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Introduction
Metformin is, together with insulin, one of the oldest antidiabetic drugs. A century after its chemical invention (1922) and more than six decades after its introduction in clinical practice (1957), it is the most frequently prescribed antidiabetic drug worldwide(1).

Since 2006 both European and American guidelines recommend metformin as the initial pharmacologic intervention in type 2 diabetes patients(2). Although this recommendation is still validated in most of the current guidelines, the position of metformin as a first-line drug is challenged by new antidiabetic drugs.

However, instead of being superseded by new drugs, metformin is increasingly used for a variety of new indications. The working mechanism of metformin, although still not fully elucidated, has a pleiotropic nature. Metformin is involved with critical mitochondrial processes.

There is an extensive body of literature about metformin but long-term randomized trials are scarce. The HOME trial, an acronym for Hyperinsulinaemia: the Outcome of its Metabolic Effects, is a Dutch placebo-controlled randomized trial in which cardiovascular and metabolic outcomes in 390 type 2 diabetes patients were studied for 4.3 years(3).

This thesis describes the results of 1 prespecified analysis and 4 post-hoc analyses of the HOME trial together with a review of the current positioning of metformin in a broader perspective. The analyses concern basic working mechanisms (effect on pancreatic beta cell function and effect on enteral vitamin absorption) and cardiovascular aspects (effect on brain natriuretic peptide, troponin, and dimethylarginines).

**History of metformin**

The history of oral antidiabetic agents, starts before World War I. It is closely linked to the progress of organic chemistry in the 20th century in which an increasing amount of biologically active molecules were synthesized.

Often these new compounds were based on naturally occurring substances. In the case of metformin, this concerns guanidine derivates. Guanidine was first synthesized from the excrements of seabirds and bats in 1861. Similar substances were found in the sperm of herrings in 1910 (called agmatin) and in the aerial parts of the legume plant goats’ rue in 1914 (called galegin).
**Goat’s rue**

Galegin derived from the legume plant goat’s rue (scientific name: *Galega officinalis* aka professor-weed, French lilac), is often referred to as the natural precursor of metformin. Although both galegin and metformin share the presence of a guanidin group, galegin is not a biguanide. Guanidine derivates (like galegin) are totally distinct from biguanides which have their own unique properties(4).

Goat’s rue has a medieval history as traditional medicine (hence the suffix officinalis which was used for medicinal plants). Medieval indications for goat’s rue include worms, epilepsy, antidote for poison, and infectious diseases among which the plague.

British pharmacopeias from the early modern period, are frequently quoted for their presumed mentioning of goat’s rue to alleviate the complaints of diabetes-induced polyuria. In those days diabetes was first recognized as a separate disease entity.

However, the two most-cited authors of these pharmacopeias in this respect, Nicholas Culpeper (1616-1654) and John Hill (1714-1775), do not explicitly mention the use of goat’s rue for diabetes. Instead they are more likely to be opposed to such a use of goat’s rue.

Culpeper associated healing plants with the signs of the zodiac. Diabetes was a Saturn-associated disease and should be treated with Saturn-associated plants like bistort or darnel. Goat’s rue was associated with Mercury instead. Moreover, Culpeper claimed to have cured his own child of diabetes with alehoof and prickly holly instead of goat’s rue. The only thing close to goats Culpeper advised for diabetes, was a powdered goat bladder or if this proved unpractical just the powdered sphincter of the goat bladder(5).

John Hill is often cited with his magnum opus: the Vegetable System. This is however no pharmacopeia but a botanical handbook in which he made his own classification system. Goat’s rue is included in volume XXI with a concise description without the mention of medical applications. However in his pharmacopeia The British Herbal, he refers to the medical use of goat’s rue with the notion that it is an outdated plant that was “once in great esteem but never deserved the praise bestowed upon it and is now with reason fallen into neglect” (6).

The main use of goat’s rue in the 19th century was as a fodder plant for livestock. It was thought not only to be nutritious but also to increase milk production. This explains the scientific name galega as “gala” means milk and “ago” means to bring on. This concept was extrapolated to humans as goat’s rue was marketed in the late 19th century as a milk stimulating agent (galactogogue) for breastfeeding women but not as a treatment for diabetes(7).
Until the discovery of insulin in 1921, diabetes, especially type 1 diabetes, remained an often fatal disease without an effective treatment other than diets. On the brink of the 20th century, the average life expectancy for a child diagnosed with diabetes was 1.3 years, diagnosed at a thirty year-old 4.1 years, and as a fifty-year-old 8 years(8).

**From Guanidine to Synthalin**

Before the invention of insulin, the hypoglycemic action of guanidines was demonstrated in 1918 by Watanabe in a chemistry lab at Yale University(9). Although his research hypothesis proved wrong (tetany after parathyroidectomy is caused by guanidine intoxication), his finding turned out to become a milestone in the development of metformin.

Triggered by the hypoglycemic action of guanidine, German researchers in Breslau (present-day Poland) experimented with guanidine derivates. In his 1926 article, Erich Frank states that he tried to dissociate the hypoglycemic effects from the toxic effects of guanidine and chose to evaluate agmatin because the group of biogenic amines to which agmatin belongs, combines low toxicity with strong biological activity(10).

However, in the process of synthesizing agmatin, a mono-guanidine derivate, the chemist Heyn discovered by chance di-guanidines, a new class of molecules in which two guanidine groups were connected by a carbon chain. The research group evaluated carbon chains with varying lengths and chose a 10 carbon chain as the most promising drug candidate. They filed a patent for it under the name Synthalin.

This turned out to be the first oral antidiabetic drug that was marketed in 1926. Although there was international attention, it never became accepted as a conventional treatment. In contrast with insulin which was almost always effective without side effects, Synthalin had gastro-intestinal side effects (like metformin) and in rare cases liver toxicity.

Maybe the most important factor for its failure was the lack of discrimination between type 1 and type 2 diabetes resulting in the use of Synthalin in a lot of type 1 diabetes patients in which the effect was disappointing. Although different diabetes phenotypes were recognized, and Synthalin was more effective in older patients, the diagnostic differentiation between type 1 and 2 diabetes did not start until 1936.

**From Synthalin to metformin**

In 1929, a few years after Synthalin was introduced, Erich Hesse und Gert Taubmann also from Breslau, started experiments with yet another class of guanidine derivates: the biguanides(11). In this molecule, which was first synthesized in 1879, two guanidine groups were directly connected without a carbon chain.
They tested the unmodified biguanide for its potential anti-inflammatory properties in rabbits. However, they observed that most rabbits unexpectedly died. Triggered by the known hypoglycemic actions of guanidine and Synthalin, they postulated that biguanides could also induce (fatal) hypoglycemia and cause the death of their rabbits. They tested their hypothesis and they found indeed that biguanide was responsible for fatal hypoglycemia in their rabbits.

Because pure biguanide turned out to be too toxic, they searched for a biguanide-derivate that was less toxic but still lowered the blood sugar. With the help of Slotta and Tschesche, they selected two biguanide compounds among which 1,1 dimethylbiguanide better known as metformin these days. Metformin proved to be the most potent molecule they tested.

Although the chemical synthesis of metformin was first described in 1922, no practical application had been tried till Hesse decided to use metformin in rabbits. He administered a single dose of 100 -150 mg/kg either oral or subcutaneous (equivalent human dose 33-50 mg/kg). This resulted in a halving of the blood glucose, close and incidentally past the neuroglycopenic threshold. Despite intervention with glucose, 2 of the 15 rabbits became cachectic and eventually died.

For more chronic use, a lower dose of 10-25 mg/kg (human equivalent of 3-8 mg/kg) was used for 14 days. This did not result in any observable damage in urine analysis (testing for liver or renal damage) and also obduction did not reveal damage in the liver, kidneys, intestines, and lungs. Also, lactate levels were measured in 11 rabbits which did not show a meaningful change. Only the rabbits that got a neuroglycopenic seizure, had an increase to 150% of their baseline lactate.

The oral or subcutaneous administration of metformin was well tolerated in contrast with pure biguanide which gave rise to blood pressure decreases of 50%. Direct intravenous administration of 200 mg/kg metformin (equivalent human dose 65 mg/kg) however proved lethal to the rabbits with a cardiorespiratory arrest.

Summarizing his experiments, Hesse judged metformin to be less toxic than pure biguanide but still too risky to use in human beings. Therefore, both Synthalin and metformin fell into oblivion as oral antidiabetic drugs just before World War II. Synthalin because it was judged not to be effective enough and because of side effects, metformin because it was regarded as too toxic for safe human application.

**Rediscovery as antimalarial and antiflu agent**

World War II sparked interest in another therapeutic indication of biguanides as
antimalarial agents. Because of the war, the access to the natural antimalarial substance quinine from the Dutch East Indies was limited. Antimalarials were vital for the armed forces because part of the war was fought in malaria-endemic regions in which more than 3 million malaria-related sick days were counted(12).

Therefore, both in the US and the UK, a massive effort was made to find a synthetic antimalarial drug. The UK researchers evaluated biguanides which led to the discovery of Paludrine in 1945. This drug was first tested on the researchers themselves and after that on Australian military volunteers.

Further tests to assess toxicity, revealed mild glucose-lowering effect in rabbits(13). But not as potent as Synthalin. In addition, another biguanide property was discovered: if two Paludrine molecules were connected with a chain of six carbon atoms, the resulting molecule (a bisbiguanide) turned out to be a superb antiseptic. This new drug derived from Paludrine was marketed under the name chlorhexidine.

An important landmark in the history of metformin was the work of a Philippine malaria researcher named Eusebio Garcia. Driven by the rapidly developing resistance against Paludrine, he experimented with other biguanide derivates to treat malaria patients. He synthesized several biguanides and tried them on animals and humans. One of the biguanides he synthesized was metformin.

By chance, Garcia noted that metformin, although not particularly effective for malaria, seemed effective for influenza especially if combined with sulfathiazole. Because of the beneficial effect against flu symptoms, he called it Flumamine. He wrote an article about Flumamine in the Journal of the Philippine Medical Association in 1950(14). Also, he presented an abstract to the XIIth International Congress of Pure and Applied Chemistry in 1951 in which he reported his experience with 200 patients.

Compared to current standards, the doses he used were small. In his journal article, the average metformin dose was 65 mg for maximal two days administered by intramuscular injection. In his abstract Garcia presented a combination therapy of 15 mg metformin and 25 mg sulfathiazole daily. This resulted in the disappearance of headaches within 10-15 minutes and gradual dryness of the nasal mucous membranes. Most cases were resolved in one or two days, some more chronic cases needed a course of six days.

In his abstract, he did not mention effects on the blood glucose. In his journal article, however, he speculates about the potential glucose-lowering effects of Flumamine, analog to Synthalin, which may contribute to the working mechanism by which biguanides can destroy malaria parasites. It was known that blood-stage malaria is
highly dependent on glucose metabolism and that Synthalin was effective against malaria-related protozoa (trypanosoma).

Surprisingly enough, Garcia does not report if he verified his hypothesis with actual blood glucose measurements. Retrospective, it is not probable that he would have detected a glucose-lowering effect with the small dose he used. Especially not because he chose parental administration which bypasses the enteral effects of metformin.

**From Flumamine to Glucophage**

Again, metformin is sinking back into oblivion. The antiflu study of Garcia does not spark international interest with regard to the treatment of flu which is self-limiting anyway. However, his paper is noticed by a Polish and a French researcher.

The Polish Janusz Supniewski was, like Garcia, investigating biguanides for their potential use as a chemotherapeutic agent. He published a study in 1954 in which he evaluated metformin primarily in animals but also in humans(15). He reports that in higher doses, metformin was lethal for some animals. In humans he found metformin to be somehow effective in viral diseases with 50-100 mg subcutaneously every four hours. Especially measles pneumonia in children did respond well to metformin.

To assess the hypoglycemic action of metformin he administered doses of 100 mg/kg to rabbits subcutaneously. Remarkably, he does not notice a relevant change in blood glucose after three hours although the German pre-war researchers had used the same dose. Possibly this may be explained by 24-hour fasting animals in the German study and no mention of fasting in the Polish rabbits.

The other researcher who noticed the report of Garcia was the French Jean Sterne. He would become the founding father of metformin. Sterne started diabetes research as a young physician in a Paris laboratory in which he participated in the testing of galegin, the mono-guanidine that is found in goat’s rue. Galegine like agmatin and Synthalin were tested as oral antidiabetic in Germany and France with disappointing results.

When Sterne read about the experience of Garcia with the biguanide Flumamine, he decided in 1956 to investigate this biguanide as an antidiabetic drug. He was encouraged by laboratory owner Jan Aron of Aron Laboratories in Paris. In contrast with Supniewski, he confirmed in animal experiments the glucose-lowering effect of metformin as discovered by Hesse. The next step was the transition to human beings.

Maybe reassured by the human administration of metformin by Garcia without apparent side effects and the fact that related biguanides were extensively used in humans as
antimalarial treatment, he decided to take the leap in 1957. He prescribed metformin to patients in his own hospital and collaborated with his Paris colleague Elie Azerad to perform a trial in the hospital of Azerad(16).

It turned out that metformin did not cause hypoglycemia in non-diabetic individuals and did not reach dangerously low neuroglycopenic levels in diabetic patients. In humans, it behaved as an antihyperglycemic drug instead of a hypoglycemic drug.

The concern of the German prewar researchers about the toxicity in humans turned out better than expected. Sterne emphasizes the unique chemical and biological aspects of biguanides which make it a class of its own and separates it from related compounds such as the more toxic guanidine derivates (including galegin) and the diguanidines (Synthalin)(16).

Sterne filed a patent in 1957 and wrote the same year an article in the Maroc Medical (17). He coined the name Glucophage (sugar eater) for his new drug which was codenamed LA-6023 during development. He proposed a starting regime of 1-5 grams daily in three or four divided doses after which a lower maintenance dose should suffice.

Further confirmation studies were performed and slowly metformin started to enter the market in Europe. But worldwide acceptance was not yet achieved. In the United States, the research group of Georges Ungar had developed a competing biguanide: phenformin(18).

With increasing clinical experience of both metformin and phenformin, it turned out that phenformin was associated with lactic acidosis which was frequently fatal. Despite dose reduction to 100 mg daily, phenformin was ultimately taken off the market in 1977. However, metformin proved to be safe if contraindications were respected.

In contrast with phenformin, lactate levels do increase slightly but remain in the normal range at therapeutic plasma concentrations(19). The incidence of lactic acidosis in metformin users is so low that its existence is debated and the majority of cases are probably more related to an underlying disease condition.

In the same year that phenformin was taken off the market, a major metformin trial was started in the UK with a 20-year follow-up (UKPDS). Eventually, metformin was allowed on the US market in 1995. Shortly thereafter, the results of the UKPDS were published which showed not only good glycemic results but also meaningful improvements in cardiovascular outcomes(20). Therefore metformin entered the 21st century as the only remaining biguanide worldwide with favorable outcomes and started its journey to become the most important oral drug in the treatment of type 2 diabetes.
Metformin timeline

1861
Synthesis of guanidine

1879
Synthesis of biguanide (Rathke)

1918
Hypoglycemic action of guanidine (Watanabe)

1922
Synthesis of biguanide metformin (Werner & Bell)

1921
Discovery of insulin

1929
Animal testing of metformin and discovery of hypoglycemic action (Hesse & Taubmann)

1926
First oral antihyperglycemic drug Synthalin (diguanide class, Frank, Nothmann & Wagner)

1945
Discovery of antimalarial biguanide Paludrine

1946
Discovery of antiseptic biguanide Chlorhexidine

1950
First human application of metformin as antiviral agent (Garcia)

1954
Human application of metformin as anti-infectious agent (Supniewski)

1956
Introduction sulfonyleurea's

1957
First human application of metformin as antihyperglycemic agent, patent on Glucophage (Sterne)

1977
Start UKPDS trial

1977
Withdrawal of phenformin

1977 - 2002
HOME trial

1977
Withdrawal of phenformin

1995
FDA approval metformin

1998
UKPDS publication

1998
UKPDS publication

2006
Metformin recommended as initial therapy in type 2 diabetes
History of presumed working mechanism

As referred to in the preceding paragraphs, the discovery of metformin is more a chance finding than a deliberate search to influence a specific pathophysiological mechanism. As the guanidine-derivates were found to lower the blood sugar, the mechanism was not understood. However, from the very beginning it was noticed that metformin had a different working mechanism than insulin.

The researchers in Breslau noticed that a Synthalin-induced hypoglycemia, in contrast with insulin-induced hypoglycemia, could not be reversed by adrenalin. This was suggestive of a glycogenic blockade in the liver. Also, obduction in animals showed that glycogen was practically lost in the livers of the treated animals(21).

In addition, it was noticed that guanidine derivates had a slower onset of action than insulin and sometimes induced short hyperglycemia before the glucose levels started to fall. This was interpreted as a temporary activation of glycogenolysis, followed by a seemingly permanent inhibition of glycogenolysis.

In the 1950s further pathophysiological insights were developed. The pathophysiological difference between type 1 and type 2 diabetes was discovered just before World War II and just after the war the function of the pancreatic alpha cell and the hormone glucagon was described. With these new insights it was speculated that biguanides might modulate pancreatic alpha cells and prevent glucagon to stimulate glycogenolysis. This hypothesis has only recently been confirmed as one of the working mechanisms of metformin(22).

However, also other theories were deployed. In animal and cell research, it was found that biguanides affected mitochondrial respiration. It was proposed that biguanides interfered with the respiratory chain, blocking electron transport. This could also explain the metabolic accumulation of citrate, pyruvate, and lactate. This concept was proved at the beginning of 2000 in which the precise mitochondrial site of action was localized to respiratory complex 1(23,24).

Shortly after the publication of this mitochondrial site of action, a second discovery was made regarding another site of action of metformin: the intracellular enzyme AMPK(25). This enzyme regulates the bioenergetic status of the cell either stimulating or slowing down metabolism. In the case of metformin, AMPK was activated leading to a slowing down of metabolism simulating a fasting state.
The partial inhibition of mitochondrial respiration and the activation of AMPK are two important working mechanisms of metformin. However, additional mechanisms have been found since. Most importantly this concerns the enteral site of action of metformin which is further discussed in the general discussion (chapter 8).

**Biguanide properties**

Regarding mitochondrial complex 1 inhibition, it seems that metformin, compared to other biguanides, has a more selective and balanced way of suppressing the mitochondrial energy flux. Other biguanides are often more potent but lead to deterioration of mitochondria resulting in lactic acidosis. Metformin is unique in reaching an equilibrium in which the energy flux is mildly and stable suppressed(26) instead of a progressive inhibition leading to permanent damage.

Like the biguanide-derivate chlorhexidine, metformin has anti-microbial properties on multiple pathogens and is being proposed as adjunctive therapy in multidrug-resistant infections(27). This could be a pathway by which metformin is able to influence the gut microbiota.

Like paludrine, metformin is being researched for potential antimalarial properties. Although not effective in the late phase of malaria as the parasites are spreading in the blood (similar to the results of Garcia), it has been shown to be effective in the initial liver replication stage of malaria(28).

And last but not least metformin has antiviral properties. It has been shown effective against hepatitis, dengue, and zika virus(29). And its effect on the current COVID-19 pandemic starts being reported. From observational evidence, metformin decreases mortality in type 2 diabetes patients with COVID-19 by 30% in contrast with insulin which doubles the mortality(30). The COVID-19 research on metformin closes a historical loop of the drug that once was called flumazine and was proposed for its antiflu properties.

**Aim and outline of this thesis**

This thesis aims to increase understanding of the working mechanisms and cardiovascular protective properties of metformin. Derived from the placebo-controlled randomized HOME trial, in this thesis 5 studies and 1 review are reported.

The analyses concern two main topics. First two mechanistic effects of metformin: its effect on pancreatic beta cell function and its enteral effects regarding the absorption of vitamin D. Second its cardiac effects regarding the NT-pro brain natriuretic peptide, high...
sensitive troponins, and its endothelial effect in regard to the nitric oxide metabolites arginine and dimethylarginines.

**Research questions**

**Related to mechanistic effects**

1 *Does metformin improve beta cell cell function?*

Because beta cell dysfunction is one of the major pathophysiological mechanisms of diabetes progression over time, preservation or even improvement of beta cell function is important.

Metformin is regarded as an insulin sensitizer and an inhibitor of hepatic glucose production and is considered neutral with respect to beta cell function. From earlier trials (UKPDS, ADOPT) it is known that beta cell function progressively declines also in metformin-treated patients. However, the rate of decline can vary between different treatments and metformin has never been evaluated versus placebo in a long-term randomized trial.

In this study, we evaluate the effect of metformin on fasting estimates of beta cell function. Because beta cell function is dependent on both insulin sensitivity and glycemic stimuli, we will correct for concurrent changes in insulin sensitivity and glycemia.

2 *Does metformin influence the absorption of vitamin D?*

The enteral working mechanisms of metformin are increasingly recognized. Similar to hepatocytes, metformin has a very high accumulation in enterocytes. Besides beneficial enteral effects like increased secretion of GLP-1 hormone and increased glucose uptake, there may be a downside to the enteral accumulation such as gastrointestinal side effects and malabsorption.

Regarding malabsorption, it has been shown that metformin causes malabsorption of vitamin B12 which is selectively absorbed in the terminal ileum(31). Also, there is malabsorption of bile acids which are necessary for the proper absorption of fat-soluble vitamins like vitamin D.

In this study, we want to evaluate the effect of metformin on the absorption of vitamin D which is, in contrast with vitamin B12, a fat-soluble vitamin and has a more proximal absorption in the small intestine.
Related to cardiovascular protective effects

3 Does metformin influence the secretion of N-type pro BNP?

Brain natriuretic peptides are hormones that are secreted in response to the cardiac volume of pressure loads. They are used as indicators of heart failure. Among type 2 diabetes patients, brain natriuretic peptide levels are elevated compared to non-diabetic individuals. This could be related to (sub)clinical structural cardiac injuries such as silent cardiac ischemia or diastolic heart failure. If metformin reduces cardiac injury, this might be reflected in decreased NT-proBNP plasma levels. In this study, we want to evaluate the effect of metformin on plasma levels of NT-proBNP.

4 Does metformin influence the concentration of high-sensitive troponines?

High-sensitive troponines are a prognostic marker of subclinical myocardial injury. Type 2 diabetes patients present with chronically elevated troponin levels. This is related to the presence of chronic coronary artery disease. In this study, we evaluate whether metformin may favorably decrease troponin levels as a reflection of its cardioprotective effects.

5 Does metformin influence the plasma concentration of arginine, ADMA, or SDMA?

Metformin has been shown to improve markers of endothelial function. An important pathway for endothelial function is the nitric oxide pathway. The amino acid arginine is the metabolic precursor of nitric oxide. In contrast, dimethylarginines (ADMA and SDMA) are toxic amino acids that inhibit the production of nitric oxide. Elevated ADMA and SDMA levels and a decreased arginine/ADMA ratio are correlated with cardiovascular disease. If the beneficial effects of metformin on endothelial function are mediated by improved nitric oxide production, this might be reflected in decreased levels of dimethylarginines or an increased arginine/ADMA ratio. In this study, we want to evaluate the effect of metformin on plasma levels of dimethylarginines and the arginine/ADMA ratio.

Review

6 Although metformin is an old drug and new diabetes drugs have been developed, it has a unique working mechanism that extends its beneficial effects beyond the treatment of type 2 diabetes. In this narrative review, we discuss the potential benefits of metformin on cardiovascular disease, cancer, cognitive dysfunction and dementia. Together with observational evidence, recent meta-analyses and randomized trials are reported to summarize the current state of knowledge.
References


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