	7	279	-0.071	0.056	0.203	0.0	0.744
	OCTN1 L503F	T	5	241	0.080	0.058	0.168	0.0	0.455

Subgroup	SNP	Effect allele	No. of studies	No. of patients	Beta	SE	P value	I ²	p(Q)
OCTN1 T306I wt/wt	OCT2 A270S	T	7	1,806	0.081	0.041	0.048	0.0	0.557
	MATE1 rs2289669	A	7	1,728	-0.020	0.028	0.469	0.0	0.554
	MATE1 rs2252281	C	5	827	-0.001	0.040	0.976	35.2	0.187
	MATE2 rs12943590	A	7	1,426	0.018	0.033	0.581	0.0	0.630
OCTN1 T306I v/v	OCT2 A270S	T	6	634	-0.017	0.067	0.803	31.7	0.198
	MATE1 rs2289669	A	7	609	-0.023	0.040	0.571	25.7	0.232
	MATE1 rs2252281	C	5	267	0.049	0.122	0.690	63.7	0.027
	MATE2 rs12943590	A	7	541	-0.145	0.094	0.121	63.1	0.012
OCTN1 L503F wt/wt	OCT2 A270S	T	5	1,367	-0.040	0.047	0.394	41.8	0.143
	MATE1 rs2289669	A	5	1,274	-0.019	0.031	0.548	0.0	0.724
	MATE1 rs2252281	C	4	583	-0.059	0.063	0.344	42.2	0.158
	MATE2 rs12943590	A	5	1,114	-0.049	0.057	0.394	53.5	0.072
OCTN1 L503F v/v	OCT2 A270S	T	5	816	0.106	0.063	0.095	40.2	0.153
	MATE1 rs2289669	A	5	768	-0.042	0.045	0.354	0.0	0.982
	MATE1 rs2252281	C	4	337	0.017	0.065	0.793	5.6	0.365
	MATE2 rs12943590	A	5	584	0.031	0.053	0.561	0.0	0.857

Table 6b. Meta-analysis results for the effects of individual single-nucleotide polymorphisms in the subgroups of patients homozygous for the wild-type or variant allele of possible confounding single-nucleotide polymorphisms - interactions between metformin kidney transporters - monotherapy group

that other transporters, which were not the subject of this study, play an important role in metformin absorption,^{34,39-41} and there still may be unidentified cation transporters in the intestine involved in metformin absorption, which could also affect metformin response. It should also be noted that variants in transporters in various tissues may play opposing roles. For example, OCT1 could mediate basolateral flux of metformin from enterocytes to the portal circulation^{7,42} and across the sinusoidal membrane of hepatocytes.⁷ Thus a reduced function OCT1 genetic variant may result in increased concentrations in enterocytes and decreased concentrations in hepatocytes.

Though the current study demonstrates that genetic variants in transporters that play a role in metformin pharmacokinetics and pharmacodynamics have no significant effect on metformin glycemic response in large cohorts of diabetic patients, there are some limitations to our study. First, despite the large sample size used in the current study, we did not have statistical power to detect an allelic effect size for HbA_{1c} reduction smaller than 0.06%-0.14% (0.7-1.5 mmol/mol, depending on SNP frequency), however these small

effect sizes would be unlikely to have any clinical importance. Second, the meta-analysis included population-based observational studies which could be confounded by a number of factors, such as patient adherence or frequency of HbA_{1c} measurements in the cohort. However, in four cohorts, including two largest cohorts, GoDARTS and PMT2-EU, adherence could be calculated from the drug dispensing records, and was added as a covariate in the model. Likewise, although we could not completely harmonize definition of on-treatment HbA_{1c} due to different frequency of HbA_{1c} measurements available in the cohorts, four studies, including two largest studies, used minimal HbA_{1c} achieved within 18 months as outcome. Third, concomitant medications, which affect metformin transport, are well established to cause changes in its pharmacokinetics,⁴³ and may obscure effects of genetic variants on metformin response. Information on prescription and over-the-counter drugs, which affect metformin pharmacokinetics such as cimetidine, were not gathered in the current study. In addition, several previous studies have shown effects of polymorphisms in transporters on metformin response in multi-ethnic cohorts including many individuals from non-European ancestries.^{18,24} Genetic variants may have different effect sizes on drug response in individuals from different ethnic backgrounds. Further studies are needed to test the effects of these variants on metformin response in individuals from non-European backgrounds. Finally, recent studies suggest that genetic variants in transcription factors that affect expression of several metformin transporters may have larger effect sizes on metformin response than genetic variants in the individual transporters themselves,⁴⁴ underscoring the need to understand not only the mechanisms of metformin transport in various tissues, but the proteins that modulate their activity and expression.

In conclusion, in our large meta-analysis including almost 8,000 individuals across ten international cohorts of the MetGen Consortium, variants in metformin transporters genes have shown no relevant contribution to variability in metformin response in patients with T2D although we cannot rule out gene-concentration or more complex multiple gene-gene interactions that may be required to account for transporter redundancy. As has been recognized now for a number of years in disease and complex trait genetics, this study shows the importance of large sample sizes, usually only available to international consortia, for robust pharmacogenetic studies. Future even larger consortia efforts are required to corroborate these findings and to unravel genetic variations that could be used as better predictors for personalized metformin therapy.

METHODS

Studies

The MetGen Consortium consists of research groups from Europe and the United States with available data for studies of metformin pharmacogenetics from population observational studies and clinical trials. The 10 MetGen cohorts with available data for the study of metformin transporter gene variants are presented in Supplementary Table S5 online. The studies were approved by relevant institutional review boards, and all participants gave written informed consent.

Single-nucleotide polymorphism selection and genotyping

Nine SNPs in five transporter genes, reported to be associated with metformin response or pharmacokinetics in previous studies, were included in the meta-analysis (Table 1). The numbers of available SNPs, genotyping methods, and the minor allele frequencies in each cohort are provided in Supplementary Table S6 online. All SNPs were in line with Hardy-Weinberg equilibrium ($P > 0.01$). The variants were first analyzed individually for association with metformin response. The reduced-function OCT1 polymorphisms, R61C and M420del, were analyzed individually, and also together as a combined genotype, according to the number of haplotypes carrying reduced-function alleles: 0, 1, or 2, in line with previous studies in patients of European ancestry.^{23,45,46}

Assessment of metformin response

Metformin response was defined as a reduction in HbA_{1c} during treatment with metformin: pretreatment minus on-treatment HbA_{1c}. As such, a positive b in the regression models indicates an association of the effect allele with greater glycemic response to metformin. Inclusion criteria for all participants included in the meta-analysis were continuous treatment with metformin for at least 3 months, pretreatment HbA_{1c} measured within 6 months prior to metformin therapy and $<14\%$, measurement of on-treatment HbA_{1c} within 18 months of metformin commencement, and no treatment with other glucose-lowering agents except stable sulfonylurea treatment before and during metformin therapy. Hence, two cohorts from randomized controlled trials, HOME and SDDS studies, in which metformin was added to insulin therapy, were not included in the meta-analysis, and the results are shown separately. On-treatment HbA_{1c} was defined as minimal HbA_{1c} measured within 18 months of

metformin commencement in the GoDARTS, PMT2-EU, Riga, and Rotterdam studies, as HbA_{1c} measured after 6 months (Košice and Sarajevo studies) or 12 months (DCS study) of metformin treatment, and as HbA_{1c} measured within the first 3–9 months of metformin therapy in the PMT1-EU study.

STATISTICAL ANALYSIS

In each cohort, the effects of individual SNPs on metformin response were assessed in additive genetic model using linear regression with reduction of HbA_{1c} as outcome (primary analysis). The pretreatment HbA_{1c}, metformin daily dose, adherence, creatinine clearance, baseline gap (time between pretreatment HbA_{1c} measurement and start of metformin therapy), and treatment group (metformin prescribed as monotherapy or dual therapy – metformin added to stable sulfonylurea treatment), were added as covariates, when available/applicable (Supplementary Table S7 online). In the HOME study, analyses were adjusted for pretreatment HbA_{1c}, metformin daily dose and creatinine clearance, and in the SDDS study, pretreatment HbA_{1c} and randomization group²³ were added as covariates.

As the effects of transporter SNPs could depend on metformin level, or they could be confounded by the effect of other variants, secondary analyses were performed to explore possible gene-dose and gene-gene interactions. For gene-dose interactions, we first examined interaction models that included SNP x dose interaction term with dose as a continuous variable and then dose coded as a dichotomized variable (low or high dose) in the following model: HbA_{1c} reduction ~ pre-treatment HbA_{1c} + adherence + creatinine clearance + baseline gap + treatment group + dose + SNP + SNP x dose.

Next, we assessed the association of SNPs with metformin response separately for patients taking low ($\leq 1,000$ mg) or high doses ($> 1,000$ mg) of metformin. The cutoff value of 1,000 mg was chosen based on the median dose in the largest cohort. The analyses were performed using the same basic regression model: HbA_{1c} reduction ~ pre-treatment HbA_{1c} + adherence + creatinine clearance + baseline gap + treatment group + dose + SNP.

To examine potential interactions between the variants, additional exploratory analyses of the effects of individual SNPs were carried out in the subgroups of individuals who were homozygous for wild-type allele and of the individuals who were homozygous for variant allele of possibly confounding SNPs, assuming that impact of the variants could be more pronounced in more extreme genotype groups. Possible 1 x 1 interactions between SNPs in

metformin liver transporters (OCT1 and MATE1), and SNPs in metformin kidney transporters (OCT2, MATE1, MATE2-K, and OCTN1) were explored.

All analyses were carried out for the total group of patients treated with metformin, including monotherapy and dual therapy group (metformin added to stable sulfonylurea therapy), and also separately for the metformin monotherapy group. The results from individual studies were combined in the meta-analysis using PLINK software under fixed-effects model (<http://pngu.mgh.harvard.edu/purcell/plink/>).⁴⁷ For gene-dose interaction models, estimates of SNP x dose interaction terms were pooled in the meta-analysis.⁴⁸ Heterogeneity across studies was assessed using the Cochran Q test and the I² heterogeneity index. In case the substantial heterogeneity was detected (*P* value for Cochran Q test < 0.10), the results of the random-effects model were presented. Power analyses were performed using Quanto (<http://biostats.usc.edu/Quanto.html>). Forest plots were created using RevMan 5.3 software (Review Manager, The Nordic Cochrane Centre, The Cochrane Collaboration).

As the primary analysis included nine SNPs and one combined OCT1 genotype, a nominal statistical significance threshold was set to 5×10^{-3} ($P = 0.05/10$), and a study-wise statistical significance threshold to 2×10^{-4} to correct for the overall number of tests performed ($P = 0.05/236$). In addition to our main hypothesis, we performed a supplemental, locus-wise meta-analysis of all common SNPs within the proximity of the transporter genes to examine the possibility that original associations reported in previous studies might be driven by other SNPs in these gene regions. A total of 3,471 variants with minor allele frequency $\geq 1\%$ were tested for association with metformin glycemic response in 6,964 participants from two MetGen cohorts with available genome-wide association study imputed data, the GoDARTS and PMT2-EU. The details of genotyping, quality control, imputation, association analysis, and metaanalysis were described in our previous study.⁴⁹ The experiment-wise significance threshold for this supplemental analysis was derived from 10,000 permutations of the phenotype in the GoDARTS data,⁴⁷ which gave a 95% cutoff for significance of $P = 1.6 \times 10^{-4}$.

ARTICLE INFORMATION

Additional Supporting Information including acknowledgments and author affiliations may be found in the online version of this article. This chapter has been modified from its original version: Table 3 has been moved from the supplements to the main article.

Conflict of Interest

The authors declared no conflict of interest.

Author Contributions

T.D., K.Z., K.M.G., and E.R.P. wrote the manuscript. T.D., K.M.G., and E.R.P. designed the research. T.D., K.Z., S.W.Y., N.v.L., C.E.d.K., M.J., S.G., L.Z., M.M.H.C., M.O., R.T., M.K., M.M.H., A.A.v.d.H., L.K., V.P., A.K., K.B., J.K., S.S., I.T., B.H.S., C.N.A.P., L.M.t.H., K.M.G., and E.R.P. performed the research. T.D., K.Z., S.W.Y., N.v.L., C.E.d.K., M.J., S.G., L.Z., M.M.H.C., and M.O. analyzed the data.

REFERENCES

- Hermann, L.S., Schersten, B., Bitzen, P.O., Kjellstrom, T., Lindgarde, F. & Melander, A. Therapeutic comparison of metformin and sulfonylurea, alone and in various combinations. A double-blind controlled study. *Diabetes Care* 17, 1100-1109 (1994).
- Kahn, S.E. et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N Engl J Med* 355, 2427-2443 (2006).
- Zhou, K. et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. *Lancet Diabetes Endocrinol* 2, 481-487 (2014).
- Giacomini, K.M. et al. International Transporter Consortium commentary on clinically important transporter polymorphisms. *Clin Pharmacol Ther* 94, 23-26 (2013).
- Foretz, M., Guigas, B., Bertrand, L., Pollak, M. & Viollet, B. Metformin: from mechanisms of action to therapies. *Cell Metab* 20, 953-966 (2014).
- McCreight, L.J., Bailey, C.J. & Pearson, E.R. Metformin and the gastrointestinal tract. *Diabetologia* 59, 426-435 (2016).
- Wang, D.S., Jonker, J.W., Kato, Y., Kusunohara, H., Schinkel, A.H. & Sugiyama, Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther* 302, 510-515 (2002).
- Kimura, N., Okuda, M. & Inui, K. Metformin transport by renal basolateral organic cation transporter hOCT2. *Pharm Res* 22, 255-259 (2005).
- Otsuka, M., Matsumoto, T., Morimoto, R., Arioka, S., Omote, H. & Moriyama, Y. A human transporter protein that mediates the final excretion step for toxic organic cations. *Proc Natl Acad Sci U S A* 102, 17923-17928 (2005).
- Masuda, S. et al. Identification and functional characterization of a new human kidney-specific H⁺/organic cation antiporter, kidney-specific multidrug and toxin extrusion 2. *J Am Soc Nephrol* 17, 2127-2135 (2006).
- Kusunohara, H. et al. Effects of a MATE protein inhibitor, pyrimethamine, on the renal elimination of metformin at oral microdose and at therapeutic dose in healthy subjects. *Clin Pharmacol Ther* 89, 837-844 (2011).
- Nakamichi, N. et al. Involvement of carnitine/organic cation transporter OCTN1/SLC22A4 in gastrointestinal absorption of metformin. *J Pharm Sci* 102, 3407-3417 (2013).
- Tamai, I. et al. Involvement of OCTN1 (SLC22A4) in pH-dependent transport of organic cations. *Mol Pharm* 1, 57-66 (2004).
- Yoon, H., Cho, H.Y., Yoo, H.D., Kim, S.M. & Lee, Y.B. Influences of organic cation transporter polymorphisms on the population pharmacokinetics of metformin in healthy subjects. *AAPS J* 15, 571-580 (2013).
- Shu, Y. et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* 117, 1422-1431 (2007).
- Shu, Y. et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther* 83, 273-280 (2008).
- Chen, Y. et al. Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenet Genomics* 19, 497-504 (2009).
- Stocker, S.L. et al. The effect of novel promoter variants in MATE1 and MATE2 on the pharmacokinetics and pharmacodynamics of metformin. *Clin Pharmacol Ther* 93, 186-194 (2013).
- Zhou, K. et al. Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes* 58, 1434-1439 (2009).
- Becker, M.L., Visser, L.E., van Schaik, R.H., Hofman, A., Uitterlinden, A.G. & Stricker, B.H. Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. *Pharmacogenomics J* 9, 242-247 (2009).
- Becker, M.L., Visser, L.E., van Schaik, R.H., Hofman, A., Uitterlinden, A.G. & Stricker, B.H. Genetic variation in the multidrug and toxin extrusion 1 transporter protein influences the glucose-lowering effect of metformin in patients with diabetes: a preliminary study. *Diabetes* 58, 745-749 (2009).
- Becker, M.L., Visser, L.E., van Schaik, R.H., Hofman, A., Uitterlinden, A.G. & Stricker, B.H. Interaction between polymorphisms in the OCT1 and MATE1 transporter and metformin response. *Pharmacogenet Genomics* 20, 38-44 (2010).
- Christensen, M.M. et al. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. *Pharmacogenet Genomics* 21,

- 837-850 (2011).
24. Choi, J.H. et al. A common 5'-UTR variant in MATE2-K is associated with poor response to metformin. *Clin Pharmacol Ther* 90, 674-684 (2011).
 25. Tkac, I. et al. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in type 2 diabetes. *Diabetes Obes Metab* 15, 189-191 (2013).
 26. Christensen, M.M. et al. A gene-gene interaction between polymorphisms in the OCT2 and MATE1 genes influences the renal clearance of metformin. *Pharmacogenet Genomics* 23, 526-534 (2013).
 27. Todd, J.N. & Florez, J.C. An update on the pharmacogenomics of metformin: progress, problems and potential. *Pharmacogenomics* 15, 529-539 (2014).
 28. Tzvetkov, M.V. et al. The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin. *Clin Pharmacol Ther* 86, 299-306 (2009).
 29. Song, I.S. et al. Genetic variants of the organic cation transporter 2 influence the disposition of metformin. *Clin Pharmacol Ther* 84, 559-562 (2008).
 30. Wang, Z.J., Yin, O.Q., Tomlinson, B. & Chow, M.S. OCT2 polymorphisms and in-vivo renal functional consequence: studies with metformin and cimetidine. *Pharmacogenet Genomics* 18, 637-645 (2008).
 31. Jablonski, K.A. et al. Common variants in 40 genes assessed for diabetes incidence and response to metformin and lifestyle intervention in the diabetes prevention program. *Diabetes* 59, 2672-2681 (2010).
 32. Christensen, M.M. et al. Endogenous glucose production increases in response to metformin treatment in the glycogen-depleted state in humans: a randomised trial. *Diabetologia* 58, 2494-2502 (2015).
 33. Koepsell, H., Lips, K. & Volk, C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* 24, 1227-1251 (2007).
 34. Chen, E.C. et al. Targeted disruption of organic cation transporter 3 attenuates the pharmacologic response to metformin. *Mol Pharmacol* 88, 75-83 (2015).
 35. Napolitano, A. et al. Novel gut-based pharmacology of metformin in patients with type 2 diabetes mellitus. *PLoS One* 9, e100778 (2014).
 36. Duca, F.A. et al. Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. *Nat Med* 21, 506-511 (2015).
 37. Forslund, K. et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* 528, 262-266 (2015).
 38. Buse, J.B. et al. The Primary Glucose-Lowering Effect of Metformin Resides in the Gut, Not the Circulation: Results From Short-term Pharmacokinetic and 12-Week Dose-Ranging Studies. *Diabetes Care* 39, 198-205 (2016).
 39. Han, T.K., Proctor, W.R., Costales, C.L., Cai, H., Everett, R.S. & Thakker, D.R. Four cation-selective transporters contribute to apical uptake and accumulation of metformin in caco-2 cell monolayers. *J Pharmacol Exp Ther* 352, 519-528 (2015).
 40. Zhou, M., Xia, L. & Wang, J. Metformin transport by a newly cloned proton-stimulated organic cation transporter (plasma membrane monoamine transporter) expressed in human intestine. *Drug Metab Dispos* 35, 1956-1962 (2007).
 41. Liang, X. et al. Metformin Is a Substrate and Inhibitor of the Human Thiamine Transporter, THTR-2 (SLC19A3). *Mol Pharm* 12, 4301-4310 (2015).
 42. Muller, J., Lips, K.S., Metzner, L., Neubert, R.H., Koepsell, H. & Brandsch, M. Drug specificity and intestinal membrane localization of human organic cation transporters (OCT). *Biochem Pharmacol* 70, 1851-1860 (2005).
 43. Stage, T.B., Brosen, K. & Christensen, M.M. A Comprehensive Review of Drug-Drug Interactions with Metformin. *Clin Pharmacokinet* 54, 811-824 (2015).
 44. Goswami, S. et al. Genetic variants in transcription factors are associated with the pharmacokinetics and pharmacodynamics of metformin. *Clin Pharmacol Ther* 96, 370-379 (2014).
 45. Tzvetkov, M.V., Saadatmand, A.R., Bokelmann, K., Meineke, I., Kaiser, R. & Brockmoller, J. Effects of OCT1 polymorphisms on the cellular uptake, plasma concentrations and efficacy of the 5-HT(3) antagonists tropisetron and ondansetron. *Pharmacogenomics J* 12, 22-29 (2012).
 46. Dujic, T., Zhou, K., Donnelly, L.A., Tavendale, R., Palmer, C.N. & Pearson, E.R. Association of Organic Cation Transporter 1 With Intolerance to Metformin in Type 2 Diabetes: A GoDARTS Study. *Diabetes* 64, 1786-1793 (2015).
 47. Purcell, S. et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81, 559-575 (2007).
 48. Manning, A.K. et al. Meta-analysis of gene-environment interaction: joint estimation of SNP and SNP x environment regression coefficients. *Genet Epidemiol* 35, 11-18 (2011).
 49. Zhou, K. et al. Variation in the glucose transporter gene SLC2A2 is associated with glycemic response to metformin. *Nat Genet* 48, 1055-1059 (2016).

Chapter 8.

General discussion and summary

BACKGROUND AND HISTORICAL PERSPECTIVE

Sixty years after the first use of metformin for diabetes,¹ and twenty years after publication of the major results of the United Kingdom Prospective Diabetes Study (UKPDS),² metformin is the preferred initial pharmacological treatment for patients with type 2 diabetes.³ Even after sixty years, there is still much to discover about the effects and mechanisms of action of metformin. The number of annual publications about metformin is growing each year, reaching almost two thousand in 2017 (Figure 1).

The HOME study

In the late 1990s when the study protocol for the HOME trial was written, sulfonylureas were still first choice for treatment of type 2 diabetes. In the Netherlands, metformin was named in the national diabetes guidelines in 1999 as preferred initial oral treatment in type 2 diabetes, but only for patients with a body mass index > 27.⁴ It was not until 2006 that metformin was named in the Dutch guidelines as first choice for oral treatment in all patients with type 2 diabetes.⁵ The primary goal of the HOME trial was to study whether metformin could lower the incidence of microvascular and macrovascular disease in type 2 diabetes. The present dissertation is the third thesis based on the results from the HOME study, with a fourth to follow. In 2004, Michiel Wulffelé published the results of the short-term effects of metformin on glucose regulation, insulin requirements and side-effects like vitamin B12 deficiency.⁶ In 2010, Jolien de Jager published her thesis, in which she described the long-term effects of metformin on glucose regulation, microvascular and macrovascular endpoints and vitamin B12 levels.⁷ To date, the HOME trial is still the largest long-term randomised placebo-controlled trial with metformin in type 2 diabetes worldwide. A recent meta-analysis named the study as the trial with the lowest risk of bias of the included studies.⁸ In addition, the HOME trial was the first RCT to evaluate the long-term effects of metformin vs placebo on the vitamin B12 status and related clinical endpoints.⁹

The Metformin Genetics Consortium (MetGen)

During the last decade, the attention of research with metformin has shifted from glucose control to other subjects like the effects of metformin in the intestines,¹⁰ the possible role of metformin in prevention from and treatment against cancer¹¹ and even longevity.¹² In addition, significant progress has been made in unravelling the mechanisms of action of metformin.¹³ Nevertheless, the considerable interindividual variability in response to met-

Figure 1. Number of publications per year about metformin (source: PubMed)

formin is still not completely understood. Genetic variation plays an important role with up to 34% of variance in HbA1c reduction explained by genetic variants.¹⁴ However, the results of studies on polymorphisms are disappointingly inconclusive, as several studies failed to replicate previously found associations, with small sample sizes and variety in ethnicity limiting conclusions to be drawn. Therefore, it was proposed during an international workshop on metformin pharmacogenomics in 2013 to create a “metformin consortium” with multiple research groups contributing expertise and samples from various ethnic groups.¹⁵ First contacts were made during a meeting at the annual convention of the European Association for the Study of Diabetes (EASD) in 2013 in Barcelona, right after a poster presentation of the results of the pharmacogenetic analysis of the HOME study.¹⁶ One month later, the MetGen Consortium was initiated. MetGen is co-led by Prof. Ewan Pearson of the University of Dundee, and Prof. Kathy Giacomini of the University of California, San Francisco. At the moment of writing, the consortium consists of a collection of numerous cohorts with over 10,000 individuals and still expanding.¹⁷

Present findings

Metformin not only decreases vitamin B12 levels, but also increases serum levels of methylmalonic acid (MMA) (HOME study)

Previous analysis of the HOME study showed that metformin is associated with a lowering of serum B12 that is progressive over time, and accompanied by an increase in serum homocysteine, which is suggestive of tissue B12 deficiency. The present study showed that, after 52 months, metformin not only reduced serum B12, but also progressively increased serum MMA: metformin, as compared to placebo, increased MMA at the end of the study with a mean difference of 0.039 $\mu\text{mol/L}$ (95%CI: 0.019 to 0.055). Expressed in gram years of exposure to metformin, MMA increased by 0.006 $\mu\text{mol/l}$ per gram year (summary mean; 95% CI 0.003 to 0.009, compared to placebo).

The increase of MMA in metformin users is associated with a significant worsening of a validated clinical neuropathy score (HOME study)

During 4.3 years, metformin had no overall effect on the neuropathy score. However, mediation analysis showed that the non-significant effect of metformin on a clinically validated neuropathy score can in fact be explained by a beneficial mediating effect through lowering of HbA1c (a decrease of a the neuropathy score of $0.04 \times 0.50 = 0.020$ per metformin gram year) and an adverse effect through increasing MMA (an increase of the neuropathy score of $0.04 \times 1.06 = 0.042$ per metformin gram year). A possible explanation may be that the harmful effects of B12 deficiency are counteracted by the protective effects of metformin by improvement of the glucose regulation, as our analysis suggested.

Metformin has no effect on vitamin D levels (HOME study)

Metformin versus placebo did not affect 25(OH)D levels adjusted for seasonal effects during 16 months (coefficient: 1.002 per month, multiplicative model; 95%CI: 0.998 to 1.006). Metformin was associated with a small increase of 25(OH)D₂ (coefficient: 1.012 per month; 95% CI 1.003 to 1.021). However, 25(OH)D₂ was only a very small fraction (3%) of total 25(OH)D. Seasonal variation had the biggest impact on 25(OH)D levels.

The use of metformin does not reduce energy intake (HOME study)

In the HOME trial, dietary intake was assessed at baseline, after 1 year and after 4.3 years, according to the dietary history method. Analysis showed that metformin did not reduce

energy intake (metformin vs placebo: -31.0 kcal/d; 95% CI, -107.4 to 45.4). In addition, linear mixed models did not show a significant effect of energy intake as explanation for the difference in weight gain between the groups. Therefore the prevention of weight gain by metformin cannot be explained by a reduction of energy intake.

Approximately one third of the difference in weight gain between metformin and placebo recipients can be explained by the reduction of insulin requirements by use of metformin (HOME study)

Mediation analysis showed that, at the end of the study, a patient receiving the actual mean dose of metformin (2050 mg) had 3.2 ± 0.5 kg less weight gain, as compared to placebo, of which approximately one third (1.2 ± 0.0 kg) could be explained by reduction of insulin intake by the use of metformin.

The ATM SNP rs11212617 not only affects the effect of metformin on HbA1c and insulin requirements, but also metformin plasma concentrations (HOME study)

We analysed the influence of the polymorphisms rs12208357 and rs622342 in the gene coding for organic cation transporter 1 (OCT1), rs2289669 in the gene coding for multidrug and toxin extrusion transporter 1 (MATE1) and rs11212617 in the ataxia telangiectasia mutated gene (ATM), on metformin's effects on HbA1c and insulin requirement (daily dose of insulin, DDI). Outcome measure was a combined HbA1c + DDI Z score. The minor allele of rs11212617 (ATM) was associated with an improved Z score (a decrease per allele of 0.11, 95%CI 0.01 to 0.20) and a lower metformin plasma concentration (-0.42 mg/ml per minor allele, 95% CI: -0.81 to -0.03). The major allele of rs2289669 (MATE1) was also associated with an improved Z score (-0.35, 95%CI -0.09 to -0.60). Metformin plasma concentration was also associated with a reduction of the Z score. However, the daily dose of metformin was the best predictor for the response to metformin.

The C allele of rs8192675 in the intron of SLC2A2, which encodes the facilitated glucose transporter GLUT2, is associated with the glucose-lowering effect of metformin in type 2 diabetes, especially among obese individuals (MetGen analysis)

A three-stage genome-wide association study (GWAS) in more than 13,000 participants was performed. The C allele of rs8192675 in the intron of SLC2A2, which encodes the facilitated glucose transporter GLUT2, was associated with a 0.17% greater metformin-induced reduc-

tion in HbA1c. Among obese individuals, C-allele homozygotes at rs8192675 had a 0.33% greater absolute HbA1c reduction than T-allele homozygotes.

Transporter gene variants contribute little to variability in glycaemic response to metformin in type 2 diabetes (MetGen analysis)

A meta-analysis was performed across the cohorts of the MetGen Consortium. Nine candidate polymorphisms in five transporter genes (OCT1, OCT2, MATE1, MATE2-K, and OCTN1) were analysed in almost 8,000 individuals. None of the variants showed a significant effect on metformin response in the primary analysis, or in the exploratory secondary analyses.

METHODOLOGICAL CONSIDERATIONS AND CRITICAL REMARKS

The HOME trial

The HOME trial included 390 patients aged 30-80 years with type 2 diabetes who were receiving treatment with insulin. The trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, the Netherlands). Patients were randomly allocated to either placebo or metformin (in identically looking boxes). Either metformin 850 mg or placebo (one to three times daily) was added to insulin therapy. No other glucose-lowering drugs were used, allowing comparison of metformin with placebo in patients receiving insulin but no other antidiabetic agents. The trial consisted of a 12 week pre-randomisation phase, in which patients were treated with insulin only and concomitant medication was discontinued and a 4.3 year long-term treatment phase, at the beginning of which patients were randomised to receive either metformin or placebo in addition to insulin therapy. Patients were recruited between November 1998 and July 1999; follow-up ended in 2003.

Collection of data and laboratory investigations

Polyneuropathy was evaluated every three months by two well-trained medical doctors. Dietary intake was assessed according to the dietary history method by four trained registered dietitians. Serum levels of MMA and vitamin D were analysed in Maastricht by the laboratory of prof.dr. Schalkwijk. Genotyping was done by prof.dr. van Schaik's laboratory at the Erasmus University Rotterdam. DNA samples were collected at baseline on IsoCode Stix and stored at basement climate. Genotyping was done using Taqman allelic discrimi-

nation assays. Dr. Matthijs Becker performed the measurement of the metformin plasma concentrations at the Erasmus University.

Statistical analysis

All analyses were post-hoc analyses. The mixed linear model analyses of chapter 2 and 3 were carried out by prof.dr. Leheret of the Louvain Academy in Mons, Belgium. The mixed linear model analyses of chapter 4 were performed by dr. Wim Krijnen of the Hanze University of Applied Sciences in Groningen. All other analyses of the HOME study were performed by myself under supervision from prof.dr. Leheret.

All chapters were written under supervision from dr. Adriaan Kooy and prof.dr. Coen Stehouwer. Chapter 3 was written in co-operation with Michel Top of Treant Zorggroep in Hoogeveen, chapter 4 was written in co-operation with Ida Miedema of the Hanze University of Applied Sciences in Groningen.

Strengths and limitations

Strengths of the HOME study include the randomised, placebo-controlled, double-blind design and its relatively long period of follow-up (4.3 years), with both short-term and long-term outcomes available, as well as the frequent serum collection and frequent testing for neuropathy. Patients in the HOME trial used no other glucose-lowering medication than metformin and insulin. For the pharmacogenetic analyses, it was possible not only to analyse the effects of metformin on glycaemic control, but also on another important effect: the reduction in insulin requirements. Furthermore, the HOME trial was held in a period in which vitamin D supplementation was uncommon, which was an advantage for the analysis of the association between the use of metformin and vitamin D levels.

However, there are some limitations. First, the power analysis of the HOME trial was primarily based on the aggregation of mortality and macrovascular and microvascular endpoints. However, this does not invalidate the current findings. Second, we studied middle-aged, Caucasian, insulin-treated individuals with a long duration of diabetes at baseline. It is not clear whether our findings can be generalised to non-Caucasians, to individuals not treated with insulin or with pre-diabetes or recently diagnosed diabetes. Third, the patients received regular dietary advice. The risk of B12 deficiency or vitamin D deficiency may be higher in older individuals and those with poor dietary habits. The analysis of the effect of metformin on MMA had additional limitations. First, for the evaluation of neuropathy, we

did not perform electrophysiological examinations, but instead used a clinically validated semiquantitative neuropathy score. Second, we did not assess other B12-related potential outcomes, notably macrocytic anaemia and cognition. A limitation for the pharmacogenetic analysis was that our selection of SNPs may only explain part of the variation. However, analysing all the variations would ultimately lead to sequencing the whole genome without knowing the relevance of the new SNPs.

The MetGen analyses

The MetGen Consortium consists of research groups from Europe and the United States with available data for studies of metformin pharmacogenetics from population-based observational studies and clinical trials. Metformin response was defined as a reduction in HbA1c during treatment with metformin.

Inclusion

Inclusion criteria were continuous treatment with metformin for at least 3 months, pre-treatment HbA1c measured within 6 months prior to metformin therapy and <14%, and treatment with metformin monotherapy or metformin as an add-on therapy to another oral agent. Hence, patients from the HOME study, in which metformin was added to insulin therapy, were not included in the meta-analyses, and results are shown separately. For the screening phase and first-stage replication of the GWAS, patients from the GoDARTS were used. The second-stage replication consisted of participants from the UKPDS study. The final replication and meta-analysis was conducted within the MetGen Consortium.

Statistical analysis

In each cohort, the effects of individual SNPs on metformin response were assessed in an additive genetic model using linear regression with reduction of HbA1c as outcome (primary analysis). The pre-treatment HbA1c, metformin daily dose, adherence, estimated glomerular filtration rate (eGFR), baseline gap (time between pre-treatment HbA1c measurement and start of metformin therapy), and treatment group (metformin prescribed as monotherapy or dual therapy), were added as covariates. In the HOME study, analyses were adjusted for pre-treatment HbA1c, metformin daily dose and eGFR. The evaluation of rs8192675 genotype by BMI group interaction was performed with linear regression by adjusting for treatment group, sex and study cohort. For the linear regression analyses, all representatives

performed the statistical analyses for their cohorts. The meta-analysis for the GWAS was performed by dr. Kaixin Zhou from the University of Dundee and dr. Sook Wah Yee from the University of California, San Francisco. The meta-analysis of the variants in pharmacokinetic transporters was performed by dr. Tanja Dujic from the University of Sarajevo.

Strengths and limitations

Strengths of the MetGen analyses are the large sample size and the ethnic diversity. First, despite the large sample size, statistical power remains limited to detect differences in HbA1c reduction smaller than 0.06–0.14%. Second, the MetGen cohorts include population-based observational studies, which could be confounded by a number of factors, such as patient adherence or frequency of HbA1c measurements in the cohort. Third, in several cohorts patients used additional oral glucose-lowering agents. Finally, results from the HOME study were shown separately, as studies in which metformin was added to insulin therapy were not included in the meta-analyses.

SUMMARY, PRACTICAL IMPLICATIONS AND FUTURE DIRECTIONS

Summary

In the first part this thesis, we describe three studies about possible side-effects of metformin. First, we show that long-term treatment with metformin, as compared to placebo, is associated with an increase of serum MMA in patients with type 2 diabetes treated with insulin. This effect of metformin increases over time and is dependent on the cumulative dose of metformin during treatment. The results are in line with earlier findings from the HOME study showing a progressive decrease of serum levels of B12 in patients treated with metformin.⁹ Furthermore, the increase in serum MMA by the use of metformin was associated with a small increase of a validated clinical neuropathy score. Taken together, these data suggest that metformin-associated decreases in B12 can, over time, lead to tissue damage. Second, analysis showed that metformin is not associated with a decrease of serum vitamin D levels. Third, we showed that a favourable side-effect of metformin, preventing weight gain, cannot be explained by reduced food intake, as we found that metformin does not affect caloric intake. However, approximately one third of the difference in weight gain between metformin and placebo recipients can be explained by the reduction of insulin requirements by use of metformin.

In the second part of the thesis, we describe three studies of the influence of pharmacogenetic variation on the effects of metformin. The most interesting finding of the first pharmacogenetic study was not that we replicated an association between the minor allele of the polymorphism rs11212617 near the ATM locus and improved treatment outcome, but that this minor allele was also associated with lower metformin plasma concentrations. An explanation could be that rs11212617 might increase the intracellular uptake of metformin thereby increasing its efficacy. The target gene of rs11212617 is unknown yet. The ATM gene is suggested as the most likely candidate, as one of the upstream targets of ATM is known to be involved in the actions of metformin¹⁸. However, the finding that the minor allele was associated with lower plasma concentrations, makes it less likely that ATM is the target gene, as ATM is not known to be involved in the pharmacokinetics of metformin. Therefore, our findings might support the theory that rs11212617 influences the expression of transporters involved in the cellular uptake of metformin in the liver or the gut. In addition, we found associations between the MATE1 polymorphism rs2289669 and the response to metformin. However, the daily dose of metformin was the best predictor of treatment outcome. The second and third pharmacogenetic studies are papers from the MetGen Consortium. A three-stage GWAS with patients from the MetGen cohorts found that the C allele of rs8192675 in the intron of *SLC2A2*, which encodes the facilitated glucose transporter GLUT2, was associated with improved response to metformin in type 2 diabetes, especially in obese patients. Finally, a meta-analysis across the cohorts of the MetGen Consortium found that none of the nine analysed candidate transporter gene variants had a significant effect on metformin response.

Practical implications

International guidelines do recognise the risk of B12 deficiency as a disadvantage of metformin.³ However, they just advise to consider periodic measurement of B12 levels, without making strong recommendations. The most recent version of the Dutch guidelines for the treatment of type 2 diabetes does not recommend screening for B12 deficiency in metformin users.¹⁹ A revision of the guidelines, which has recently been released in concept, has mostly focused on adjustment of advice on combination therapy for type 2 diabetes.²⁰ However, recommendations about side-effects of metformin will remain unchanged until the next revision (personal communication by email with the Dutch guideline committee, December 18, 2017). However, our findings are an important indication that metformin-related B12 deficiency is clinically relevant. Monitoring of B12 in long-term users of metformin should

Figure 2. Flowchart for screening for B12 deficiency in metformin users
* As there is still no consensus about cut-off values for B12 and MMA, it is also optional to use local cut-off values. **Especially in patients with neuropathy or anaemia.

be considered, especially in patients with neuropathy or macrocytic anaemia. I propose a flowchart for screening for B12 deficiency in metformin users (figure 2), and advise to measure B12 levels one year after the start of metformin use. In addition, I advise to consider testing for MMA when B12 levels are in the intermediate range. Depending on the outcome, one can decide to start with B12 supplementation, or to repeat measurement of B12 after one or five years. I have included a proposal for cut-off values of B12 and MMA levels.^{21–23} However, as there is still no consensus about cut-off values for B12 and MMA, it is also optional to use local cut-off values.^{21–23} Ideally, this would require further research.

Future directions

Sixty years after its first clinical use, there is still much to discover about metformin. Nowadays, metformin is well established as the initial choice for oral treatment of type 2 diabetes. However, beyond comparisons with sulfonylureas and insulin, there are no long-term data comparing metformin as first-line treatment with newer glucose-lowering agents like SGLT2 inhibitors and GLP-1 receptor agonists. In addition, there remains uncertainty about

whether metformin really does reduce the risk of cardiovascular disease among patients with type 2 diabetes, mainly due to a lack of RCTs. As metformin is off-patent and as preferred initial treatment ‘ethically almost inevitable’ in the treatment of type 2 diabetes, 3 new large placebo-controlled trials with metformin are difficult to organise. However, there are several upcoming trials for individuals without type 2 diabetes. The Bethesda Diabetes Research Center, with dr. Adriaan Kooy as principle investigator, is about to start with the *Pregnancy Outcomes: Effects of Metformin* (POEM) study, a three-phase randomised controlled multi-centre trial with metformin in gestational diabetes. In addition, various in vitro and in vivo studies are ongoing, exploring potential effects of metformin on cancer prevention and treatment.¹¹ Furthermore, three large RCTs on the effects of metformin on ageing are about to start with recruitment.¹² It will be interesting to see whether these trials will lead to new and broader indications for the use of metformin. Furthermore, time will tell whether metformin will remain the first-line treatment, or whether the newer classes of glucose-lowering agents will take over the place of metformin as the number one choice for the treatment of type 2 diabetes. Finally, it will be fascinating to find out what direction the research in pharmacogenetics will take. Important findings, as described in this thesis, about the influence of variation in the encoding gene of the facilitated glucose transporter GLUT2, suggest that in the future stratified medicine based on genetic profiles may be a real possibility. Worldwide cooperations like the MetGen Consortium are needed to find and replicate new pharmacogenetic associations. These possibilities require further study, perseverance and perhaps most important of all, imagination.

REFERENCES

1. Sterne J. Du nouveau dans les antidiabétiques. La NN diméthylamine guanyl guanidine (N.N.D.G.). Maroc Med. 1957;36:1295-6.
2. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 1998;352(9131):854-65.
3. American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2018. Diabetes Care. 2018;41(Suppl 1):S73-85.
4. Wiersma TJ, Heine RJ, Rutten GE. Summary of the practice guideline ‘Diabetes mellitus type 2’ (first revision) of the Dutch College of General Practitioners. Ned Tijdschr Geneesk. 1999;143(33):1688-91.
5. Bouma M, Rutten GE, de Grauw WJ, Wiersma T, Goudswaard AN, Nederlands Huisartsen Genootschap. Summary of the practice guideline ‘Diabetes mellitus type 2’ (second revision) from the Dutch College of General Practitioners. Ned Tijdschr Geneesk. 2006;150(41):2251-6.
6. Wulfele MG. Effects of Metformin in Patients with Type 2 Diabetes Intensively Treated with Insulin [dissertation]. 2004.
7. de Jager J. The Effects of Metformin on Metabolism and Cardiovascular Disease in Type 2 Diabetes [dissertation]. 2010.
8. Griffin SJ, Leaver JK, Irving GJ. Impact of metformin on cardiovascular disease: a meta-analysis of randomised trials among people with type 2 diabetes. Diabetologia. 2017;60(9):1620-9.
9. de Jager J, Kooy A, Leher P, et al. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. BMJ. 2010;340:c2181.
10. McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. Diabetologia. 2016;59(3):426-35.
11. Heckman-Stoddard BM, DeCensi A, Sahasrabudhe VV, Ford LG. Repurposing metformin for the prevention of cancer and cancer recurrence. Diabetologia. 2017;60(9):1639-47.
12. Valencia WM, Palacio A, Tamariz L, Florez H. Metformin and ageing: improving ageing outcomes beyond glycaemic control. Diabetologia. 2017;60(9):1630-8.
13. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia. 2017;60(9):1577-85.
14. Zhou K, Donnelly L, Yang J, et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. Lancet Diabetes Endocrinol. 2014;2(6):481-7.
15. Pawlyk AC, Giacomini KM, McKeon C, Shuldiner AR, Florez JC. Metformin pharmacogenomics: current status and future directions. Diabetes. 2014;63(8):2590-9.
16. Out M, Becker ML, van Schaik RH, et al. SNPs Related to the Glucose Lowering Effect of Metformin: Do they Affect Plasma Concentrations of Metformin (MPC) and their Effects on Insulin Requirements (DDI)? (Abstract). Diabetologia. 2013;56 Suppl 1:388.
17. The Metformin Genetics Consortium; available at: <http://www.pgrn.org/metgen.html>.
18. GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group, Wellcome Trust Case Control Consortium 2, Zhou K, et al. Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. Nat Genet. 2011;43(2):117-20.
19. Rutten GEHM, De Grauw WJC, Nijpels G, et al. NHG-Standaard Diabetes mellitus type 2 (derde herziening). Huisarts Wet. 2013;56:515.
20. Diabetes mellitus type 2 in de tweede lijn (concept). Geïnitieerd door de Nederlandse

Internisten Vereniging, in samenwerking met Diabetesvereniging Nederland. 2018;.

21. Bailey RL, Carmel R, Green R, et al. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B-12. *Am J Clin Nutr.* 2011;94(2):552-61.
22. Bailey RL, Durazo-Arvizu RA, Carmel R, et al. Modeling a methylmalonic acid-derived change point for serum vitamin B-12 for adults in NHANES. *Am J Clin Nutr.* 2013;98(2):460-7.
23. van Wijngaarden JP, Dhonukshe-Rutten RAM, Brouwer-Brolsma EM, et al. Vitamin B12 Intake and Related Biomarkers: Associations in a Dutch Elderly Population. *J Nutr Health Aging.* 2017;21(10):1268-76.

Chapter 9.

Samenvatting

Valorisatie

Affiliaties coauteurs en beoordelingscommissie

Dankwoord

Curriculum vitae

Lijst met publicaties

Samenvatting

ACHTERGROND EN HISTORISCH PERSPECTIEF

Zestig jaar nadat metformine voor het eerst werd voorgeschreven voor diabetes¹ en twintig jaar na de publicatie van de resultaten van de United Kingdom Prospective Diabetes Study (UKPDS)² is metformine de eerste keus voor medicamenteuze behandeling voor patiënten met diabetes mellitus type 2.³ Metformine wordt jaarlijks meer dan honderd miljoen keer voorgeschreven. Zelfs na zestig jaar is er nog veel te ontdekken aan de effecten van metformine en de manier waarop metformine werkt. Het aantal jaarlijkse wetenschappelijke publicaties over metformine neemt nog altijd toe, tot bijna tweeduizend in 2017 (figuur 1).

De HOME studie

Het studieprotocol voor de Hyperinsulinemia: the Outcome of its Metabolic Effects (HOME) studie werd eind jaren negentig geschreven. Destijds golden sulfonyleureumderivaten nog als de eerste keus bij behandeling van diabetes mellitus type 2. In Nederland werd metformine in 1999 voor het eerst opgenomen als voorkeursbehandeling in de richtlijnen, maar alleen voor patiënten met een body mass index boven de 27 kg/m².⁴ Pas in 2006 werd metformine in een herziening van de Nederlandse richtlijnen genoemd als eerste keuze voor medicamenteuze behandeling bij alle patiënten met diabetes mellitus type 2.⁵ Het primaire doel van de HOME studie was om te onderzoeken of metformine beschermt tegen microvasculaire of macrovasculaire schade bij diabetes mellitus type 2. Dit is het derde proefschrift dat gebaseerd is op de resultaten van de HOME studie, met een vierde op komst. In 2004 publiceerde Michiel Wulffelé de kortetermijnresultaten van metformine op glucoseregulatie, insulinebehoefte en vitamine B12.⁶ In 2010 publiceerde Jolien de Jager haar proefschrift, waarin ze de langetermijneffecten van metformine beschreef op glucoseregulatie, microvasculaire en macrovasculaire eindpunten en vitamine B12-gehalte.⁷ De HOME studie is wereldwijd nog altijd de grootste lange termijn placebogecontroleerde studie met metformine bij diabetes mellitus type 2. Een recente meta-analyse noemde de studie als het onderzoek met het laagste risico op bias van de geïncludeerde metformine onderzoeken.⁸ Bovendien was de HOME studie het eerste gerandomiseerde langetermijnonderzoek naar de effecten van metformine op vitamine B12.⁹

Figuur 1. Aantal jaarlijkse publicaties over metformine (bron: PubMed).

Het Metformin Genetics Consortium (MetGen)

Gedurende het laatste decennium is de aandacht van het onderzoek met metformine verschoven van glucoseregulatie naar andere onderwerpen zoals de effecten van metformine in de darm,¹⁰ de mogelijke rol van metformine bij preventie en behandeling van kanker¹¹ en zelfs veroudering.¹² Bovendien er is vooruitgang geboekt bij het ontrafelen van de werkingsmechanismen van metformine.¹³ Niettemin is de aanzienlijke variabiliteit in reactie op metformine nog steeds niet volledig begrepen. Genetische variatie speelt een belangrijke rol. Zo kan 34% van de variatie in HbA1c-reductie verklaard worden door genetische verschillen.¹⁴ De resultaten van studies naar polymorfismen zijn echter teleurstellend, omdat verschillende onderzoeken eerder gevonden associaties niet konden bevestigen, mede doordat deze studies vaak beperkt werden door kleine aantallen patiënten en variëteit in etniciteit. Daarom werd in 2013 tijdens een internationale workshop over de farmacogenetica van metformine een voorstel gedaan om een “metformine-consortium” in het leven te roepen, bestaande uit meerdere onderzoeksgroepen met inclusie van studies met patiënten van verschillende afkomst.¹⁵ De eerste contacten werden gelegd tijdens een bijeenkomst op het

jaarlijkse congres van de European Association for the Study of Diabetes (EASD) in 2013 in Barcelona, direct na een posterpresentatie van de resultaten van de farmacogenetische analyse van de HOME-studie.¹⁶ Een maand later werd het MetGen Consortium opgericht. MetGen wordt geleid door prof.dr. Ewan Pearson van de universiteit van Dundee en prof. dr. Kathy Giacomini van de universiteit van Californië, San Francisco. Op het moment van schrijven bestaat het consortium uit een verzameling van talrijke cohorten met meer dan 10.000 personen, een aantal dat nog altijd toeneemt.¹⁷

Huidige bevindingen

Metformine verlaagt niet alleen het vitamine B12-gehalte, maar verhoogt ook de serumspiegels van methylmalonzuur (MMA) (HOME-studie)

Eerdere analyse van de HOME studie toonde aan dat metformine geassocieerd is met een verlaging van serum B12, progressief in de tijd en vergezeld gaand met een toename van het serum homocysteïne, wat suggestief is voor B12-tekort op weefselniveau. De huidige studie toonde aan dat metformine na 52 maanden niet alleen het serum-B12 verlaagde, maar ook het MMA verhoogde: metformine verhoogde het MMA aan het einde van de studie in vergelijking met placebo met een gemiddeld verschil van 0,039 $\mu\text{mol/l}$ (95% betrouwbaarheidsinterval 0,019 tot 0,055). Uitgedrukt in gramjaren metformine nam het MMA toe met 0,006 $\mu\text{mol/l}$ per gram jaar (summary mean; 95% betrouwbaarheidsinterval 0,003 tot 0,009, in vergelijking met placebo).

De toename van MMA bij metforminegebruikers gaat gepaard met een significante verslechtering van een gevalideerde score voor klinische neuropathie (HOME-studie)

Gedurende 4,3 jaar leidde metformine in vergelijking met placebo niet tot meer neuropathie. Echter, mediation-analyse toonde aan dat dit nuleffect van metformine op de neuropathie kan worden verklaard door een gunstig effect op neuropathie door verlaging van het HbA1c (een afname van de neuropathiescore van $0,04 \times 0,50 = 0,020$ per metformine gram jaar) en een nadelig effect door het verhogen van MMA (een toename van de neuropathiescore van $0,04 \times 1,06 = 0,042$ per metformine gramjaar). Dit betekent dat de schadelijke effecten van een dalend B12-gehalte wordt geneutraliseerd door de beschermende effecten van metformine door verbetering van de glucoseregulatie.

Metformine heeft geen effect op vitamine D-spiegels (HOME-onderzoek)

Metformine had in vergelijking met placebo geen effect op de 25(OH)D-spiegels, gecorrigeerd voor seizoenseffecten gedurende 16 maanden (coëfficiënt: 1,002 per maand, multiplicatief model, 95% betrouwbaarheidsinterval: 0,998 tot 1,006). Metformine was geassocieerd met een kleine toename van 25(OH)D₂ (coëfficiënt: 1,012 per maand, 95% betrouwbaarheidsinterval 1,003 tot 1,021). 25(OH)D₂ besloeg echter slechts een zeer kleine fractie (3%) van het totaal 25(OH)D. Seizoensvariatie had de grootste impact op 25(OH)D-spiegels.

Het gebruik van metformine vermindert de energie-inname niet (HOME-studie)

In de HOME-studie werd de voedingsinname beoordeeld bij aanvang van de studie, na 1 jaar en na 4,3 jaar volgens de diet history methode. Analyse toonde aan dat metformine de energie-inname niet verminderde ($-31,0$ kcal/d; 95% betrouwbaarheidsinterval $-107,4$ tot $45,4$). Bovendien toonden lineair mixed model analyses geen significant effect van energie-inname als verklaring voor het verschil in gewichtstoename tussen de groepen. Daarom kan het voorkomen van gewichtstoename door metformine niet worden verklaard door een vermindering van de energie-inname.

Ongeveer een derde van het verschil in gewichtstoename tussen metformine en placebo-ontvangers kan worden verklaard door de vermindering van de insulinebehoefte door gebruik van metformine (HOME-studie)

Mediation-analyse toonde aan dat aan het einde van het onderzoek een patiënt die de actuele gemiddelde dosis metformine (2050 mg) ontving $3,2 \pm 0,5$ kg minder gewichtstoename had, in vergelijking met placebo, waarvan ongeveer een derde ($1,2 \pm 0,0$ kg) kan worden verklaard door een verlaging van de insuline-inname door het gebruik van metformine.

De ATM SNP rs11212617 heeft niet alleen invloed op het effect van metformine op HbA1c en insulinebehoefte, maar ook op metformine plasmaconcentraties (HOME-studie)

We analyseerden de invloed van de polymorfismen rs12208357 en rs622342 in het gen dat codeert voor organic cation transporter 1 (OCT1), rs2289669 in het gen dat codeert voor multidrug and toxin extrusion transporter 1 (MATE1) en rs11212617 in het ataxia telangiectasia mutated gene (ATM), op de effecten van metformine op HbA1c en insulinebehoefte (dagelijkse dosis insuline, DDI). Uitkomstmaat was een gecombineerde HbA1c + DDI Z-score. Het minor allel van rs11212617 (ATM) was geassocieerd met een verbeterde Z-score (een afname per

allel van 0,11, 95% betrouwbaarheidsinterval 0,01 tot 0,20) en een lagere plasmaconcentratie van metformine (−0,42 mg/ml per minor allel, 95% betrouwbaarheidsinterval: −0,81 tot −0,03). Het major allel van rs2289669 (MATE1) was ook geassocieerd met een verbeterde Z-score (−0,35, 95% betrouwbaarheidsinterval −0,09 tot −0,60). De plasmaconcentratie van metformine was ook geassocieerd met een verlaging van de Z-score. De dagelijkse dosis metformine was echter de beste voorspeller voor de respons op metformine.

Het C-allel van rs8192675 in het intron van SLC2A2, dat codeert voor de glucosetransporter GLUT2, is geassocieerd met het glucoseverlagende effect van metformine bij diabetes mellitus type 2, vooral bij obese personen (MetGen-analyse)

Een drie-fasen genomewide associatiestudie (GWAS) met meer dan 13.000 deelnemers toonde aan dat het C-allel van rs8192675 in het intron van *SLC2A2*, coderend voor de glucosetransporter GLUT2, was geassocieerd met een 0,17% sterkere verlaging van het HbA1c door metformine. Bij obese personen met twee C-allelen was daalde het HbA1c met gemiddeld 0,33% meer dan bij personen met homozygotie voor het T-allel.

Variatie van transportergenen draagt weinig bij aan de variabiliteit in glycemische respons op metformine bij diabetes mellitus type 2 (MetGen-analyse)

Een meta-analyse werd uitgevoerd in de cohorten van het MetGen Consortium. Negen kandidaat-polymorfismen in vijf transportergenen (OCT1, OCT2, MATE1, MATE2-K en OCTN1) werden in bijna 8000 individuen geanalyseerd. Geen van de varianten toonde een significant effect op de respons van metformine in de primaire analyse of in de oriënterende secundaire analyses.

SAMENVATTING, PRAKTISCHE IMPLICATIES EN TOEKOMSTPERSPECTIEF

Samenvatting

In het eerste deel van dit proefschrift beschrijven we drie studies over mogelijke bijwerkingen van metformine. Ten eerste tonen we aan dat langdurige behandeling met metformine, in vergelijking met placebo, geassocieerd is met een toename van serum-MMA bij patiënten met diabetes mellitus type 2 behandeld met insuline. Dit effect van metformine neemt in de loop van de tijd toe en is afhankelijk van de cumulatieve dosis metformine. De resultaten zijn in lijn met eerdere bevindingen uit de HOME-studie die een progressieve afname van de

serumconcentraties van B12 bij patiënten behandeld met metformine aantonen. Bovendien was de toename in serum-MMA door het gebruik van metformine geassocieerd met een kleine toename van een gevalideerde klinische neuropathie score. Deze uitkomsten suggereren dat de afname van B12 door metformine na verloop van tijd kan leiden tot weefselschade. Ten tweede toonden we aan dat metformine niet geassocieerd is met een verlaging van serum vitamine D-spiegels. Ten derde hebben we laten zien dat een gunstig neveneffect van metformine, het voorkomen van gewichtstoename, niet kan worden verklaard door verminderde voedselinname, aangezien onze analyse toonde dat metformine de calorie-inname niet beïnvloedt. Wel kan ongeveer een derde van het verschil in gewichtstoename tussen metformine en placebo-ontvangers verklaard worden door de vermindering van de insulinebehoefte bij gebruik van metformine.

In het tweede deel van het proefschrift beschrijven we drie studies naar de invloed van farmacogenetische variatie op de effecten van metformine. In de eerste farmacogenetische analyse repliceerden we een associatie tussen het minor allel van het ATM-polymorfisme rs11212617 en het effect van metformine. De meest interessante bevinding was dat dit minor allel niet alleen geassocieerd was met een verbeterde behandeluitkomst, maar ook met lagere plasmaconcentraties van metformine. Een verklaring zou kunnen zijn dat rs11212617 de intracellulaire opname van metformine verhoogt, waardoor de werkzaamheid toeneemt. Het target-gen van rs11212617 is nog niet bekend. Het ATM-gen wordt gesuggereerd als de meest waarschijnlijke kandidaat, omdat ATM mogelijk betrokken is bij de acties van metformine.¹⁸ De bevinding dat het minor allel geassocieerd was met lagere plasmaconcentraties, maakt het echter minder waarschijnlijk dat ATM het target-gen is, omdat van ATM niet bekend is dat het betrokken is bij de farmacokinetiek van metformine. Daarom zouden onze bevindingen de theorie kunnen ondersteunen dat rs11212617 de expressie beïnvloedt van transporters die betrokken zijn bij de cellulaire opname van metformine in de lever of de darm. Daarnaast vonden we associaties tussen het MATE1-polymorfisme rs2289669 en de respons op metformine. De dagelijkse dosis metformine was echter de beste voorspeller van het behandelresultaat. De tweede en derde farmacogenetische studies zijn publicaties van het MetGen Consortium. Een GWAS met patiënten uit de MetGen-cohorten vond dat het C-allel van rs8192675 in het intron van *SLC2A2*, coderend voor de glucosetransporter GLUT2, was geassocieerd met een verbeterde respons op metformine, in het bijzonder bij obese patiënten. Ten slotte vond een meta-analyse van het MetGen Consortium bij geen van de negen geanalyseerde transportergenvarianten een significant effect op de respons van metformine.

Praktische implicaties

Internationale richtlijnen erkennen het risico op B12-deficiëntie als een nadeel van metformine.³ Ze adviseren echter om te overwegen B12-waarden te meten, zonder krachtige aanbevelingen te doen. De meest recente versie van de Nederlandse richtlijnen voor de behandeling van diabetes type 2 beveelt screening op B12-deficiëntie tekort bij metforminegebruikers niet aan.¹⁹ De herziening van de richtlijn die inmiddels in conceptvorm verschenen is heeft vooral de adviezen over combinatietherapie aangepast.²⁰ Echter, we hebben begrepen dat de rubriek met aanbevelingen over bijwerkingen van metformine ongewijzigd zal blijven tot de volgende herziening (persoonlijke communicatie per e-mail met de Nederlandse richtlijncommissie, 18 december 2017). Onze bevindingen zijn echter een belangrijke aanwijzing dat metformine-gerelateerde B12-deficiëntie klinisch relevant is. Monitoring van B12 bij langdurig gebruik van metformine moet worden overwogen, vooral bij patiënten met neuropathie of macrocytaire anemie. Ik stel een stroomdiagram voor om te screenen op B12-tekort bij gebruikers van metformine (figuur 2) en adviseer om de B12-waarden één jaar na het begin van het gebruik van metformine te meten. Daarnaast adviseer ik om het MMA te testen wanneer B12-waarden alleen onvoldoende duidelijkheid geven. Afhankelijk van de uitkomst kan men besluiten om te beginnen met B12-suppletie of om de B12-meting na één of vijf jaar te herhalen. Ik heb een voorstel opgenomen voor afkapwaarden van B12- en MMA-niveaus.^{21–23} Aangezien er echter nog geen consensus is over afkapwaarden voor B12 en MMA is het ook optioneel om lokale afkapwaarden te gebruiken.^{21–23} Idealiter vergt dit voorstel nader onderzoek ter bevestiging.

Toekomstperspectief

Zestig jaar na het eerste klinische gebruik is er nog veel te ontdekken over metformine. Tegenwoordig is metformine goed ingeslepen als de eerste keuze voor orale behandeling van type 2 diabetes. Afgezien van vergelijkingen met sulfonyleureumderivaten en insuline zijn er geen lange-termijn data die metformine als eerste keus behandeling vergelijken met nieuwere glucoseverlagende middelen zoals SGLT2-remmers en GLP-1 receptoragonisten. Daarnaast blijft er onzekerheid bestaan over de vraag of metformine het risico op cardiovasculaire aandoeningen bij patiënten met diabetes type 2 vermindert, vooral als gevolg van een gebrek aan gerandomiseerde langetermijnonderzoeken. Aangezien metformine uit patent is en als voorkeursbehandeling ‘ethisch bijna onvermijdelijk’ bij de behandeling van diabetes type 2,³ zijn nieuwe grote placebogecontroleerde onderzoeken met metformine moeilijk uit

Figuur 2. Stroomdiagram voor screening op B12-deficiëntie bij metforminegebruikers
* Aangezien er nog geen consensus is over afkapwaarden voor B12 en MMA, is het ook optioneel om lokale afkapwaarden te gebruiken. ** Vooral bij patiënten met neuropathie of anemie.

te voeren. Er zijn echter verschillende studies op komst voor mensen zonder diabetes type 2. Het Bethesda Diabetes Research Center met dr. Adriaan Kooy als hoofdonderzoeker staat op het punt te beginnen met de *Pregnancy Outcomes: Effects of Metformin* (POEM) studie, een multicenter gerandomiseerde driefase studie met metformine in zwangerschapsdiabetes. Daarnaast lopen er diverse in vitro en in vivo studies naar de mogelijke effecten van metformine op voorkomen en behandelen van kanker.¹¹ Bovendien staan drie grote studies naar effecten van metformine op veroudering op het punt om te beginnen met inclusie.¹² Het zal interessant zijn om te zien of deze onderzoeken zullen leiden tot nieuwe indicaties voor het gebruik van metformine. De tijd zal leren of metformine de eerstekeusbehandeling zal blijven of dat de nieuwe glucoseverlagende middelen de plaats van metformine bij behandeling van type 2 diabetes over zullen nemen. Ten slotte zal het fascinerend zijn om te volgen welke richting het onderzoek in de farmacogenetica zal uitgaan. Bevindingen zoals beschreven in dit proefschrift over de invloed van genetische variatie op effecten van medicatie suggereren dat in de toekomst gestratificeerde geneeskunde op basis van genetische profielen een reële mogelijkheid kan zijn. Wereldwijde samenwerkingsverbanden

zoals het MetGen Consortium zijn nodig om nieuwe farmacogenetische associaties te vinden en te repliceren. Deze mogelijkheden vereisen verdere studie, doorzettingsvermogen en misschien wel het belangrijkste, verbeeldingskracht.

REFERENCES

1. Sterne J. Du nouveau dans les antidiabétiques. La NN dimethylamine guanyl guanidine (N.N.D.G.). Maroc Med. 1957;36:1295-6.
2. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 1998;352(9131):854-65.
3. American Diabetes Association. 8. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2018. Diabetes Care. 2018;41(Suppl 1):S73-85.
4. Wiersma TJ, Heine RJ, Rutten GE. Summary of the practice guideline 'Diabetes mellitus type 2' (first revision) of the Dutch College of General Practitioners. Ned Tijdschr Geneesk. 1999;143(33):1688-91.
5. Bouma M, Rutten GE, de Grauw WJ, Wiersma T, Goudswaard AN, Nederlands Huisartsen Genootschap. Summary of the practice guideline 'Diabetes mellitus type 2' (second revision) from the Dutch College of General Practitioners. Ned Tijdschr Geneesk. 2006;150(41):2251-6.
6. Wulfele MG. Effects of Metformin in Patients with Type 2 Diabetes Intensively Treated with Insulin [dissertation]. 2004.
7. de Jager J. The Effects of Metformin on Metabolism and Cardiovascular Disease in Type 2 Diabetes [dissertation]. 2010.
8. Griffin SJ, Leaver JK, Irving GJ. Impact of metformin on cardiovascular disease: a meta-analysis of randomised trials among people with type 2 diabetes. Diabetologia. 2017;60(9):1620-9.
9. de Jager J, Kooy A, Lehert P, et al. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. BMJ. 2010;340:c2181.
10. McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. Diabetologia. 2016;59(3):426-35.
11. Heckman-Stoddard BM, DeCensi A, Sahasrabudhe VV, Ford LG. Repurposing metformin for the prevention of cancer and cancer recurrence. Diabetologia. 2017;60(9):1639-47.
12. Valencia WM, Palacio A, Tamariz L, Florez H. Metformin and ageing: improving ageing outcomes beyond glycaemic control. Diabetologia. 2017;60(9):1630-8.
13. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia. 2017;60(9):1577-85.
14. Zhou K, Donnelly L, Yang J, et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. Lancet Diabetes Endocrinol. 2014;2(6):481-7.
15. Pawlyk AC, Giacomini KM, McKeon C, Shuldiner AR, Florez JC. Metformin pharmacogenomics: current status and future directions. Diabetes. 2014;63(8):2590-9.
16. Out M, Becker ML, van Schaik RH, et al. SNPs Related to the Glucose Lowering Effect of Metformin: Do they Affect Plasma Concentrations of Metformin (MPC) and their Effects on Insulin Requirements (DDI)? (Abstract). Diabetologia. 2013;56 Suppl 1:388.
17. The Metformin Genetics Consortium; available at: <http://www.pgrn.org/metgen.html>.
18. GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group, Wellcome Trust Case Control Consortium 2, Zhou K, et al. Common variants near ATM are associated with glycaemic response to metformin in type 2 diabetes. Nat Genet. 2011;43(2):117-20.
19. Rutten GEHM, De Grauw WJC, Nijpels G, et al. NHG-Standaard Diabetes mellitus type 2((derde herziening)). Huisarts Wet. 2013;56:515.
20. Diabetes mellitus type 2 in de tweede lijn (concept). Geïnitieerd door de Nederlandse Internisten Vereniging, in samenwerking met Diabetesvereniging Nederland. 2018;.
21. Bailey RL, Carmel R, Green R, et al. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B-12. Am J Clin Nutr. 2011;94(2):552-61.
22. Bailey RL, Durazo-Arvizu RA, Carmel R, et al. Modeling a methylmalonic acid-derived change point for serum vitamin B-12 for adults in NHANES. Am J Clin Nutr. 2013;98(2):460-7.
23. van Wijngaarden JP, Dhonukshe-Rutten RAM, Brouwer-Brolsma EM, et al. Vitamin B12 Intake and Related Biomarkers: Associations in a Dutch Elderly Population. J Nutr Health Aging. 2017;21(10):1268-76.

Valorisatie

Metformine staat al jaren bekend om zijn gunstige effecten bij de behandeling van diabetes mellitus type 2. Bijwerkingen, vooral maag-darm klachten, zijn meestal mild en onschuldig. Wel wordt metformine al tientallen jaren gelinkt aan een tekort aan vitamine B12. Hoofdstuk 2 beschrijft niet alleen belangrijke aanwijzingen dat metformine ook op weefselniveau een tekort aan vitamine B12 veroorzaakt, maar dat dat ook gepaard gaat met een toename van zenuwschade. In dit proefschrift adviseer ik daarom om metforminegebruikers te screenen op vitamine B12-deficiëntie. Een voorstel hoe een dergelijke screening kan verlopen geef ik in de beschouwing (hoofdstuk 8). Een screening heeft echter ook nadelen, bijvoorbeeld de kosten die er aan verbonden zijn. De meest praktische en wellicht kosteneffectieve oplossing zou zijn om vitamine B12 toe te voegen aan metformine.

We hebben een serieuze poging gedaan om een dergelijk preparaat te laten ontwikkelen met een traject richting patentering vanuit het Bethesda Diabetes Research Center te Hoogeveen. Helaas stuiten we daarbij op een aantal beperkingen van de hedendaagse gezondheidszorg. Om de meerwaarde van een nieuw medicijn aan te tonen is een fase 3 onderzoek nodig. Fase 3 onderzoeken zijn vanzelfsprekend belangrijk om ervoor te zorgen dat alleen effectieve en veilige nieuwe medicijnen op de markt verschijnen. De vraag is of de eis om fase 3 onderzoek uit te voeren bij het ontwikkelen van een “Metformine Plus” tablet zijn doel niet voorbij schiet. Het uitvoeren van een dergelijk onderzoek is kostbaar. Patenten waarin de bereiding van een tablet met metformine en vitamine B12 beschreven wordt blijken bovendien gemakkelijk te omzeilen. Hierdoor is de kans om de ontwikkelkosten terug te verdienen te klein en het ontwikkelen van Metformine Plus daarmee onaantrekkelijk voor een fabrikant. Deze beperkingen maken een voor de hand liggende en kosteneffectieve oplossing helaas moeilijk haalbaar.

Hoofdstuk 3 beschrijft dat metformine geen invloed heeft op het vitamine D gehalte, waarmee screening op vitamine D-deficiëntie niet geïndiceerd is bij metforminegebruikers.

Eén van de gunstige effecten van metformine is de bescherming tegen gewichtstoename. Tot dusver werd gedacht dat deze bescherming verklaard kan worden doordat patiënten door het gebruik van metformine minder eetlust krijgen. Hoofdstuk 4 beschrijft echter dat patiënten in de metforminegroep niet minder gaan eten dan patiënten in de placebogroep en dat er dus een andere verklaring moet zijn. Nog altijd zijn niet alle mechanismen van metformine bekend. Wellicht kan verder onderzoek naar het beschermende effect van

metformine tegen gewichtstoename bijdragen in de ontwikkeling van nieuwe medicijnen in de moeilijke strijd tegen overgewicht.

De prevalentie van obesitas en morbide obesitas, en daarmee diabetes mellitus type 2, neemt nog altijd toe. De afgelopen decennia heeft de behandeling zich teveel geconcentreerd op negatieve voedingsadviezen (wat een patiënt dient te vermijden) en de medicamenteuze behandeling van diabetes mellitus type 2. Gelukkig komen er de laatste tijd steeds meer initiatieven die gericht zijn op voorkomen en/of genezen van diabetes mellitus type 2, zoals “Keer Diabetes Om”,¹ “Arts en Voeding”² en “Voeding Leeft”.³ Bij deze initiatieven wordt niet alleen aandacht besteed aan positieve voedingskeuzes, bewegingsadviezen en cognitieve gedragstherapie, maar wordt ook aangestuurd op zeer noodzakelijke maatschappelijke veranderingen.

Hoofdstuk 5 tot en met 7 beschrijven resultaten van analyses naar de invloed van genetische variatie op de effecten van metformine. Bij genetische studies wordt meestal gedacht aan grote aantallen patiënten. Hoofdstuk 5 toont aan dat een genetische studie ook in een gerandomiseerde placebogecontroleerde trial als de HOME studie toch heel interessante bevindingen kan opleveren, terwijl dit soort onderzoek doorgaans in grote populatiestudies plaatsvindt. Vanzelfsprekend geeft de samenwerking van het Metformine Genetics (MetGen) consortium een veel grotere power. Hoofdstuk 7 beschrijft dat het al dan niet hebben van een variant in het *SLC2A2* gen een verschil in behandeling kan geven ter grootte van het effect van 550 mg metformine of de helft van het effect van een DPP4-remmer. Mijn verwachting is dat in de diabeteszorg binnen tien jaar op grote schaal gebruik gemaakt zal gaan worden van genetische profielen, waarmee inschatting gemaakt kan worden of patiënten beter zullen reageren op metformine of bijvoorbeeld een GLP1-analoog en SGLT2-remmer. Hoewel dit misschien nog als toekomstmuziek of wellicht science fiction mag klinken, toonde recente berichtgeving aan dat in het Leids Universitair Medisch Centrum inmiddels de eerste stappen in de dagelijkse praktijk gezet worden.⁴

REFERENCES

1. Keer Diabetes Type 2 om; available at: <https://keerdiabetesom.nl/>. Accessed April/23, 2018.
2. Arts en Voeding; available at: <http://www.artsenvoeding.nl/>. Accessed April/23, 2018.
3. Voeding Leeft; available at: <https://voedingleeft.nl/>. Accessed April/23, 2018.
4. Leids ziekenhuis gaat dna-informatie van patiënt delen; available at: <https://www.volkskrant.nl/>.

nl/wetenschap/leids-ziekenhuis-gaat-dna-informatie-van-patient-delen-dit-kan-de-werking-van-medicijnen-verbeteren~a4557148/. Accessed January/16, 2018.

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Sinds mijn start als vasculair internist in het Bethesdaziekenhuis heeft mijn promotieonderzoek een belangrijke bijrol gespeeld niet alleen in mijn eigen leven, maar ook in het leven van de mensen om me heen. Graag wil ik dan ook van de gelegenheid gebruik maken om hen te bedanken.

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<https://stats.stackexchange.com/questions/423/what-is-your-favorite-data-analysis-cartoon>

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Curriculum vitae

CURRICULUM VITAE

Mattijs Out werd geboren op 10 april 1976 te Krimpen aan den IJssel. In 1994 behaalde hij het VWO diploma aan het Erasmiaans Gymnasium te Rotterdam. In datzelfde jaar begon hij met de studie Geneeskunde aan de Medische Faculteit van de Erasmus Universiteit te Rotterdam. In 1998 behaalde hij zijn doctoraalexamen Geneeskunde. Zijn afstudeeronderzoek "Preconditioning rat hearts by adenosine A1 or A3 receptor activation" verrichtte hij op de afdeling Cardiochemie van de Erasmus Universiteit. In afwachting van zijn coschapen heeft hij nog een half jaar doorgewerkt als student-assistent aan dit onderzoek. Na het afleggen van het Artsexamen begin 2001 heeft hij in afwachting van een reis van zes maanden door Canada en Zuidoost-Azië gedurende een half jaar gewerkt als bedrijfsarts bij de Arbo Unie West Nederland in Rotterdam. In maart 2002 begon hij als AGNIO op de afdeling Algemene Interne Geneeskunde van het Erasmus MC (destijds nog Dijkzigt Ziekenhuis geheten). In datzelfde ziekenhuis begon hij in 2003 de opleiding tot internist (opleiders prof.dr. H.A.P. Pols en prof.dr. J.C.L.M. van Saase). Vanaf 2005 vervolgde hij zijn opleiding in het Ikazia Ziekenhuis te Rotterdam (opleider dr. A. Dees). In 2007 maakte hij de overstap naar het Leids Universitair Medisch Centrum, waar hij als fellow opgeleid werd in de vasculaire geneeskunde (opleiders prof.dr. M.V. Huisman en prof.dr. J.A. Romijn). In 2009 behaalde hij zijn registratie als internist en vasculair geneeskundige, waarna hij op 1 februari 2009 met zijn gezin verhuisde naar Beilen om aan de slag te gaan als vasculair internist in het Bethesda Ziekenhuis. Via het Bethesda Diabetes Research Center startte hij daar met zijn promotieonderzoek. Op 1 juni 2018 maakte hij de overstap naar de afdeling Interne Geneeskunde van het Medisch Spectrum Twente te Enschede. Sinds 1 mei woont hij in Enschede met zijn vrouw Inge en hun kinderen Ingmar en Hannah.

Lijst met publicaties

PUBLICATIES BEHOREND BIJ DIT PROEFSCHRIFT:

1. **Out M**, Miedema I, Jager-Wittenaar HA, Van der Schans CP, Krijnen WP, Lehert P, Stehouwer CDA, Kooy A. Metformin-associated prevention of weight gain in insulin-treated type 2 diabetic patients cannot be explained by decreased energy intake: A post hoc analysis of a randomized placebo-controlled 4.3-year trial. *Diabetes Obes Metab.* 2018;20(1):219-23.
2. **Out M**, Kooy A, Lehert P, Schalkwijk CA, Stehouwer CDA. Long-term treatment with metformin in type 2 diabetes and methylmalonic acid: Post hoc analysis of a randomized controlled 4.3 year trial. *J Diabetes Complications.* 2018;32(2):171-8.
3. **Out M**, Top WMC, Lehert P, Schalkwijk CA, Stehouwer CDA, Kooy A. Long-term treatment with metformin in type 2 diabetes and vitamin D levels: A post hoc analysis of a randomised placebo-controlled trial. *Diabetes Obes Metab.* 2018 Apr 17. doi: 10.1111/dom.13327. [Epub ahead of print].
4. **Out M**, Becker ML, Van Schaik RH, Lehert P, Stehouwer CDA, Kooy A. A gene variant near ATM affects the response to metformin and metformin plasma levels - A post hoc analysis of an RCT. *Pharmacogenomics.* 2018 May 23. doi: 10.2217/pgs-2018-0010. [Epub ahead of print].
5. Zhou K, Yee SW, Seiser EL, van Leeuwen N, Tavendale R, Bennett AJ, Groves CJ, Coleman RL, van der Heijden AA, Beulens JW, de Keyser CE, Zaharenko L, Rotroff DM, **Out M**, Jablonski KA, Chen L, Javorsky M, Zidzik J, Levin AM, Williams LK, Dujic T, Semiz S, Kubo M, Chien HC, Maeda S, Witte JS, Wu L, Tkac I, Kooy A, van Schaik RH, Stehouwer CD, Logie L, MetGen Investigators, DPP Investigators, ACCORD Investigators, Sutherland C, Klovins J, Pirags V, Hofman A, Stricker BH, Motsinger-Reif AA, Wagner MJ, Innocenti F, Hart LM, Holman RR, McCarthy MI, Hedderson MM, Palmer CN, Florez JC, Giacomini KM, Pearson ER. Variation in the glucose transporter gene SLC2A2 is associated with glycemic response to metformin. *Nat Genet.* 2016;48(9):1055-9.
6. Dujic T, Zhou K, Yee SW, van Leeuwen N, de Keyser CE, Javorsky M, Goswami S, Zaharenko L, Hougaard Christensen MM, **Out M**, Tavendale R, Kubo M, Hedderson MM, van der Heijden AA, Klimcakova L, Pirags V, Kooy A, Brosen K, Klovins J, Semiz S, Tkac I, Stricker BH, Palmer C, 't Hart LM, Giacomini KM, Pearson ER. Variants in Pharmacokinetic Transporters and Glycemic Response to Metformin: A Metgen Meta-Analysis. *Clin Pharmacol Ther.* 2017;101(6):763-72.

OVERIGE PUBLICATIES:

1. De Jonge R, **Out M**, Maas WJ, De Jong JW. Preconditioning of rat hearts by adenosine A1 or A3 receptor activation. *Eur J Pharmacol.* 2002;441(3):165-72.
2. Mutsaerts HJ, **Out M**, Goedhart PT, Ince C, Hardeman MR, Romijn JA, Rabelink TJ, Reiber JH, Box FM. Improved viscosity modeling in patients with type 2 diabetes mellitus by accounting for enhanced red blood cell aggregation tendency. *Clin Hemorheol Microcirc.* 2010;44(4):303-13.
3. **Out M**, Kooy A. Metformine: nieuwe inzichten in effecten en werkingsmechanismen. *Focus Vasculair.* 2017;2:48-52.
4. Rotroff DM, Yee SW, Zhou K, Marvel SW, Shah HS, Jack JR, Havener TM, Hedderson MM, Kubo M, Herman MA, Gao H, Mychaleckyi JC, McLeod HL, Doria A, Giacomini KM, Pearson ER, Wagner MJ, Buse JB, Motsinger-Reif AA, **MetGen Investigators**, ACCORD/ACCORDion Investigators. Genetic Variants in CPA6 and PRPF31 Are Associated With Variation in Response to Metformin in Individuals With Type 2 Diabetes. *Diabetes.* 2018 Apr 12. pii: db171164. doi: 10.2337/db17-1164. [Epub ahead of print].

PRESENTATIES BEHOREND BIJ DIT PROEFSCHRIFT:

1. **Out M**, Kooy A, Lehert P, Schalkwijk CA, Stehouwer CDA. Metformin Treatment Is Associated With Increased Serum Levels of Methylmalonic Acid in Patients with Type 2 Diabetes Treated With Insulin: A Placebo-Controlled 4-Year Trial. *Diabetes* 2013;62:A275 (poster ADA 73rd Scientific Sessions 2013).
2. **Out M**, Kooy A, Becker ML, Van Schaik RH, Lehert P, Stehouwer CDA. SNPs Related to the Glucose Lowering Effect of Metformin: Do they Affect Plasma Concentrations of Metformin (MPC) and their Effects on Insulin Requirements (DDI)? *Diabetes* 2013;62:A298 (poster ADA 73rd Scientific Sessions 2013).
3. **Out M**, Kooy A, Lehert P, Schalkwijk CA, Stehouwer CDA. Metformin Treatment Is Associated With Increased Serum Levels of Methylmalonic Acid in Patients with Type 2 Diabetes Treated With Insulin: A Placebo-Controlled 4-Year Trial. *Diabetologia* 2013;56 Suppl 1:386 (poster 49th EASD Annual Meeting 2013).
4. **Out M**, Kooy A, Becker ML, Van Schaik RH, Lehert P, Stehouwer CDA. SNPs Related to the Glucose Lowering Effect of Metformin: Do they Affect Plasma Concentrations of Metformin (MPC) and their Effects on Insulin Requirements (DDI)? *Diabetologia* 2013;56 Suppl 1:388 (poster 49th EASD Annual Meeting 2013).

5. **Out M**, Miedema I, Jager-Wittenaar HA, Van der Schans CP, Krijnen WP, Lehert P, Stehouwer CDA, Kooy A. Metformin-associated prevention of weight gain in insulin-treated type 2 diabetic patients cannot be explained by decreased energy intake. *Diabetologia* 2014;57 Suppl 1:320 (poster 50th EASD Annual Meeting 2014).
6. **Out M**, Kooy A, Lehert P, Schalkwijk CA, Stehouwer CDA. Metformin, methylmalonic acid and the risk of neuropathy: a randomised placebo-controlled trial. *Diabetologia* (2015) 58 (Suppl 1):S110–S111 (oral presentation 51st EASD Annual Meeting 2015)
7. **Out M**, Kooy A. Metformine, nieuwe inzichten in een beproefd medicijn (invited lecture at the Annual Dutch Diabetes Research Meeting 2016).
8. **Out M**, Kooy A, Lehert P, Schalkwijk CA, Stehouwer CDA. Metformin, methylmalonic acid and the risk of neuropathy: a randomised placebo-controlled trial. *Ned Tijdschr Diab* 2016;14(4):112 (oral presentation at the Annual Dutch Diabetes Research Meeting 2016).
9. **Out M**, Becker ML, Van Schaik RH, Lehert P, Stehouwer CDA, Kooy A. Metformin and pharmacogenetics: the efficacy of polymorphisms on daily dose of insulin, glycemic control and metformin plasma levels – a post-hoc analysis of a 4.3 year RCT. *Ned Tijdschr Diab* 2016;14(4) (oral presentation at the Annual Dutch Diabetes Research Meeting 2016).
10. **Out M**, Becker ML, Van Schaik RH, Lehert P, Stehouwer CDA, Kooy A. Metformin and pharmacogenetics: the efficacy of polymorphisms on daily dose of insulin, glycemic control and metformin plasma levels – a post-hoc analysis of a 4.3 year RCT (poster 6th meeting EASD Study Group on Genetics of Diabetes 2017).
11. **Out M**, Top WMC, Lehert P, Schalkwijk CA, Stehouwer CDA, Kooy A. Long-term treatment with metformin in type 2 diabetes and vitamin D levels: A post hoc analysis of a randomised placebo-controlled 4.3 year trial. *Ned Tijdschr Diab* 2017;15(4):105 (oral presentation at the Annual Dutch Diabetes Research Meeting 2017).