EFFECTS OF NARINGENIN ON METFORMIN DISPOSITION IN A DIABETIC RAT MODEL

By

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Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy (PhD) in Pharmacology

Department of Pharmacology, Discipline of Pharmaceutical Sciences

School of Health Sciences, College of Health Sciences

University of KwaZulu-Natal, Durban, South Africa

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Title: **EFFECTS OF NARINGENIN ON METFORMIN DISPOSITION IN A DIABETIC RAT MODEL**

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As the candidate's supervisor, I have approved this dissertation for submission

Signed: Name: Date:

PREFACE

The experimental work described in this thesis was conducted at the Biomedical Resources Unit and the Molecular and Clinical Pharmacology Research laboratory, Department of pharmacology, School of Health Sciences, University of Kwazulu-Natal, Durban, South Africa from January 2016 to November 2018 under the supervision of Dr P.M.O OWIRA and Pr M. Faadiel ESSOP.

This study represents the original work of the author and has not been submitted to another university in any other form. The use of information from other sources has been duly acknowledged in the text and reference section.

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MOFO MATO Edith Pascale

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Dr OWIRA P.M.O (Supervisor)

DECLARATIONS

DECLARATION: PLAGIARISM

I, **MOFO MATO Edith Pascale** declare that

1. The research reported in this thesis, except where otherwise indicated, is my own original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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a) Their words have been re-written but the general information attributed to them has been referenced.

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PUBLICATIONS

- **Edith Pascale Mofo Mato, Magellan Guewo-Fokeng, M. Faadiel Essop, and Peter Mark Oroma Owira** (2018). Genetic polymorphisms of organic cation transporters 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes mellitus: a systematic review protocol. BMC Systematic Reviews, 7, 105. http://doi.org/10.1186/s13643-018-0773-y
- **Edith Pascale Mofo Mato, Magellan Guewo-Fokeng, M. Faadiel Essop, and Peter Mark Oroma Owira** (2018). Genetic polymorphisms of organic cation transporter 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes: A systematic review. Medicine, 97(27), e11349. http://doi.org/10.1097/MD.0000000000011349.
- **Edith Pascale Mofo Mato, M. Faadiel Essop, Peter Mark Oroma Owira** (2019)**.** Effects of naringenin on renal expression of organic cation transporter 1 and 2 proteins and metformin disposition in diabetic rats. Journal of Functional Foods, 59:1-7. https://doi.org/10.1016/j.jff.2019.05.021.
- **Edith Pascale Mofo Mato, M. Faadiel Essop, Peter Mark Oroma Owira.** Naringenin exacerbates metformin-associated metabolic/lactic acidosis by upregulating hepatic organic cation transporter 1 protein expression. Biochemical Pharmacology. Manuscript BCP- S- 18- 01848. Under Review

CONFERENCE AND PRESENTATIONS

- Edith Pascale Mofo Mato, M. Faadiel Essop, Peter Mark Oroma Owira. Effects of naringenin on metformin disposition in a diabetic rat model. University of Kwazulu-Natal, College of Health Science Research Symposium, 5 - 6 October 2017 Durban, South Africa.

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DEDICATION

It is with my deepest gratitude that I dedicate this thesis to Dr Jaimendra P. Singh without whom none of this work would be possible

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THESIS OUTLINE

This thesis is presented in 5 chapters.

- **Chapter one:** this chapter provides an introduction and a comprehensive literature review. It also highlights the study rationale and provides information on the aims and specific objectives addressed in the study.
- **Chapter two:** provides a published systematic review and its protocol. This review highlights and summarizes the overall effects of organic cation transporter 1 (OCT1) polymorphisms on therapeutic responses to metformin while the protocol describes the objectives and the methods used to carry out the review.
    - **Article Title**: Genetic polymorphisms of organic cation transporters 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes mellitus: A systematic review protocol. Systematic Reviews 2018; 7: 105.
    - **Article Title**: Genetic polymorphisms of organic cation transporter 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes: A systematic review. Medicine (Baltimore) 2018; 97(27): e11349.

Authors: Edith Pascale Mofo Mato, Magellan Guewo-Fokeng, M. Faadiel Essop, Peter Mark Oroma Owira.

- **Chapter three:** investigates the effects of naringenin on OCT1 expression and metformin associated metabolic/ lactic acidosis.

Manuscript title: Naringenin exacerbates metformin-associated metabolic/lactic acidosis by upregulating hepatic organic cation transporter 1 protein expression. Manuscript BCP- S- 18- 01848.

Authors: Edith Pascale Mofo Mato, M. Faadiel Essop, Peter Mark Oroma Owira

- **Chapter four**: In this chapter, we investigated the effects of naringenin on metformin clearance and renal organic cation transporters expression.

Article title: Effects of naringenin on renal expression of organic cation transporter 1 and 2 proteins and metformin disposition in diabetic rats. Journal of Functional Foods 2019; 59:1-7.

- **Chapter five**: This chapter provides a general discussion and a conclusion of the study.

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ABBREVIATIONS

ABCC8 : ATP Binding Cassette subfamily C member 8

ACAT : Acyl-coA Cholestérol Acyl Transférase

ACC: Acetyl-CoA Carboxylase

AGEs: Advanced Glycation End products

AMP: Adenosine Monophosphate

AMPK: Adenosine Monophosphate-activated Protein Kinase

AT1R : Angiotensin II Type-1 Receptor

ATP : Adenosine Triphosphate

cAMP: Cyclic Adénosine Monophosphate

CAPN10 : Calpain 10

CHT : Choline Transporters

CLr : Renal Clearance

CPT-1 : Carnitine Palmitoyltransferase 1

CREB: C-AMP Response Element-binding protein

CRTC2: CREB Regulated Transcription Coactivator 2

CYP450 : Cytochrome P450

DM: Diabetes Mellitus

eGFR : estimated Glomerular Filtration Rate

ERK1 : Extracellular signal-regulated protein Kinase 1

F2,6BP: Fructose- 2,6-bisphosphate

FDA : Food and Drug Administration

G6Pase: Glucose-6-phosphatase

GDM: Gestational Diabetes Mellitus

GFR : Glomerular Filtration Rate

GLUT- 4 : Glucose Transporter type 4

HMGB1 : High Mobility Group protein B1

HMG-CoA : 3-hydroxy-3-méthylglutaryl-coenzyme A

HO : Heme Oxygenase

IDF: International Diabetes Federation

IL : Interleukin

KCNJ11 : Potassium Voltage-Gated Channel Subfamily J Member 11

LAMTOR1: Late Endosomal/Lysosomal Adaptor, MAPK And mTOR Activator 1

LDL : Low Density Lipoprotein

LDLr : Low Density Lipoprotein receptor

LKB1: Liver Kinase B1

MALA : Metformin Associated Lactic Acidosis

MAPKs : Mitogen-activated Protein Kinase

MATE : Multidrug and Toxin Extrusion

mGPD: mitochondrial Glycerophosphate Dehydrogenase

MODY: Maturity Onset Diabetes of the Young

NADH: Nicotinamide Adenine Dinucleotide reduced form

NF-kB : Nuclear Factor-kappa B

NMDA : N-méthyl-D-aspartate

Nrf2 : Nuclear factor erythroid 2–related factor 2

OATP1A2 : Organic Anion Transporting Polypeptide 1 A2

OATP1B1 : Organic Anion Transporting Polypeptide 1 B1

OCTN1 : Organic cation / carnitine transporter 1

OCT: Organic Cation Transporter

PEPCK: Phosphoenolpyruvate Carboxykinase

PgP : P-glycoprotein

PKA: Protein kinase A

PKC : Protein kinase C

PMAT : Plasma membrane Monoamine Transporter

PPARg : Peroxisome Proliferator-Activated Receptor gamma

PPARα : Peroxisome Proliferator-Activated Receptor alpha

Pyr K: Pyruvate Kinase

RAGE: Advanced Glycation End products receptors

ROS : Reactive Oxygen Species

SERT : Serotonin Transporter

SIK2: Salt Inducible Kinase 2

SLC : Solute Carrier

SLC22A1: Solute carrier family 22 member 1

SNPs : Single Nucleotide Polymorphism

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

TCA : Tricarboxylic Acid

TCF7L2 : Transcription factor 7-like 2

TEA : Tetraethylammonium

TMHs : Transmembrane α-helices

TNF : Tumor Necrosis Factor

UCP-2 : Uncoupling Protein 2

VLDL : Very Low Density Lipoprotein

ABSTRACT

Introduction

Diabetes mellitus (DM) is one of the largest global health emergencies of the 21st century. It is a major cause of blindness, kidney failure, cardiovascular diseases, lower limb amputation and accounted for 10,7 % of global all-cause mortality among people aged between 20 and 79 years old. Metformin is currently the most widely prescribed anti-diabetic drug. It exists as a hydrophilic cation at physiological pH. As such, membrane transporters play a substantial role in its oral absorption, hepatic uptake, and renal elimination. Among these transporters, organic cation transporters OCT 1 (SLC22A1) and OCT 2 (SLC22A2) are known to be important determinants of the pharmacokinetics of metformin. Naringenin, which is a plant-derived compound found in citrus fruits and vegetables, has been presumed to interact with conventional drugs and influence their disposition by modification of drug-metabolizing enzymes and transporters. The aim of this study was to investigate the effects of naringenin on organic cations transporters OCT1 and OCT2 protein expression and subsequently on metformin disposition in streptozotocin- induced diabetic rats.

Methods

Forty-nine male Sprague Dawley rats 250–300 g body weight (BW) were randomly divided into 7 experimental groups (n = 7). They were orally treated daily with 3.0 ml/kg body weight (BW) of distilled water (group 1) or 250 mg/kg BW of metformin (groups 3, 6 and 7) or 60 mg/kg BW of naringenin (groups 2, 5 and 7) dissolved in distilled water. Groups 4, 5, 6 and 7 were given a single intraperitoneal injection of 60 mg/kg BW of streptozotocin to induce diabetes. Animal body weights and water intake were recorded daily. Fasting blood glucose (FBG) and glucose tolerance tests (GTT) were subsequently done. Urine samples were

collected from rats kept in individual metabolic cages for 24 hours, to determine output, electrolytes, albumin, creatinine and metformin levels. Thereafter, the animals were sacrificed by halothane overdose and blood was collected via cardiac puncture. Liver and kidneys were excised, rinsed in normal saline, blotted dry, weighed, snap frozen in liquid nitrogen and stored at -80°c for analysis of OCT 1 and OCT 2 protein expression by Western blot. OCT 1 and OCT 2 proteins were extracted and separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Then, the gel was blotted electrophoretically onto a nitrocellulose membrane which was then probed with a primary antibody and ultimately an enzyme conjugated secondary antibody and substrate to visualize the bands representing the target proteins.

Results

Diabetic rats treated with naringenin and metformin either alone or in combination exhibited weight gain, improved creatinine clearance and reduced polydipsia, albuminuria, serum creatinine and blood urea nitrogen compared to untreated diabetic rats. By contrast, metformin with/without naringenin did not significantly ameliorate hyperglycemia in diabetic rats. Treatment with naringenin increased hepatic uptake and renal clearance of metformin in diabetic rats compared to untreated groups. In addition, naringenin significantly increased lactate concentrations and metabolic acidosis in rats treated with metformin compared to those that were not treated with metformin. Furthermore, diabetic rats exhibited lower OCT1 and OCT2 protein expressions but naringenin treatment significantly increased hepatic OCT1 and renal OCT2 protein expressions in the presence of metformin.

Conclusion

Collectively, our data suggest that metformin disposition could be affected by naringenin through the upregulation of OCT1 and OCT2 protein expressions. Upregulation of OCT1

expression may be associated with metformin-induced lactic acidosis while increased renal OCT2 expression might facilitate metformin excretion and reduce the risk of lactic acid. However, increased renal excretion of metformin by naringenin may not be sufficient to avert metformin-induced lactic acidosis.

CHAPTER ONE

Introduction and Literature review

1.1 Introduction

Diabetes mellitus is a chronic degenerative metabolic disease that has reached epidemic proportions in the past 30 years with an estimated worldwide prevalence of 423 million people. This "epidemic" is a consequency of combination of social, behavioral, and genetic factors [1, 2]. Treatment of diabetes is designed not only to achieve blood glucose control, but to also prevent micro- and macrovascular complications. The essential components of such treatment are dietary modulation, physical activity and pharmacological treatment with insulin and/or oral anti-diabetic agents. Although there are several oral anti-diabetic agents with different mechanisms of action, metformin is currently the most widely prescribed anti-diabetic medication [3].

Compared to other anti-diabetic drugs, metformin specifically reduces hepatic gluconeogenesis without increasing insulin secretion or inducing weight gain or risk of hypoglycemia. In addition to its efficacy in treating diabetes, metformin may also have wider non-glycemic therapeutic effects in diabetic nephropathy, cardiovascular diseases, polycystic ovary syndrome and the prevention or treatment of some types of cancer [4, 5]. The mechanisms underlying these effects are complex and still not fully understood. Metformin acts via both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms; mostly through a mild and transient inhibition of the mitochondrial respiratory chain complex I. However, it likely also acts by other mechanisms such as the inhibition of mitochondrial glycerophosphate dehydrogenase and by impacting on the lysosome [6, 7].

Metformin exists as a cation at physiological pH, a state that limits its permeability across lipid bilayer membranes. Therefore the oral absorption, hepatic uptake and renal excretion of metformin are largely mediated by Organic cation transporters (OCTs) [8]. Organic cation transporters belong to the solute carriers (SLC) 22 family of membrane proteins. They mainly mediate bi-directional facilitated movement of a variety of lipophilic cations based on their

electrogenic properties but independent of a Na^+ ion or proton gradient [9]. Three members of the OCT subfamily have thus far been cloned in humans, including OCT1, OCT2 and OCT3 that are encoded by SLC22A1, SLC22A2, and SLC22A3 genes, respectively [10].

Two OCTs (OCT1 and OCT2) possess 70% amino acid sequence homology. In rodents, OCT1 is expressed in the liver, kidney, and small intestine [11], whereas in humans it is primarily expressed in the basolateral membrane of hepatocytes where it is involved in the hepatic metabolism/biliary excretion of substrates [12]. Organic cation transporter 2 has a substrate specificity similar to that of OCT1 but is predominantly expressed in the basolateral membrane of the proximal tubule epithelium in both rodent and human kidneys [13]. It is involved in the uptake of many xenobiotics from the bloodstream into renal epithelial cells [14]. Although the precise mechanism(s) of action of metformin remains unclear, it is believed that hepatic uptake is an essential step in reducing hepatic glucose production as well as the occurrence of life-threatening side effects such as lactic acidosis [15]. Indeed, inhibition of the mitochondrial respiratory chain complex I by metformin, leads to the increased cytosolic redox state and decreased mitochondrial redox state [16]. Thus, an increased cytosolic redox state could impair conversion of lactate to pyruvate, leading to decreased gluconeogenesis and accumulation of lactate in the blood. Moreover, a number of single nucleotide polymorphisms (SNPs) were identified in SLC22A1 and SLC22A2 genes. Some of these SNPs are associated with altered in vitro and in vivo transport activity and reduced metformin bioavailability [17].

Pharmacokinetic studies demonstrated that the co-administration of certain drugs with grape fruit juice can markedly increase the drug bioavailability. For instance, grapefruit juice can cause mechanism-based inhibition of intestinal CYP3A4 in humans, and may also inhibit P-glycoprotein-mediated efflux transport of drugs such as cyclosporine to increase its oral bioavailability [18]. Naringenin is a major and active flavanone glycoside of citrus fruits such

as grapes and oranges. It is suggested to possess antioxidant, anti-atherogenic, anti-inflammatory, hepatoprotective and anti-hyperglycemic activities [19]. Here, it exerts its anti-hyperglycemic effects by inhibition of gluconeogenesis through upregulation of AMPK hence metformin like-effects [20]. Although there are many studies that investigated the effects of grapefruit juice on drug transporters, the effects of naringenin on such transporters are indeed limited. Nevertheless, it has been shown that naringin and its aglycone naringenin are capable of inhibiting the organic anion transporting polypeptide 1B1 (OATP1B1)-mediated uptake of dehydroepiandrosterone sulfate and the OATP1A2-mediated uptake of fexofenadine in stably transfected HeLa cells [19]. As far as we are aware, no study has previously investigated the effects of naringenin on OCT1 and OCT2. Moreover, reduced expression of OCT1 and OCT2 was reported in experimental diabetic rats and is associated with plasma metformin accumulation and reduced glucose lowering effects. In light of this, we hypothesized that naringenin could upregulate OCT1 and OCT2 expression in diabetic rats treated with metformin thus potentiating metformin efficacy [21].

1.1.1 **Rationale**

Membrane transporters are now widely acknowledged as important determinants of drug absorption, excretion, and, in many cases, extent of drug entry into target organs. Therefore, altered drug transporter function whether due to genetic polymorphisms, drug-drug interactions, or environmental factors such as dietary constituents, may change the oral bioavailability and resulting in unexpected life-threatening conditions and toxicity [22].

Naringenin is the most abundant flavanone in citrus fruits and therefore frequently ingested on a global scale. Due to an increased interest in alternative medicines, the use of flavonoid-containing supplements for health maintenance has become more popular raising the potential interactions with conventional drug therapies [23]. Of note, furanocoumarins in grapefruit juice were found to increase the oral bioavailability of felodipine, nimodipine and

cyclosporine. Subsequently, this juice has been found to interact with more than twenty five medications from a wide range of therapeutic categories [24]. In addition, Owira and Ojewole have previously demonstrated that grape fruit juice may exacerbate metformin-induced lactic acidosis in diabetic rats but the mechanism has not been elucidated [25]. Thus, an investigation of naringenin effects on metformin disposition offers great potential regarding diabetic patients' treatment modalities. Such studies could indeed assist in predicting potential toxicities or adverse-drugs reactions associated with the co-administration of naringenin-containing supplements/natural products with metformin.

1.1.2 **Aim and Objectives**

Aim

To investigate the effects of naringenin on metformin disposition in streptozotocin-induced diabetic rat models

Objectives

- Demonstrate the importance of OCT1 protein on metformin pharmacodynamics
- Study combined effect of naringenin and metformin in glycemic control in diabetic rats;
- Investigate the effects of naringenin on OCT1 expression and hepatic uptake of metformin in streptozotocin-induced diabetic rats and
- Investigate the effects of naringenin on OCT1 and OCT2 expression and renal excretion of metformin in streptozotocin-induced diabetic rats.

Literature Review

1.2 Diabetes mellitus

1.2.1 Definition and symptoms

Diabetes Mellitus (DM) describes a metabolic disorder of multiple etiologies, characterised by chronic hyperglycemia, with disturbances in carbohydrates, proteins and lipids metabolism, resulting from defects in insulin secretion, insulin action, or both [26]. Symptoms of chronic hyperglycemia include polydipsia, polyuria, weight loss, polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia [25].

1.2.2 Epidemiology

International Diabetes Federation (IDF) estimated that approximately 425 million people worldwide (or 8.8% of adults aged 20-79 years) were living with diabetes in 2017 (Figure 1) and if these trends continue then by 2045, approximately 629 million persons (20-79 years) will be diagnosed with DM. In Sub-Sahara Africa, approximately 9.8-27.8 million adults are currently estimated to have DM while, in South Africa it is about 1.8 (1.1 –3.6) million [2].

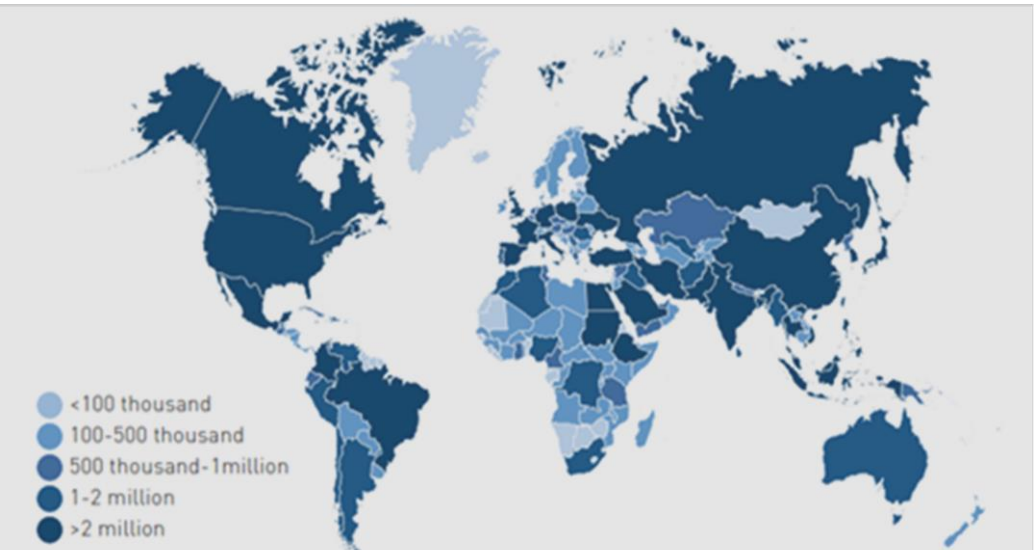

Figure 1: Global diabetes mellitus prevalence (IDF Atlas, 8th edition 2017).

1.2.3 **Classification of diabetes**

Classification of diabetes is complex and has been the subject of much consultation, debate and revision stretching over many decades, but it is now widely accepted by consensus that there are three main types: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM). There are also some less common types of diabetes which include monogenic diabetes and secondary diabetes [2].

1.2.3.1 **Type 1 diabetes mellitus**

Type 1 diabetes mellitus also known as insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune condition characterised by the destruction of the ß cells of the endocrine pancreas, due to interactions between genetic susceptibility, perturbed immunology and environmental factors [27, 28]. The proportion of DM patients diagnosed with T1DM is estimated to be 5%–10% with an annual increase of 3.8–5.6% [29–31]. A small percentage of affected patients with no evidence of autoimmunity are classified as type 1B, and the pathogenesis in these cases is considered idiopathic [32]. Insulin replacement has been the mainstay therapy for T1DM but new therapies including immunotherapy, islet transplantation, and stem cell/precursor cell transplantation are increasingly utilised [33]. However, numerous defects such as the absence of sustainable efficacy, adverse-effects, immunosuppression, and the scarcity of donors limit their extended applications [34].

1.2.3.2 **Type 2 diabetes mellitus**

Type 2 diabetes mellitus also known as non-insulin-dependent diabetes mellitus (NIDDM), consists of an array of dysfunctions characterised by hyperglycemia resulting from the combination of peripheral resistance to insulin, impaired insulin secretion by the pancreatic β-cells, and excessive or inappropriate glucagon secretion [35]. Insulin resistance is a state where target tissues such as skeletal muscles, adipose tissues, and the liver fail to respond adequately to insulin. This results in decreased glucose utilisation in the muscle and adipose

tissue and increased gluconeogenesis in the liver. Hyperglycemia appears when the β-cells fail to compensate for insulin resistance with excess insulin output [36]. Multiple genetic and environmental factors contribute to both insulin resistance and impaired insulin secretion. Well known environmental risk factors include physical inactivity, a high-calorie diet, obesity, and certain drugs such as glucocorticoids [37]. Studies have identified more than 83 genetic variants including variants in peroxisome proliferator activated receptor gamma (PPARG), transcription factor 7 like 2 (TCF7L2), ATP binding cassette subfamily C member 8 (ABCC8), calpain 10 (CAPN10) and potassium voltage-gated channel subfamily J member 11 (KCNJ11) to be associated with T2DM [38, 39].

1.2.3.3 **Gestational diabetes mellitus**

Gestational diabetes mellitus is defined as any degree of glucose intolerance with onset or first recognition during pregnancy, although international guidelines recommended differentiation between probable T2DM identified early in pregnancy that has not been previously diagnosed and GDM that develops later in pregnancy [40, 41]. Approximately 7% of all pregnancies are complicated by GDM [42]. During normal pregnancy, there is a decrease in insulin sensitivity which could be the result of maternal obesity with varying degree of adipocytokine production, or increased production of diabetogenic placental hormones. Gestational diabetes mellitus occurs when pancreatic β-cells cannot compensate for the increased levels of insulin resistance [43]. Although GDM normally disappears after delivery, women who have been previously diagnosed with GDM are at a greater risk of developing gestational diabetes in subsequent pregnancies and T2DM later in life. Infants born to mothers with GDM also have a higher risk of developing T2DM in their teens or early adulthood [44].

1.2.3.4 **Others specific diabetes**

There are also unusual causes of diabetes that result as a consequence of other medical conditions such as diseases of the exocrine pancreas (e.g. pancreatitis, cystic fibrosis, hemochromatosis), endocrinopathies (e.g. Cushing syndrome, acromegaly, pheochromocytoma), genetic defects of the β-cell function (e.g. maturity onset diabetes of the youth), genetic defects of insulin action, rare forms of auto-immune mediated diabetes and infections. Some drugs like glucocorticoids, neuroleptics, alpha-interferons, pentamidine have been also associated with diabetes [45].

1.2.4 **Complications of diabetes mellitus**

Chronic hyperglycemia in diabetes is associated with long-term complications, dysfunction, and failure of various organs leading to ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (macrovascular), retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputation, and Charcot joints, and autonomic neuropathy causing gastrointestinal genitourinary, and sexual dysfunction (microvascular) [46]. Hyperglycemia promotes diabetes-related complications through at least five main mechanisms: an increased flux of glucose and other sugars through the polyol pathway; an increased intracellular Advanced Glycation End products (AGE's) formation; interaction between AGEs and their receptors (RAGEs) leading to intracellular signaling which disrupts cell function [47]; persistent activation of protein kinase C (PKC) isoforms [48]; and an increased hexosamine biosynthetic pathway activity [49]. Among these biochemical pathways, AGE formations are thought to be the major pathological pathway that is strongly involved in the development and progression of different diabetes-related complications [47].

1.2.5 **Glycation end products formation under hyperglycemic conditions**

Glycation end products are formed by the Maillard process, a non-enzymatic reaction between reducing sugars and the amino groups of proteins, lipids and nucleic acids that contribute to the aging of macromolecules [50, 51]. Under hyperglycemic and/or oxidative stress, the protein glycation process starts with a nucleophilic addition between free ε-amino or NH-terminal groups of proteins and the carbonyl group of glucose (or other reducing sugars such as fructose, galactose, mannose, pentose, xylulose) to form a reversible Schiff base [52]. The latter can rearrange into a stable, irreversible ketoamine or Amadori product that can undergo dehydration and rearrangement to produce highly reactive dicarbonyls (or AGE precursors) such as methylglyoxyl, glyoxyl, and 3-deoxyglucosone [53–55]. The Schiff base is highly prone to oxidation and free radical generation leading to formation of dicarbonyls by the so-called Namiki pathway of the Maillard reaction that occurs early in the glycation process [56]. AGEs can also be altered by glycoxidation to produce N-carboxy-methyl-(lysine) or pentosidine from lipids, also called advanced lipoxidation end products. With the polyol pathway, fructose can be converted to fructose-6-phosphate. Subsequently, fructose-6-phosphate can be converted to fructose-1,6-bis-phosphate. The latter can be then be further metabolized to generate glyceraldehyde-3-phosphate and methylglyoxyl (Figure 2) [55].

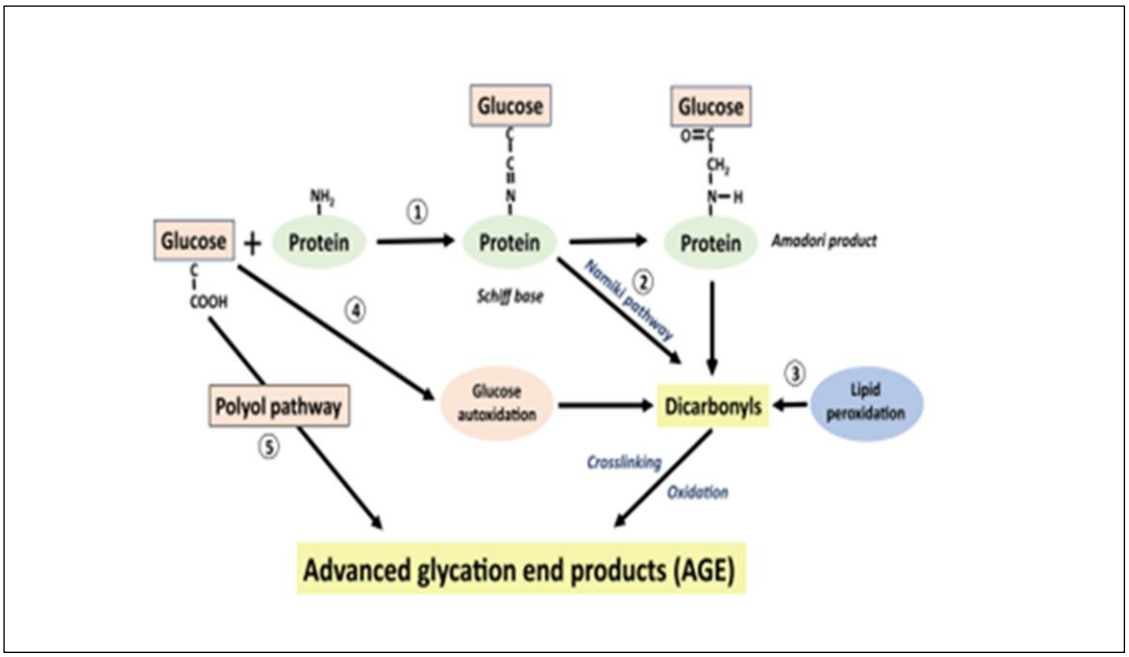

Figure 2: Formation of Advanced Glycation End Products (AGE's) [55].

1.2.5.1 **Glycation end products and diabetes complications**

The main mechanisms involved in the tissue damage caused by AGEs are: intracellular glycation, cross-linking formation and interactions with AGEs receptors (RAGE) [57].

The intracellular accumulation of AGEs play important roles as stimuli for modifying the function of intracellular proteins [58] as well as altering cytoplasmic and nuclear factors, including the proteins involved in regulating gene transcription [59]. Glycation end products accumulate in most sites of diabetes-related complications, including the kidney, retina, and atherosclerotic plaques [60]. Glycation of proteins interferes with their normal functions by disrupting the molecular conformation, altering the enzymatic activity, reducing degradation capacity, and interfering with receptor recognition [61].

1.2.5.2 **Cross linking**

The formation of AGEs causes cross-linking of collagen molecules to each other. This leads to chemical and physical changes in the collagen's structure, loss of collagen elasticity, and subsequently functional changes typical of chronic diabetic complications. For instance patients with T2DM display a 3-fold increased fracture risk depending on the skeletal site and severity of disease [62, 63]. Although the mechanism is not well understood, excessive formation of non-enzymatic cross-links (assessed by pentosidine, a surrogate marker of AGEs) may be a major cause of bone fragility in aging, osteoporosis and DM [64, 65].

1.2.5.3 **Interaction between glycation end products and its receptors**

The interaction between RAGE and AGEs leads to cellular signaling, including activation of nuclear factor-kappa B (NF-kB) through the mitogen-activated protein kinases (MAPKs) pathways, increased cytokine and adhesion molecule expression, induction of oxidative stress, and an increase in cytosolic reactive oxygen species (ROS) [66]. It is worth noting that in addition to RAGEs, a number of RAGE ligands have also been identified in diabetic patients, including members of the S100 calgranulin family and high mobility group box 1 (HMGB1). The interaction of such ligands with RAGEs suggests that the latter is involved not only in diabetes-related complications, but may also be a primary contributor to diabetes onset (both types) [67].

In support of such concepts, the deletion of Ager (gene encoding RAGE) in mouse models prevented the development of obesity and insulin resistance [68]. Therefore, RAGE targeting and associated signaling pathways could lead to the development of novel drug targets to treat diabetes and its complications [69].

1.2.6 **Treatment of diabetes**

The most effective management of diabetes mellitus demands a multidisciplinary approach, involving both lifestyle modifications with diet and exercise and pharmacologic therapies as necessary to meet individualised glycemic goals. Thus insulin is essential for the treatment of T1DM patients because there is a defect in insulin secretion. However, treatment of T2DM patients is more complex because of a defect in both insulin secretion and insulin action can exist. Therefore such treatment should target multiple defects and follow a patient-centered and individualized strategy that considers factors beyond glycemic control, including microvascular events and cardiovascular disease risk factors [70, 71]. Lifestyle modifications must be combined with oral hypoglycemic agents (Table 1).

The appropriate treatment for diabetes should be selected considering the clinical characteristics of the patient, efficacy of the drug, side-effects, and cost. In general, metformin is preferred as an initial monotherapy for T2DM because of its excellent blood glucose-lowering effect, relatively low side-effects, long-term proven safety, low risk of hypoglycemia, and low weight gain [72]. If metformin is contraindicated or not well tolerated as a first-line treatment, other appropriate medications should be selected depending of the clinical situation. If the glycemic control is not achieved by monotherapy, a combination therapy with different mechanisms of action should be initiated promptly [73]. When used as an add-on to insulin therapy in T2DM, metformin can reduce insulin dose requirements; limit insulin-induced weight gain and cardiovascular risks. Hence there has been a growing interest in exploring the role of metformin as an adjunct to injectable insulin therapy in T1DM, with the aim of improving insulin sensitivity and glycemic control, limiting the insulin dose and weight gain and the long-term possibility of cardiovascular risk reduction [74].

Table 1: Oral anti-diabetic medications [75].

Class Generic name	Mechanism of action	Adverse effects
Sulfonyureas Aceto-hexamide, Chlorpropamide, Tolazamide, Tolbutamide, Glyburide, Glibenclamide, Glipizide, Glimepiride	Inhibit adenosine triphosphate-sensitive potassium channel (K-ATP channel) in pancreatic beta cells and alter cellular resting membrane potential, causing an influx of calcium and the stimulation of insulin secretion.	Hypoglycemia, flatulence, dizziness, diarrhea, vomiting
Meglitinides Repaglinide, Nateglinide	Regulate K-ATP channels in pancreatic beta cells, thereby causing an increase in insulin secretion.	Weight gain, headache, infection, hypoglycemia, upper respiratory tract
Biguanides Metformin	Increase hepatic AMPK activity thus reducing gluconeogenesis and lipogenesis as well as increasing insulin-mediated glucose uptake by skeletal muscles.	Gastrointestinal upset, nausea, vomiting, palpitation, headache lactic acidosis, skin rash
Thiazolidinediones (TZDs) Rosiglitazone Pioglitazone	Bind to PPAR gamma to increase peripheral uptake of glucose and decrease hepatic glucose production.	Edema, sinusitis, hypoglycemia, cardiac failure headache bone fracture, myalgia
Alpha-glucosidase inhibitors Acarbose, Miglitol, Voglibose	Competitively inhibit alpha-glucosidase enzymes in the intestine (digesting dietary starch) thus inhibiting polysaccharide reabsorption as well as the breakdown of sucrose to glucose and fructose.	Flatulence, diarrhea, abdominal pain, increased serum transaminases

Class Generic name	Mechanism of action	Adverse effects
Dipeptidyl-peptidase-4 (DDP4) inhibitors Sitagliptin, Saxagliptin, Vildagliptin, Linagliptin, Alogliptin	Inhibit the enzyme dipeptidyl peptidase 4 (DPP- 4) and prolong the action of glucagon-like peptide. This inhibits glucagon release, increases insulin secretion, and decreases gastric emptying thus decreasing blood glucose levels.	Hypoglycemia, nasopharyngitis, urinary tract infection
Glucagon like peptide-1 (GLP-1) agonists Exenatide, Liraglutide, Dulaglutide	Mimic the effects of certain intestinal hormones (incretins) involved in the control of blood sugar.	Nausea, diarrhea, vomiting
Amylin analogs Pramlintide	Decrease glucagon secretion, slow gastric emptying, reduce satiety.	Nausea, vomiting, headache
Sodium glucose cotransporters2 (SGLT2) inhibitors Dapagliflozin, canagliflozin Empagliflozin	Inhibit sodium-glucose cotransporter 2 (SGLT-2) in proximal tubules of renal glomeruli, causing inhibition of 90% glucose reabsorption and resulting in glycosuria in persons with diabetes, which in turn lowers plasma glucose levels.	Dyslipidemia, urinary tract infection, nausea, fungal vaginosis, bone fracture, renal impairment
Cycloset Bromocriptine	Sympatholytic dopamine D2 receptor agonist that resets the hypothalamic circadian rhythm which might have been altered by obesity. This results in the reversal of insulin resistance and the decrease in glucose production.	Dizziness, fatigue, headache constipation, weakness nausea

1.3 **Metformin**

Metformin [3-(diaminomethylidene)−1,1-dimethylguanidine] is a synthetic oral hypoglycemic drug that is a member of the biguanides family [76]. Chemically, metformin is composed of two guanidine groups joined together with the loss of ammonia. The chemical structures of guanidine and metformin are presented in Figure 3. Biguanides are derived from isoamylene guanidine, which has been found in *Galega officinalis* (French lilac, or Goat's rue) [77] although anti-hyperglycemic effects of guanidine itself proved to be too toxic for clinical use. Of note, two diguanides (synthalin A and synthalin B) were initially evaluated but their marked toxicity could not be dissociated from therapeutic effects [78]. By the late 1950s, attention shifted to metformin and two other biguanides, i.e. buformin and phenformin. Here metformin exhibits a superior safety profile and this is primarily because of the risk of lactic acidosis (which can be fatal) as a result of phenformin or buformin treatment [79].

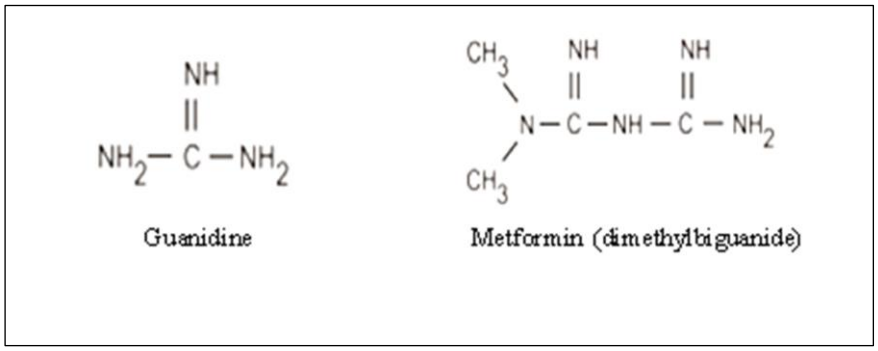

Figure 3: Chemical structures of guanidine and metformin.

1.3.1 **Mechanism of action of metformin**

The cellular and molecular mechanisms underlying the action of metformin are complex and remain an area of active investigations. Physiologically, metformin has been shown to decrease blood glucose levels by suppressing hepatic gluconeogenesis and stimulating glucose uptake in skeletal muscle and adipose tissues [80]. However, not all of its effects can be explained by this mechanism and there is increasing evidence of a key role for the gut [7].

At the molecular level, metformin has been shown to act via both AMPK-dependent and AMPK independent mechanisms, by inhibition of mitochondrial respiratory chain complex I, by inhibition of mitochondrial glycerophosphate dehydrogenase, and a mechanism involving the lysosome. Thus during the last ten years there has been a strong shift from metformin improving glycemia by acting on the liver via AMPK activation, to a more complex understanding of its multiple modes of action [77].

1.3.1.1 **AMPK-dependent mechanism**

The various mechanisms targeting the mitochondrial respiratory complex I are illustrated in Figure 4. (1) Uptake of metformin into hepatocytes is catalyzed by OCT1 [81]. Being positively charged, the drug accumulates in cells and in mitochondria because of the membrane potentials that exist across the plasma and mitochondrial inner membranes [82]. (2) Metformin inhibits complex I and interrupts mitochondrial respiration and decreases proton-driven synthesis of ATP, causing cellular energetic stress and elevation of the AMP:ATP ratio. These changes result in allosteric activation of AMPK, a primary metabolic sensor [83]. (3) Alternatively, AMPK may be activated by a lysosomal mechanism that requires axin and late endosomal/lysosomal adaptor, MAPK and mTOR activator 1 (LAMTOR1) [84]. (4) Increases in AMP: ATP ratio also inhibit fructose-1,6-bisphosphatase, resulting in the acute inhibition of gluconeogenesis [85], while also inhibiting adenylate cyclase and lowering cAMP production [86]. (5) Activated AMPK phosphorylates both Acetyl-CoA carboxylases (ACC) 1 and 2 isoforms, inhibiting fat synthesis and promoting fat oxidation instead, thus reducing hepatic lipid stores and enhancing hepatic insulin sensitivity [87].

1.3.1.2 **AMPK-independent pathway**

Though AMPK was once considered the primary executor of metformin’s anti-diabetic actions, genetic loss of-function studies in mice indicated that hepatic expression of AMPK

and its upstream activating kinase (LKB1) may not be absolutely required for suppression of gluconeogenesis by metformin [6]. (6) An AMPK-independent mechanism has since been proposed, where metformin antagonizes glucagon-dependent cyclic AMP (cAMP) signaling [86]. (7) Glucagon activates adenylyl cyclase to produce cAMP and stimulate cAMP-dependent protein kinase (PKA) signaling. PKA activation causes a decrease in fructose-2,6-bisphosphate (F2,6BP), an allosteric activator of phosphofructokinase (PFK) and inhibitor of fructose-1,6-bisphosphatase (FBPase). (8) PKA also phosphorylates and inactivates the liver isoform of the glycolytic enzyme pyruvate kinase (Pyr K) and (9) phosphorylates the transcription factor cAMP response element binding protein (CREB), thus inducing transcription of the genes encoding the gluconeogenic enzymes phosphoénolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). (10) Phosphorylation of CREB-regulated transcriptional coactivator-2 (CRTC2) by AMPK, or by AMPK-related kinases such as salt-inducible kinase 2 (SIK2), causes CRTC2 to be retained in the cytoplasm, antagonising the effects of PKA on the transcription of PEPCK and G6Pase [88, 89].

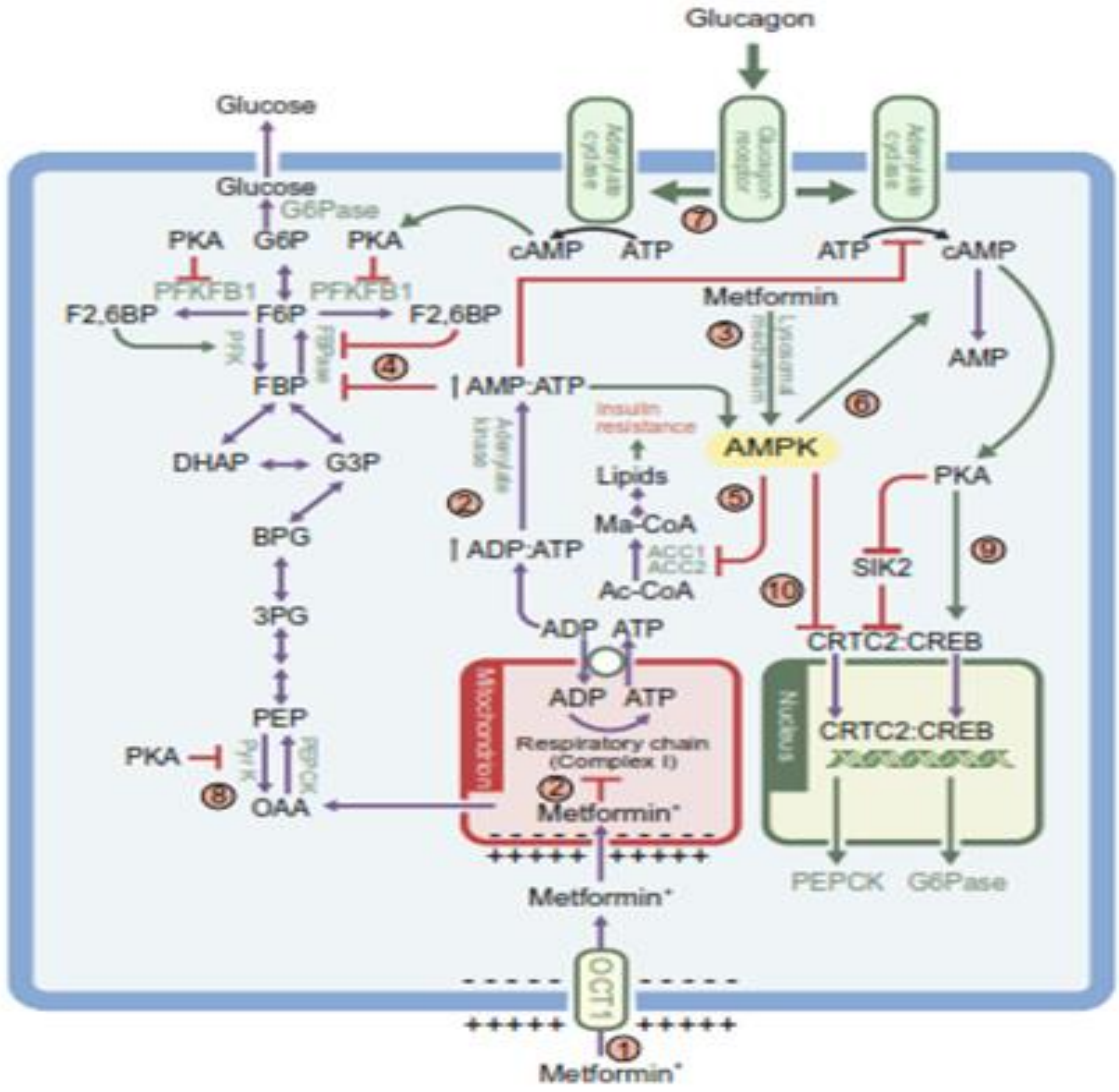

Figure 4: Multiple mechanisms by which metformin affects liver metabolism [7].

1.3.1.3 **Inhibition of mitochondrial glycerophosphate dehydrogenase**

In addition to targeting complex I, Madiraju *et al* (2014) have shown that metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mGPD) [90]. Mitochondrial glycerophosphate dehydrogenase is a ubiquinone-linked enzyme in the mitochondrial inner membrane best characterized as part of the glycerol phosphate shuttle that transfers reducing equivalents from cytosolic nicotinamide adenine dinucleotide reduced form (NADH) into the mitochondrial electron transport chain.

Interestingly, inhibition of complex I and mGPD both results in decreased NADH oxidation. Decreased electron chain activity suppresses tricarboxylic acid (TCA) cycle flux and decreases mitochondrial ATP synthesis. These actions result in increased AMPK signaling, decreased cAMP/PKA signaling, decreased gluconeogenesis and increased glycolysis (Figure 5) [80].

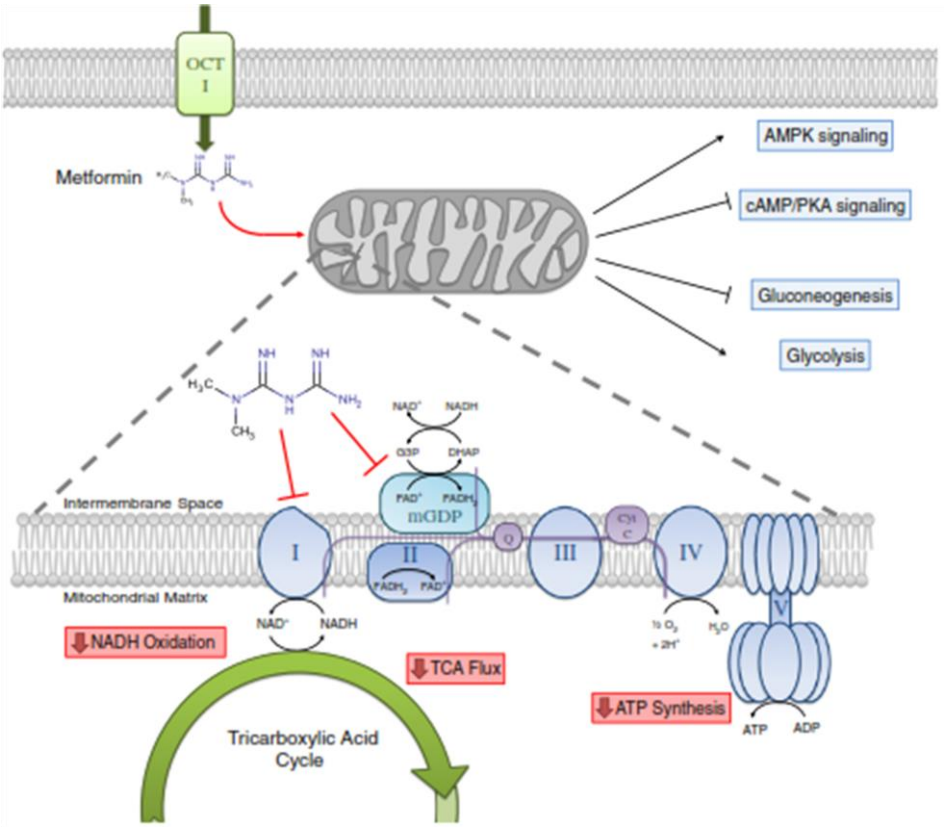

Figure 5: Cellular consequences of metformin action in the mitochondria [80].

1.3.2 **Metformin Pharmacokinetics**

Metformin is not metabolised and approximately 80% is excreted unchanged in the urine with a half-life of approximately 5 hours in patients with normal renal function [5, 91, 92]. The population mean renal clearance (CLr) and apparent total clearance after oral administration

(CL/F) of metformin were estimated to be 510±130 ml/min and 1140±330 ml/min, respectively, in healthy subjects and diabetic patients with good renal function [5]. The oral bioavailability of metformin, including administration of a tablet formulation, controlled release formulation, and an oral solution ranges from approximately 40 to 60% [93]. After a single oral dose, metformin is rapidly distributed in many tissues including the intestine, liver and kidneys. Absorption from the stomach is likely to be negligible and it therefore appears that the absorption of metformin is confined very largely to the small intestine with negligible absorption from large intestine [94]. Metformin is not a ligand for membrane-bound receptors. At physiological pH levels, metformin exists almost entirely as a monoprotonated cation and transport across biological membrane involves an active uptake process via solute carrier organic transporters [5].

1.3.3 **Drug transporters involved in metformin disposition**

Metformin, an organic cation, is actively transported via organic cation transporters primarily in the intestine, liver and kidneys [7].

1.3.3.1 **Gastrointestinal uptake of metformin**

After oral administration, metformin is efficiently taken up across the apical surface of enterocytes [95]. Several transporters are involved in the transport of metformin including Carnitine/Organic cation transporter 1 (OCTs/SLC22A, OCTN1/SLC22A4), plasma membrane monoamine transporter (PMAT/SLC29A4), serotonin transporter (SERT/SLC6A4) and high affinity choline transporter (CHT/SLC5A7) [96]. Metformin is primarily absorbed by the PMAT which is localized on the tips of the mucosal epithelial layer. Plasma membrane monoamine transporter is the major transporter responsible of the uptake of metformin from the gastrointestinal tract. Plasma membrane monoamine transporter mediated metformin transport is greatly stimulated by acidic pH [97]. Because of their basolateral locations and low expression levels in the human enterocytes, OCT1 and OCT2 may play a limited role in

intestinal absorption of metformin. Organic cation transporter 1 may facilitate the transfer of metformin into the interstitial fluid [8, 98, 99]. Organic cation transporter 3 is the third member of the OCT family and displays a relatively higher expression level in the intestine. It is localized on the apical membrane of human enterocytes and may therefore be (in part) a carrier of metformin into enterocytes [100, 101]. Organic cation/carnitine transporter 1 which is localized on apical membranes of small intestine in mice and humans can also mediate metformin uptake in gastrointestinal tract [102]. Serotonin transporter is expressed in both apical and basolateral membranes of enterocytes, although expression on the apical side is predominant in the intestine [101]. Han *et al*. (2015) used inhibitors on caco-2 cell monolayers to demonstrate the contribution of SERT and CHT in the uptake of metformin from intestines [97]. As CHT was barely detectable, direct evidence of its in vivo contribution to metformin uptake is still lacking [103].

1.3.3.2 **Transport and uptake in the liver**

The primary mediator of hepatic metformin uptake is OCT1 and possibly OCT3 [97, 104]. Both OCT1 and OCT3 are present in highest levels on the sinusoidal (basolateral) hepatocyte membranes in rat and human liver [105]. The greatly diminished hepatic uptake of metformin in OCT1 knockout mice indicates that the latter is a major transporter in liver [106]. OCT-mediated transport is electrogenic, independent of a Na^+ ion or proton gradient, and may occur in either direction across the plasma membrane [107]. Metformin is also a substrate of multidrug and toxin extrusion 1 (MATE1) encoded by the gene SLC47A1 [9, 108]. Multidrug and toxin extrusion 1 is expressed in the luminal membrane of kidney and liver and was revealed to be responsible for the tubular and biliary secretion of metformin [109].

1.3.3.3 **Transport and uptake in the kidneys**

Several cation transporters are present in the kidney, namely OCT1, OCT2 and OCT3 [110]. Metformin uptake from circulation into renal epithelial cells is primarily facilitated by OCT2

which is the most expressed and located in the basolateral membrane of renal proximal tubules cells [111]. Organic cation transporter 1 has been detected in the apical membranes (luminal sides) in the proximal and distal tubules and may play an important role in the metformin reabsorption in the kidney tubules [110, 112]. The localization of OCT3 is still unclear. Renal excretion of metformin from the tubule cell to the lumen is mediated by MATE1 (SLC47A1) and MATE2-K (SLC47A2). Multidrug and toxin extrusion 1 and MATE2-K are expressed in the apical membrane of the renal proximal tubule cells [108, 113]. Plasma membrane monoamine transporter protein is present in the human kidney, and the transporter is likely to be localized on the apical membranes of renal epithelial cells. Plasma membrane monoamine transporter may use luminal proton gradient to drive organic cation reabsorption in the kidney [114].

1.3.3.4 **Transport and uptake in peripheral tissues**

The metformin uptake in peripheral tissues is mediated by OCT1 and OCT3 [107]. However, OCT3 is the most highly expressed, particularly in skeletal muscles and adipose tissues [115]. In skeletal muscle, metformin increases glucose uptake by enhancing expression of PKC and translocation of glucose transporter 4 (GLUT4) [116].

1.4 **Organic cation transporters**

Organic cation transporters belong to the solute carriers (SLC) 22A superfamily of polyspecific membrane proteins that play a central role in transportation of organic cations, anions and zwitterions. It thus plays a major role in cellular organic ions homeostasis [8, 117]. Three members of the OCT subfamily have been identified including OCT1 (encoded by SLC22A1), OCT2 (encoded by SLC22A2), and OCT3 (encoded by SLC22A3) [118].

1.4.1 **General structure**

The OCT proteins are usually 500–600 amino acids in length and, predicted to contain 12 transmembrane α-helices (TMHs) with intracellular N- and C-termini as depicted in Figure 6. Potential N-glycosylation sites have been found in the large extracellular loop between the first and second TMHs [119]. Multiple putative phosphorylation sites have been observed in the large intracellular loop between TMHs 6 and 7. Organic cation transporters 1 and OCT2 have been identified to possess 70% amino acid sequence identity to each other and approximately 50% identity with that of OCT3 [8, 120].

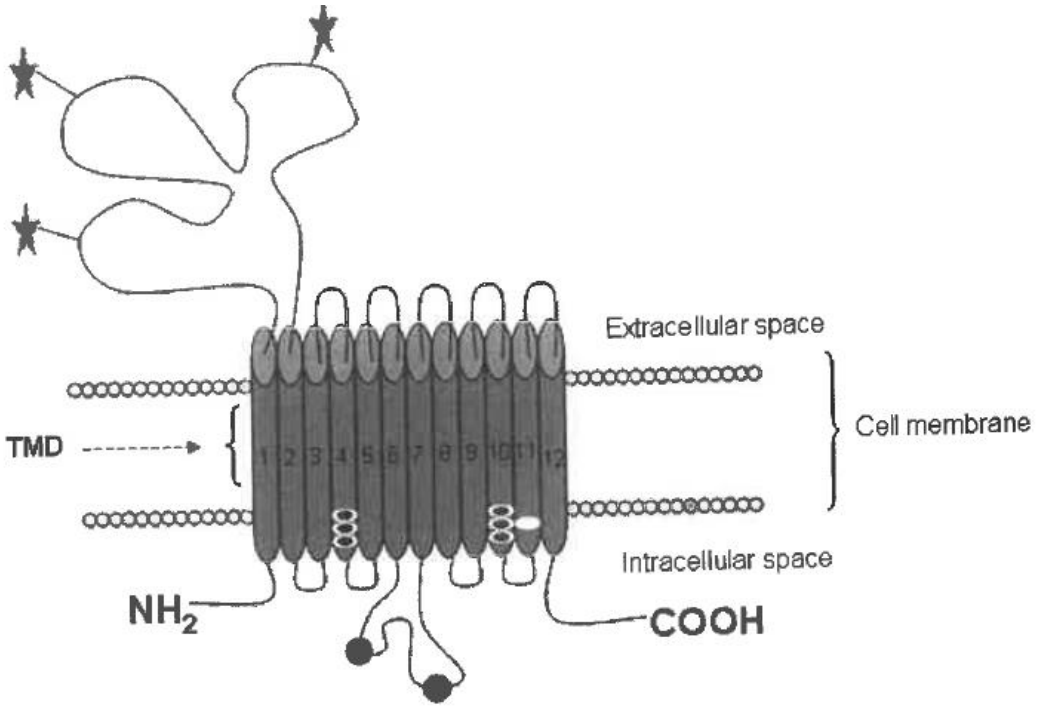

Figure 6: An illustration of human OCT1 structure [121].

1.4.2 **Tissue distribution, localization and function**

1.4.2.1 **Organic cation transporter 1**

Organic cation transporter 1 exhibits broad tissue distribution. It is expressed in epithelial cells, in some neurons and basophilic granulocytes. In humans it is most strongly expressed in

the liver whereas in rodents it is strongly expressed in the kidney and small intestine. In human and rat liver OCT 1 is located to the sinusoidal membrane of the hepatocytes [122]. In mouse small intestine OCT 1 is located in the basolateral membrane of enterocytes, and in rat kidney Oct 1 is located in the basolateral membrane of epithelial cells in the S1 and S2 segments of proximal tubules. However, in the trachea and bronchi of human, rat and mouse, OCT 1 is located in the luminal membrane of epithelial cells [8]. The major function of OCT1 is most likely to mediate the uptake of organic cations in hepatocytes as the initial step of biliary secretion [14]. Moreover, in humans it may contribute to the reabsorption of organic cations from the primary urine as OCT1 has been detected at the luminal membrane of proximal and distal tubules. By contrast, OCT 1 together with OCT 2 mediate the initial step of tubular secretion of organic cations in rodents. Moreover, OCT 1 may be involved in the absorption of organic cations in the small intestine and in the passage of substrates over the blood–brain barrier [123].

1.4.2.2 **Organic cation transporter 2**

OCT 2 has a more restricted expression pattern than OCT 1 and OCT 3. It is most strongly expressed in the kidney, but also in a variety of other organs including lung, small intestine, brain, skin and choroid plexus. In human kidney OCT 2 is expressed in all three segments of proximal tubules whereas in rat kidney it is located to the S2 and S3 segments [124, 125]. Similar to OCT 1, OCT 2 is localized to the basolateral membrane of epithelial cells in renal proximal tubules and small intestine. It is responsible for the cellular uptake of organic cations as the first step of tubular secretion. In several regions of the brain (e.g. hippocampus) it is likely involved in the control of extracellular neurotransmitter concentrations and may facilitate the transport of several drugs across the blood–brain barrier [126]. In the lung, OCT 2 may mediate epithelial release of acetylcholine during non-neuronal cholinergic regulation [127].

1.4.2.3 **Organic cation transporter 3**

Organic cation transporter 3 is expressed in a broad variety of tissues, including brain, heart, liver, skeletal muscle, lung, kidney, small intestine, skin, mammary gland, and placenta. In the liver, OCT3 is expressed at the sinusoidal membrane of hepatocytes [123], while in the brain it is expressed in several regions including cortex, hippocampus, and substantia nigra. Organic cation transporter 3 mediates (together with OCT 1) the initial step in biliary excretion of organic cations in the liver, while it contributes to the regulation of aminergic neurotransmission in the brain. A deficiency of OCT 3 in the brain of mice leads to behavioral alterations in response to stress and anxiety [128].

1.4.3 **Substrate specificities of organic cation transporters**

In addition to endogenous substrates, numerous drugs and other xenobiotics are transported by OCTs proteins, including anti-diabetic and anti-cancer drugs (Tables 2 – 4). Organic cation transporter proteins translocate organic cations across the plasma membrane in either direction and in an electrogenic manner. They operate independently of Na^+, and are independent of proton gradients when the effect of proton gradients on the membrane potential is excluded [102].

Table 2: Drug substrates of OCTs.

Class	Drugs	References
Antidiabetic	Metformin	[8]
Antiviral	Lamivudine, Aquinavir, Nelfinavir	[129, 130]
Anesthetic	Midazolam	[131]
Antimicrobial	Ciprofloxacin	[132]
Antineoplastic	Cisplatin, Oxaliplatin	[133]
Antimalarial	Quinine	[134]
Antiemetic	Tropisetron	[135]
Tyrosine Kinase Inhibitors	Imatinib	[136, 137]
Histamine H2 Receptor Antagonist	Cimetidine, Ramitidine	[131, 138]
Anti-demential	Memantine	[139]
NMDA receptors	Amantadine	[99]
Anti-hypotensive	Etilefrine	[99]
Anti-arrhythmic	Quinidine	[140]

Table 3: Neurotransmitter substrates of OCTs.

Category	Compounds	References
Vitamins	Choline	[141]
monoamine neurotransmitters	Serotonin	[126, 142]
	Histamine	
	Catecholamines (Dopamine	
	Norepinephrine	
	Epinephrine)	
polyamine neurotransmitters	Agmantine	[143, 144]
	Prutescine	
	Spermidine	

Table 4: Xenobiotic substrates of OCTs.

Category	Xenobiotics	References
Herbicides	Paraquat	[145]
Quaternary ammonium compounds	Tetraethylammonium (TEA)	[131, 146]
Alkaloids	Berberine	[147, 148]
	Nicotine	
Fluorescent Dyes	Ethidium Bromide	[149–151]
	4-[4(dimethylamino)-styryl]-N-methylpyridinium (ASP)	
	4',6-Diamidino-2-Phenylindole (DAPI)	
Neurotoxins	1-methyl-4-phenylpyridinium (MPP+)	[101, 152]
	Aflatoxin B	

1.4.4 **Clinical importance of organic cation transporters and regulation**

The importance of transporters in influencing the pharmacokinetics of drugs has become increasingly evident [153]. Here the targeted genetic disruption of a drug transporter in mice (OCT knockout mice models) provided the first direct evidence of the significance of drug transporters to metformin disposition *in vivo*. For example, OCT 1 (−/−) and OCT 2 (−/−) mice are viable and display no obvious phenotypic abnormalities [141, 154]. However, OCT 1 (−/−) mice show dramatically reduced hepatic uptake of tetraethylammonium (TEA) and metformin [155], whereas renal excretion of metformin is virtually unchanged compared with OCT 1 (+/+) mice [81]. In addition, distribution in the small intestine is also decreased in OCT 1 (-/-) mice. In mouse hepatocytes the deletion of OCT 1 results in a reduction in the effects of metformin on AMPK phosphorylation and gluconeogenesis. When mice are administered metformin, the blood lactate concentration significantly increases in OCT 1 (+/+) mice, whereas only a slight increase was observed in OCT 1 (−/−) mice. Thus OCT1 is responsible for the hepatic uptake of metformin, and the liver seems to be the key organ responsible for lactic acidosis [15]. In OCT 1 and OCT 2 double knock-out OCT 1/2 (−/−) mice, renal excretion of TEA is abolished and its plasma levels are substantially increased [15]. Considering the differences in renal OCT expression between mice (OCT1 and OCT 2) and human (OCT2), a combined deficiency of OCT 1 and OCT 2 in mice may better reflect the effect of OCT 2 deficiency in humans. Metformin clearance is also reduced 4.5-fold from renal blood flow to the unbound glomerular filtration rate and the volume of distribution is reduced 3.5-fold in OCT 1/2 (−/−) mice [156]. The broad substrate specificity and strategic localization of OCT 1 and OCT 2 in the major excretory organs suggest that these proteins are essential in the removal of cationic toxins and waste products from the body via the liver, kidney and intestine. Therefore alterations of their activity and expression may cause toxicity and alter their pharmacological effects [81].

1.4.5 **Metformin-associated lactic acidosis and contraindications**

Metformin is excreted unmodified by the kidneys. In renal impairment, it may be more prone to elevated plasma metformin concentrations. Such accumulation of metformin is related to increased plasma lactate levels and is typically necessary to cause metformin-associated lactic acidosis (MALA) [157].

Lactic acidosis (LA) is a life-threatening condition characterized by hyperlactatemia (resting blood lactate concentration > 5 mmol/l), acidosis (low pH in body tissues and blood acidosis (serum pH < 7.35), and electrolyte disturbances with an increased anion gap [158]. Metformin-associated lactic acidosis is a grave but infrequent complication, typically associated with 50% mortality. Reported predisposing factors to MALA include inappropriate drug dosing, significant kidney impairment (GFR < 30), sepsis, hypovolemia, excessive alcohol intake, hepatic insufficiency, age greater than 80 years, or acute/decompensated congestive heart failure [159].

Phenformin and buformin are two biguanides that are more associated with many cases of lactic acidosis in diabetic patients and by the end of the 1970 s they were withdrawn for use in humans as a result of safety concerns. However, the role of metformin in lactic acidosis is still a matter of serious debate [160]. Epidemiological studies provide conflicting results with some suggesting that its use is associated with a higher incidence of lactic acidosis while others show no association [161, 162]. In a Cochrane review and meta-analysis on lactic acidosis with metformin use, Salpeter *et al.* (2010) pooled data from 347 comparative trials and cohort studies. Here no cases of lactic acidosis were found among 70490 metformin patient users, nor among 55451 non-metformin patients. The upper limit of the 95% confidence interval (95% CI) for the incidence of lactic acidosis per 100 000 patient-years was 4.3 cases in the metformin group and 5.4 cases in the non-metformin group. The authors concluded that there was no evidence to suggest that metformin was associated with increased

risk of lactic acidosis, or even with higher levels of plasma lactate [163]. In most MALA case reports additional risk factors for lactic acidosis were present [159, 164]. Moreover, mortality in patients with MALA appears to be lower than patients with non-metformin-associated lactic acidosis [160]. Such evidence highlights the rarity of metformin-associated lactic acidosis and support the cautious expansion of metformin use to avoid depriving a substantial number of T2DM patients from its therapeutic benefits. In April 2016 the Food and Drug Administration (FDA) revised its warnings regarding metformin usage in some patients with reduced kidney function [165]. According to current guidelines, the dose of metformin should be reviewed if the estimated glomerular filtration rate (eGFR) falls to 0.45 mL/min/1.73 m^2 and the drug should be stopped in patients with an eGFR of 0.30 mL/min/ 1.73 m^2 [166]. However, the efficacy of metformin varies considerably from patient to patient and this may in part be due to its pharmacogenomics.

1.4.6 **The pharmacogenomics of metformin**

Despite its widespread use, metformin is not a panacea. Clinical practice indicates that there are considerable variations in the response to metformin, with about 35% of patients failing to achieve initial glycemic control on metformin monotherapy [167, 168]. A considerable amount of pharmacogenetic research has demonstrated that genetic variation is one of the major factors affecting metformin response [169]. Moreover, it has been demonstrated that genetic polymorphisms in gene encoding drug-metabolising enzymes and drug transporters contribute to inter-individual variability in the pharmacokinetics/pharmacodynamics profiles of clinical important drugs [105]. The most studied transporter regarding the impact of genetic variation on metformin action has been OCT1. The gene encoding OCT1 is highly polymorphic, with a number of coding missense single nucleotide polymorphisms (SNPs) that affect its activity [170]. Several nonsynonymous variants in the OCT1 gene (R61C/rs12208357, G401S/rs34130495, M420del/rs72552763 and/or G465R/rs34059508) exhibit reduced transport activity, resulting in decrease hepatocellular uptake of metformin

[155]. By contrast, some other synonymous variants of OCT1 gene (Met408Val/ rs628031) are associated with a significantly decreased glucose-lowering response to metformin in healthy volunteers. On the other hand, no remarkable differences were found in the prevalence of OCT1 variants between metformin responders and non-responders [105]. Concerning polymorphisms affecting drug-metabolising enzymes, most studies have been focused on phase 1 enzymes such as the cytochrome P450 (CYP) monooxygenases [171]. Metformin is not metabolized and is excreted unchanged therefore, data on the clinical implication of genetic variations of drug-metabolising enzymes on metformin are scarce [172]. However, polymorphisms on CYP isoforms that have been extensively studied like CYP2C9 have been reported to account for~ 40% of the interindividual and interethnic pharmacokinetic differences in responses to sulphonylureas [173-175].

Focusing on co-treatments and an interest of our laboratory, several lines of evidence based on *in vivo* animal models and some clinical studies have emerged and support the concept that plant-derived flavonoids can elicit beneficial effects on diabetes by improving glycemic control, lipid profile, and anti-oxidant status. Here naringin (or its aglycone, naringenin) is a key focus of our laboratory as it is a plant flavonoid that exhibits anti-diabetic, anti-viral, anti-inflammatory, anti-oxidant, anti-dyslipidemic and cardioprotective effects [19, 176].

1.5 **Naringenin**

1.5.1 **Physico-chemical characteristics**

Naringin belongs to flavanones subclasses that are the most common flavonoid compounds present in citrus fruits, tomatoes, some berries and mint [19]. It is hydrolyzed to its aglycone, naringenin by intestinal bacteria naringinase complex. Naringin (molecular formula: C27H32O14) - like all flavonoids - has a chemical structure based on 15 carbon atoms, and 3 rings of which 2 are benzene rings connected with a 3-carbon chain. Two rhamnose units are attached to its aglycon portion - naringenin ($C_{15}H_{12}O_5$) - at the 7-carbon position (Figure 7)

[31]. Naringenin appears to be more biologically potent than naringin because its sugar moiety causes steric hindrance of the scavenging group. Of note, the clinical utility of naringenin is limited by its low solubility and minimal bioavailability (less than 5%) owing to its largely hydrophobic ring structure [177].

After oral intake, naringin is hydrolyzed in the small intestine and colon, respectively, and the released naringenin is rapidly converted into its respective glucuronide, sulfate and sulfoglucuronide, during its passage across the small intestine and liver. Finally, the bioactive forms (metabolites) are distributed in plasma to various cell sites and significant quantities can also be found in urinary excretions [178–180].

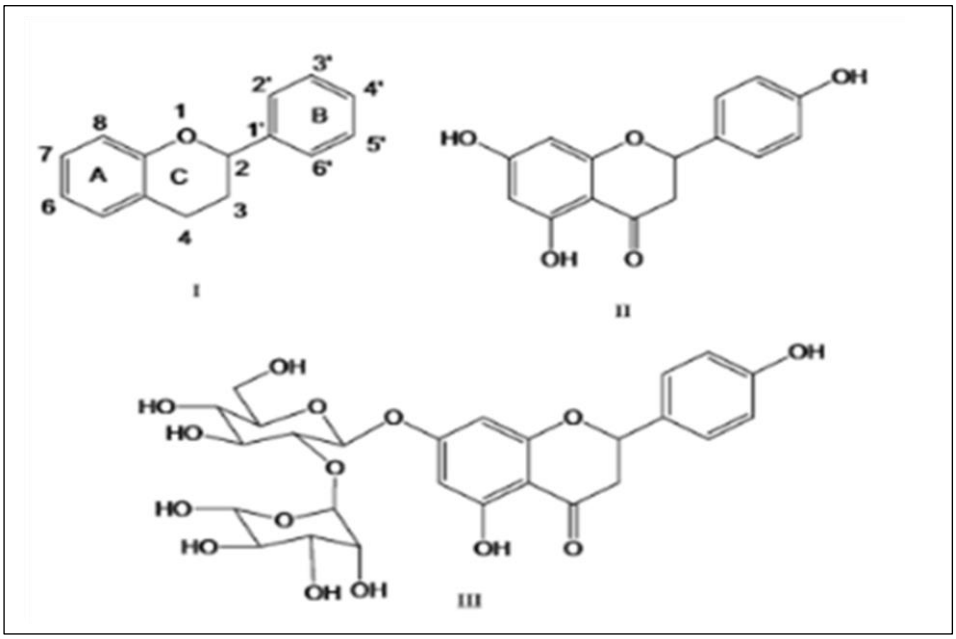

Figure 7: Chemical structures of flavonoids (I), naringin (II) and naringenin (III) [181].

1.5.2 **Biological activities and molecular mechanisms of action of naringenin**

1.5.2.1 **Anti-hyperglycemic effects**

Although naringenin has been demonstrated to have a wide range of biochemical effects on hyperglycemia, there is currently no unifying mechanism of its action. It was reported that

naringenin is beneficial for improving hyperlipidemia and hyperglycemia in T2DM animals by regulating fatty acid and cholesterol metabolism. In particular affecting the gene expression of glucose-regulating enzymes [182, 183], including the downregulation of hepatic PEPCK and G6Pase [184].

The *in vivo* anti-hyperglycemic action of naringenin and its derivatives can also be a consequence of extra-hepatic action [185]. Here it inhibits intestinal glucose uptake and renal glucose reabsorption by inhibition of Na^+ glucose co-transporters. Naringenin and naringin-like metformin do not appear to be insulin secretagogues and instead exert their anti-hyperglycemic effects without insulin. However, they may also enhance insulin secretion both *in vivo* and *in vitro* by relieving oxidative stress in pancreatic β-cells [186–188]. Furthermore, naringin can increase insulin sensitivity by enhancing tyrosine phosphorylation [189], suggesting its capacity to relieve insulin resistance and also enhance glucose uptake in skeletal muscles. This may likely occur by it antagonizing glucagon signaling pathways (similarly to metformin) [190, 191]. It was also found that naringenin increases glucose uptake by skeletal muscle cells in an AMPK-dependent manner [190] and it may exert this effect in a manner similar to metformin. However, the gastrointestinal side-effects of metformin resulting from the inhibition of mitochondrial glycerophosphate deshydrogenase are not shared with naringenin which so far displays no known toxicities in either experimental animals or clinically.

1.5.2.2 **Anti-inflammatory effects**

Naringenin can reduce oxidative stress, inhibit leukocyte recruitment, activate macrophage nuclear factor erythroid 2-related factor 2 (Nrf2) which induces heme oxygenase (HO-1) expression and also suppresses activation of nuclear factor-kappa B (NF-κB) in macrophages. This causes production of pro-hyperanalgesic cytokines such as interleukin-33 (IL-33), tumor necrosis factor (TNF-α), IL-1ß and IL-6 [192–195]. Naringenin has further been reported to

prevent cholesterol-induced systemic inflammation, atherosclerosis, and platelet activation, and also able to suppress the production of pro-inflammatory cytokines (murine endotoxemia model) by inhibiting NF-κB and MAP kinase signaling pathways [196–198].

1.5.2.3 **Anti-oxidant effects**

Naringenin has been shown to boost anti-oxidant capacity in vivo by increasing hepatic concentrations of catalase, superoxide dismutase and glutathione peroxidase [199]. This conferred reno-protective effects by relieving oxidative stress in streptozotocin-induced diabetes [200] and also ameliorated daunorubicin-induced nephrotoxicity by mitigating angiotensin receptor 1 (AT1R), extracellular signal-regulated kinases (ERK1)/2-NFκB p65 mediated inflammation [201]. Antioxidant effects of naringenin appear to be largely mediated by direct scavenging of free radicals due to the phenolic hydroxyl groups attached to the flavonoid structure [202–204]. By scavenging radicals naringenin can inhibit LDL oxidation [205] and thus it may – theoretically – trigger anti-atherosclerotic effects.

1.5.2.4 **Anti-hyperlipidemic effects**

Naringenin decreases plasma lipids and cholesterol in high-cholesterol-diet–fed rats [64]. The cholesterol-lowering effect of naringenin was observed in low density lipoprotein receptor (LDLr) knockout mice by preventing production and increasing oxidation of free fatty acids, preventing hepatic steatosis, and overproduction of very low-density lipoproteins (VLDL) [65]. Moreover, naringenin significantly reduces hepatic 3-hydroxy3-methylglutamyl CoA (HMG-CoA) reductase and acyl-CoA cholesterol acyltransferase (ACAT) activities that catalyze rate-limiting steps in the cholesterol biosynthetic pathway [206]. In addition, naringenin can prevent dyslipidemia by limiting apoprotein B overproduction through activation of hepatic PPARα gene transcription and upregulation of fatty acid oxidation effectors CPT-1 and UCP-2 [207–209].

1.5.2.5 **Anti-tumor effects**

The anti-neoplastic potential of naringenin is currently gaining recognition. Here it can induce apoptosis via up-regulation of Fas/FasL expression, activation of caspase cascades, and inhibition of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) survival signaling pathways in the tested cells [210]. Naringenin could also inhibit the outgrowth of metastases after surgery via regulating host immunity in breast cancer cells [211]. Moreover, human myeloid leukemia HL-60 cells treated with naringenin with or without caspase inhibitors initiated the caspase cascade through an intrinsic apoptotic pathway [212]. Studies on human acute lymphoblastic leukemia MT-4 cells also showed that naringenin-treated cells showed different cell cycle profiles, with accumulation in G2/M phase [213].

1.5.3 **Naringenin and drug disposition**

There are not many studies that evaluated the effects of naringenin in drug disposition. However, some have been performed with grape fruit juice which is known to contain naringenin as its most relevant compounds [20, 214, 215]. Pharmacokinetic studies demonstrated that grape fruit juice can increase the bioavailability of drugs (array of therapeutic classes). Examples include some calcium channel blockers, statins and benzodiazepines [17]. The elevation in a drug's area-under-the-plasma concentration-time curve with grape fruit juice ingestion is the result of irreversible inhibition of cytochrome P450 (CYP) 3A [214]. On the other hand, grapefruit juice can also decrease the bioavailability of some drugs (e.g. fexofenadine) [215]. The putative mechanism underlying such interactions is a reduction in drug uptake transport via inhibition of organic anion transporting polypeptides (OATPs) by grape fruit flavonoids [20]. Naringin and its aglycone (naringenin) are capable of inhibiting the OATP1B1-mediated uptake of dehydroepiandrosterone sulfate and the OATP1A2-mediated uptake of fexofenadine in stably transfected HeLa cells [20, 216]. In addition, *in vitro* investigations reported an inhibition of the protein transporter P-glycoprotein (P-gp) activity by grape fruit juice extracts, and grape

fruit juice constituents [214]. Modulation of intestinal P-gp-dependent transport by grapefruit juice may lead to changes in bioavailability of drugs that are substrates of P-gp itself. This occurs by its effects on pre-systemic clearance. However, three studies of intestinal biopsy samples demonstrated that grape fruit juice ingestion does not alter the mRNA and protein expression levels of P-gp [217]. This is similar to grape fruit juice's effect on OATP expression and suggests that grapefruit juice -mediated inhibition of P-gp would be short-lived [218]. To our knowledge, no study has previously investigated the effects of naringenin on metformin transporters OCT1 and OCT2.

1.6 References

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CHAPTER TWO

This chapter provides a protocol and a systematic review

Protocol:

Edith Pascale Mofo Mato, Magellan Guewo-Fokeng, M. Faadiel Essop, and Peter Mark Oroma Owira (2018). Genetic polymorphisms of organic cation transporters 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes mellitus: a systematic review protocol. BMC Systematic Reviews,7: 105. http://doi.org/10.1186/s13643-018-0773-y

Systematic review:

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Systematic Reviews

PROTOCOL Open Access

Genetic polymorphisms of organic cation transporters 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes mellitus: a systematic review protocol

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Abstract

Background: Metformin is one of the most commonly used drugs for type 2 diabetes mellitus (T2DM). Despite its efficacy and safety, metformin is frequently associated with highly variable glycemic responses, which is hypothesized to be the result of genetic variations in its transport by organic cation transporters (OCTs). This systematic review aims to highlight and summarize the overall effects of OCT1 polymorphisms on therapeutic responses to metformin and to evaluate their potential role in terms of interethnic differences with metformin responses.

Methods/design: We will systematically review observational studies reporting on the genetic association between OCT1 polymorphisms and metformin responses in T2DM patients. A comprehensive search strategy formulated with the help of a librarian will be used to search MEDLINE via PubMed, Embase, and CINAHL for relevant studies published between January 1990 and July 2017. Two review authors will independently screen titles and abstracts in duplicate, extract data, and assess the risk of bias with discrepancies resolved by discussion or arbitration of a third review author. Mined data will be grouped according to OCT1 polymorphisms, and their effects on therapeutic responses to metformin will be narratively synthesized. If sufficient numbers of homogeneous studies are scored, meta-analyses will be performed to obtain pooled effect estimates. Funnel plots analysis and Egger's test will be used to assess publication bias. This study will be reported according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines.

Discussion: This review will summarize the genetic effects of OCT1 polymorphisms associated with variabilities in glycemic responses to metformin. The findings of this study could help to develop genetic tests that could predict a person's response to metformin treatment and create personalized drugs with greater efficacy and safety.

Systematic review registration: Registration number: PROSPERO, CRD42017079978

Keywords: Genetic polymorphisms, Glycemic response, Metformin, OCT1, Type 2 diabetes mellitus

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Background

Metformin is recommended by major clinical practice guidelines as first-line oral anti-hyperglycemic drug for use as monotherapy in individuals with newly diagnosed T2DM [1]. It specifically decreases hepatic gluconeogenesis without enhancing insulin secretion, inducing weight gain, or increasing the risk of hypoglycemia [2].

The precise molecular mechanisms underlying metformin's action are not well understood. It was initially suggested that one of the key actions of metformin is to activate AMP-activated protein kinase (AMPK) through a decrease in hepatic energetic status (increasing AMP: ADP and/or ADP/ATP concentration ratios) or through an upstream AMPK kinase (LKB1), leading to lowered transcription of gluconeogenic genes [3]. However, recent investigations in conditional AMPK knockout mice showed that metformin inhibits hepatic gluconeogenesis in an LKB1- and AMPK-independent manner via a decrease in hepatic energetic status [4, 5]. This preferential action of metformin in hepatocytes is due to the predominant expression of organic cation transporter 1 (OCT1), the main transporter responsible for hepatic uptake of metformin [6, 7].

OCT1 belongs to the solute carriers (SLC) 22A superfamily of polyspecific membrane proteins that play a central role in transportation of organic cations, anions, and zwitterions thus playing a major role in the cellular organic ions homeostasis [8, 9]. In human, OCT1 is most strongly expressed in the liver, whereas in rodents, OCT1 is also strongly expressed in the kidney and small intestine. In human and rat liver, OCT1 is located to the sinusoidal membrane of the hepatocytes [10]. The major function of OCT1 most likely is mediating the uptake of organic cations in hepatocytes as the initial step of biliary secretion [11].

Despite its widespread use, there are considerable variations in response to metformin therapy ranging from improvement in HbA1c levels (by up to 4%) to estimates of ~ 35% failure to achieve the glycemic goal (HbA1c level less than 7%) [12]. These variabilities in metformin efficacy clearly suggest the implication of individual genetic imprints [13]. Since genetic variations in some drug transporters can dramatically alter the pharmacokinetics and pharmacodynamics of many drugs, studies conducted in different population groups have suggested that OCT1 genetic polymorphisms could affect metformin responses [14, 15]. However, there is no consensus about its precise effect; both positive and negative findings have been reported [14, 16–19]. Consequently, there is need to review the existing studies linking OCT1 variants and metformin responses in order to (a) summarize the overall effects of OCT1 polymorphisms on therapeutic responses elicited by metformin intake and (b) to evaluate the potential role of such polymorphisms in terms of interethnic differences in response to metformin therapy.

Previous literatures

Preliminary studies have reported the effects of OCT1 variants on metformin responses in T2DM individuals [16, 20–24]. In a meta-analysis carried out by Dujic et al. (2017), in order to clarify the significance of genetic variations of metformin transporter genes on glycemic response to metformin, nine candidate variants in membrane transporter genes (thereof 3 variants from OCT1) were analyzed in 7968 individuals across the cohorts of the Metformin Genetics consortium (MetGen). The authors show that the candidate variants in membrane transporter genes showed no significant effect on metformin response assessed as HbA1c reduction in patients with T2DM [14].

Why we will conduct this systematic review?

The previous meta-analysis studied the effects of three candidate OCT1 variants on glycemic response to metformin in 7968 MetGen participants of European ancestry.

- Our study will not be restricted on pre-specified OCT1 variants. We will analyze every OCT1 variant identified that could be related to metformin response. Of note, Seitz et al. performed a global scale population analysis of OCT1 variants and identified 85 variants in 52 worldwide population groups that included sub-Saharan Africa, the Middle East and North Africa, Central Asia, East Asia and Oceania, Europe, and America [15].
- Our study will add studies that were not included in the previous meta-analysis [17, 22–26].
- Genetic variation frequencies differ among different ethnicities, which may be associated with variation of susceptibility to adverse drug reactions among the different populations [27]. In this study, we will also compare the allele frequency distribution of OCT1 genetic variants among different ethnicities.

Objective

The objective of this systematic review is to highlight and summarize the overall effects of OCT1 polymorphisms on therapeutic responses to metformin and to evaluate their potential roles in terms of interethnic differences with metformin therapy.

Methods

This systematic review protocol is reported following the Preferred Reporting Items for the Systematic Reviews and Meta-analysis Protocols (PRISMA-P) 2015 Checklist (Additional file 1) [28].

Type of participants

Participants included in eligible studies must be diagnosed with T2DM and treated with metformin monotherapy for at least 3 months.

Most guidelines (American Diabetes Association, European Association for the Study of Diabetes, American Association of Clinical Endocrinologists) for the management of T2DM recommend an initial approach consisting of lifestyle changes and monotherapy, preferably with metformin. This recommendation is applicable if a patient is diagnosed with an initial HbA1c less than 9% and no existing contraindications (eGFR < 30 ml/min/1.73 m2) [29]. Three-month period on metformin monotherapy is reasonable to assess for glycemic improvement. Treatment modification is recommended when the HbA1c target (HbA1c > 7%) is not achieved or maintained by metformin monotherapy at maximal tolerated dose over 3 to 6 months [2].

Type of exposure

We will include studies in which participants were genotyped to investigate genetic variants of OCT1.

Comparators

The comparators are the responders and non-responders to metformin treatment. Response to metformin will be graded based on HbA1c level. Non-responders will constitute patients whose HbA1c levels declined by less than 1% after 3 months of treatment. Responders will be cases where HbA1c levels decreased by 1% or more after 3 months of treatment.

Outcomes

The primary outcome is the clinical effect of OCT1 polymorphisms on metformin response. Genetic polymorphisms of OCT1 will include single-nucleotide polymorphisms (SNPs), deletions, duplications, and copy-number variants. Where possible, effect estimate will include odds ratio and relative risk for the genetic variant effects in responders compared to non-responders.

Secondary outcomes include effects of OCT1 polymorphisms on fasting plasma glucose (FPG) and postprandial plasma glucose (PPG) after treatment with metformin monotherapy. FPG and PPG will be compared between responders and non-responders. The incidence of gastrointestinal side-effects will be also compared between responders and non-responders where possible.

Eligibility criteria

Inclusion criteria

- Cross-sectional, case-control, and cohort studies assessing the genetic effects of OCT1 variants on metformin responses (HbA1c, fasting plasma glucose levels, post-prandial glucose levels, and gastrointestinal side effects including nausea, vomiting, and diarrhea) in T2DM individuals, published between January 1990 and July 2017 without any geographical restriction
- Studies published in English or French
- Studies in which participants received metformin monotherapy as initial anti-hyperglycemic therapy for at least 3 months

Exclusion criteria

We will not consider:

- Letters, reviews, case reports, editorials, and comments
- Studies conducted with normal or pre-diabetes participants. Pre-diabetes is defined according to the following criteria: impaired fasting blood glucose (IFG) values between 100 and 125 mg/dL after at least 8 h of fasting, and/or glucose intolerance (ITG) when glycaemia values are between 140 and 199 mg/dL 2 h post oral administration of a 75 g glucose load (OGTT), and/or if the values of glycosylated hemoglobin (HbA1c) are between 5.7 and 6.4%
- Studies in which participants received only one dose of metformin
- Studies in which participants also have other conditions like chronic gastrointestinal diseases, chronic liver disease, cholelithiasis, chronic pancreatitis, inflammatory bowel disease, gastroduodenal ulcer, chronic kidney disease, and endocrine disorders
- Studies in which relevant data on metformin responses is lacking or impossible to extract

Search strategy for identifying relevant studies

We will search the following electronic databases: MEDLINE via PubMed, Embase, and CINAHL from January 1990 to July 2017 without any geographical restrictions. The choice of 1990 as onset date was made on the basis that OCT1 was cloned and functionally characterized in the early–mid-1990s [30, 31]. The search strategy based on the combination of relevant terms will be designed by a librarian. The main search strategy conducted in MEDLINE via PubMed is shown in Table 1. This search strategy will be adapted for possible extension to other databases and will be updated as we progress through the review. We will also manually search reference lists from relevant studies and contact experts in the field in order to identify additional eligible studies.

Data collection and analysis

Selection of studies for inclusion in the review

Two review authors (EPMM and MGF) will independently identify articles and sequentially screen their titles

Table 1 Search strategy in MEDLINE/PubMed

Search	Search terms
#1	"type 2 diabetes" OR Diabetes (MeSH Terms)
#2	"genetic markers" OR "genetic polymorphism" OR "Single nucleotide polymorphism" OR "Polymorphism" OR "variant" OR "gene" OR "allele" OR Genetic (MeSH Terms)
#3	"Organic Cation Transporter 1" OR "OCT 1" OR solute carrier family 22 organic cation transporter, member 1 (MeSH Terms)
#4	#1 AND #2 AND #3

and abstracts for eligibility. Thereafter, full texts of articles deemed potentially eligible will be retrieved. Further, these review authors will independently assess eligibility for inclusion in the review based on the inclusion and exclusion criteria. Any disagreements between the two review authors will be resolved by consensus and arbitration of a third review author if necessary. A PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis) flow diagram [32] will document the process of literature selection and reasons for exclusion.

Data extraction and management

Two review authors (EPMM and MGF) will independently extract data in accordance with the methods outlined in the Cochrane Handbook for systematic reviews of interventions. A data extraction form will be designed. Approximatively 10% of the eligible studies will be randomly selected and used as pilot in the data extraction sheet in order to ensure its suitability.

Data will be collected on the first author name, year of publication, geographical location (country where the study was performed), study design, sample size, participant characteristics (mean or median age, age range, proportion of males), duration of treatment with metformin monotherapy, relevant OCT1 polymorphism, minor allelic frequencies in each population with Hardy Weinberg equilibrium if available, and primary outcome measurements (measure of metformin response after treatment with metformin). Gastrointestinal side effects, glycated hemoglobin A1 (HbA1c) levels, fasting plasma glucose (FPG), and post-prandial plasma glucose (PPG) concentrations after the treatment with metformin will be used as indices for metformin responses. Any disagreements between the two review authors will be resolved through discussion and by consulting a third author if necessary. Should any article be duplicated, we will contact the corresponding author and include the more relevant version. For managing missing data, we will contact the corresponding author of the respective studies in an attempt to obtain the required details. If no correspondence is received, the study will be included in the systematic review and discussed in the narrative summary.

Data analysis including assessment of heterogeneity

Study characteristics and the effect estimates of OCT1 polymorphisms on metformin responses will be presented in full, in tabular form. Since this effect varies from one study to another, we will derive the pooled estimate of each polymorphism investigated in multiple studies (≥ 2), by using a random effect model.

Effects of potential confounding variables associated with metformin responses including metformin dosing, duration of treatment with metformin monotherapy, lifestyle changes, and drugs interactions will be dealt by using multivariable meta-regression analysis. All statistical analyses will be carried out using Stata statistical software version 14 (Stata Corporation, College Station, Texas, USA).

Statistical heterogeneity among the included studies will be assessed by the X^2 test on Cochrane's Q statistic. A P value less than 0.1 will indicate significant heterogeneity. The I^2 statistic test will be further used to quantify the heterogeneity in the measure of association across studies. Values of 25%, 50%, and 75% for I^2 will represent, respectively, low, medium, and high heterogeneity [33]. Where substantial heterogeneity is detected, a subgroup analysis will be performed to investigate the possible sources of heterogeneity using the following grouping variables: metformin dosing, sample size, lifestyle changes, practice of physical activity, and genotyping methods. If included studies differ significantly in design, sampling, and outcome measures, a narrative synthesis of the findings will be provided.

Subgroup analysis

We will conduct a subgroup analysis based on metformin dosing, sample size, lifestyle changes, and the genotyping method used to detect genetic variants. For the factor metformin dosing, < 1.500 mg vs > 1.500 mg will be compared. For the sample size, small vs large will be compared. For lifestyle changes, be on diet (yes vs no) and practice of physical activity (yes vs no) will be compared. For the genotyping methods, used Taqman vs others methods will be compared. Pooled odds ratios (ORs) and 95% confidence intervals (CI) in each subgroup will be calculated. The heterogeneity between subgroups will be detected by using the X^2 test on Cochrane's Q statistic.

Assessment of publication and reporting biases

We will assess publication bias by using standard approaches including Funnel Plots and Egger tests if enough eligible studies are available [34]. The reporting quality of each study will be independently assessed by two review authors (EPMM and MGF) using the STREGA (Strengthening the Reporting of Genetic Association

Studies) statement [35] which offers guidelines for reporting of individual genetic association studies.

Assessment of methodological and evidence qualities

Studies deemed fit for inclusion in the systematic review will be scored for methodological quality using the Newcastle-Ottawa assessment scale (NOS) [36]. Each domain will be rated with "high," "low," or "unclear" with regard to the risk of bias, with free text explanations.

We will also use the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology to assess the quality of evidence for each outcome. Quality rating of overall evidence will be downgraded according to five factors: risk of bias, inconsistency, indirectness, imprecision, and publication bias. In addition and where appropriate, the reasons to upgrade the evidence quality will include a large magnitude of effects, a dose-response gradient, and plausible residual confounding that would reduce a demonstrated effect or suggest a spurious effect when results show no effect. We will integrate downgrading and upgrading factors to obtain an overall quality of evidence for each outcome of interest. Overall quality of evidence will be then ranked as high, moderate, low, or very low as specified by the GRADE approach [37, 38].

Discussion

Type 2 diabetes a chronic degenerative metabolic disease represents a major medical and public health problem. After lifestyle changes failure, metformin is prescribed as first-line treatment for T2DM. However, metformin is not a panacea. Clinical practice indicates that there are considerable inter-individual variations in metformin response, with about 35% of patients failing to achieve initial glycemic control on metformin monotherapy [39, 40]. Several lines of evidence based on pharmacogenetic research have demonstrated that genetic variation is one of the major factors affecting metformin responses [41]. In addition, it is well known that genetic polymorphisms in gene encoding drug-metabolism enzymes and drug transporters contribute to interindividual variabilities in the pharmacokinetics/pharmacodynamics profiles of clinical drugs [42]. The most studied transporter regarding the impact of genetic variation on metformin action has been OCT1. The gene encoding OCT1 is highly polymorphic, with a number of coding missense single nucleotide polymorphisms that affect its activity and function [43]. This study will systematically review worldwide reports that have investigated an association between any OCT1 genetic polymorphism and therapeutic response to metformin in T2DM patients. Understanding the diversity of genetic markers associated with drug response across different global populations is essential to set ethnicity-specific reference for adverse drug reactions and identify patient groups whose genetic characteristics put them at special risk from either excessive or reduced pharmacologic effects of metformin. In terms of limitations, definitive conclusions may not be possible due to the small sample size and heterogeneity of study design and protocols.

Additional file

Additional file 1: PRISMA-P 2015 checklist. (DOC 82 kb)

Abbreviations

AMPK: Adenosine monophosphate-activated protein kinase; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; LKB1: Liver kinase B1; OCT1: Organic cation transporter 1; OCTs: Organic cation transporters; PPG: Postprandial plasma glucose; PRISMA: Preferred Reporting Items for Systematic Review and Meta-Analysis; STREGA: Strengthening the Reporting of Genetic Association Studies; TDM: Type 2 diabetes mellitus

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Availability of data and materials

All available data can be obtained by contacting the corresponding author.

Authors' contributions

EPMM conceived and designed the protocol and drafted and revised the manuscript for intellectual content. MGF designed the protocol and revised the manuscript for intellectual content. MFE supervised and revised the manuscript for intellectual content. PMOO supervised and revised the manuscript for intellectual content. All authors had full access to the data. EPMM is the guarantor of the review. All authors approved the final version of the submitted manuscript.

Ethics approval and consent to participate

Ethical approval is not required for this study as it is a systematic review.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Genetic polymorphisms of organic cation transporter 1 (OCT1) and responses to metformin therapy in individuals with type 2 diabetes

A systematic review

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Abstract

Background: Metformin is one of the most commonly used drugs for the treatment of type 2 diabetes mellitus (T2DM). Despite its widespread use, there are considerable interindividual variations in metformin response, with about 35% of patients failing to achieve initial glycemic control. These variabilities that reflect phenotypic differences in drug disposition and action may indeed be due to polymorphisms in genes that regulate pharmacokinetics and pharmacodynamics of metformin. Moreover, interethnic differences in drug responses in some cases correspond to substantial differences in the frequencies of the associated pharmacogenomics risk allele.

Aim: This study aims to highlight and summarize the overall effects of organic cation transporter 1(OCT1) polymorphisms on therapeutic responses to metformin and to evaluate the potential role of such polymorphisms in interethnic differences in metformin therapy.

Methods: We conducted a systematic review according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. We searched for PubMed/MEDLINE, Embase, and CINAHL, relevant studies reporting the effects of OCT1 polymorphisms on metformin therapy in T2DM individuals. Data were extracted on study design, population characteristics, relevant polymorphisms, measure of genetic association, and outcomes. The presence of gastrointestinal side effects, glycated hemoglobin A1 (HbA1c) levels, fasting plasma glucose (FPG), and postprandial plasma glucose (PPG) concentrations after treatment with metformin were chosen as measures of the metformin responses. This systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO).

Results: According to the data extracted, a total of 34 OCT1 polymorphisms were identified in 10 ethnic groups. Significant differences in the frequencies of common alleles were observed among these groups. *Met408Val (rs628031)* variant was the most extensively explored with metformin responses. Although some genotypes and alleles have been associated with deleterious effects on metformin response, others indeed, exhibited positive effects.

Conclusion: Genetic effects of OCT1 polymorphisms on metformin responses were population specific. Further investigations in other populations are required to set ethnicity-specific reference for metformin responses and to obtain a solid basis to design personalized therapeutic approaches for T2DM treatment.

Abbreviations: AMPK = adenosine monophosphate activated protein kinase, BMI = body mass index, FPG = fasting plasma glucose, HbA1c = glycosylated hemoglobin, LKB1 = liver kinase B1, OCT1 = organic cation transporter 1, OCTs = organic cation transporters, OR = odds ratio, PPG = postprandial plasma glucose, SLC22A1 = solute carrier family 22 member 1, SNP = single nucleotide polymorphism, T2DM = type 2 diabetes mellitus, WHO = World Health Organization.

Keywords: genetic polymorphisms, glycemic response, metformin, OCT1, type 2 diabetes mellitus

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Data availability statement: All data are all contained in supporting information files

The authors have no funding and no conflicts of interest to disclose.

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1. Introduction

Metformin is the first-choice oral anti-hyperglycemic drug for use as monotherapy in individuals with newly diagnosed type 2 diabetes mellitus (T2DM).[1,2] It has several beneficial effects on cardiovascular risk factors, cancer, and polycystic ovary syndrome.[3,4] Moreover, metformin specifically reduces hepatic gluconeogenesis without increasing insulin secretion, inducing weight gain or risk of hypoglycemia.[1,5] The precise molecular mechanisms of metformin action are not well understood. It was initially suggested that a key action of metformin was to activate AMP-activated protein kinase (AMPK) through a decrease in hepatic energy status (i.e., increasing AMP: ADP and/or ADP/ATP concentration ratio) or through an upstream modulator, liver kinase B1 (LKB1), thereby leading to a reduction in gluconeogenic gene transcription.[6] However, recent investigations in conditional AMPK knockout mice demonstrated that metformin inhibits hepatic gluconeogenesis in an LKB1- and AMPK-independent manner via a decrease in the hepatic energy state.[7] Emerging evidence also indicates that inhibition of mitochondrial glycerophosphate dehydrogenase (mGPD), a critical enzyme in the glycerophosphate shuttle, could be the primary mechanism of metformin-induced inhibition of gluconeogenesis.[8] This preferential action of metformin in hepatocytes is due to the predominant expression of organic cation transporters 1 (OCT1) that are responsible of hepatic uptake of metformin. OCT1 belongs to the Solute Carrier family *(SLC22A)* and is localized in the sinusoidal membrane of rat and human hepatocytes.[9] Other reported locations of human OCT1 include the lateral membrane of intestinal epithelial cells,[10] the luminal (apical) membrane of ciliated cells in the lung, and of tubule epithelial cells in the kidney.[11] The human *SLC22A1* gene encoding OCT1 consists of 11 exons, has been mapped to chromosome 6q26 and spans about 37 kb. OCT1 is highly polymorphic in ethnically diverse populations and mediate differences in transporter function.[12] This helps provide a possible mechanism to account for interindividual variations in the metformin responses.[13] Moreover, carriers with loss of function OCT1 polymorphisms displayed decreased hepatic metformin exposure after intravenous injection of ^{11}C metformin.[14] Many studies have identified genetic polymorphisms in the *SLC22A1* gene among different populations groups but there are still contradictory reports on the effects of OCT1 polymorphisms on metformin-related therapeutic responses.[15] In light of this, it is crucial to obtain a greater understanding of the influence of OCT1 polymorphisms in the context of variable responses elicited by metformin treatment. This systematic review therefore summarizes the overall effects of OCT1 polymorphisms on metformin-related therapeutic responses and also evaluates its potential role in terms of interethnic differences in this instance.

2. Methods

2.1. Literature search strategy

We searched the following electronic databases PubMed/MEDLINE, Embase, and CINAHL from January 1990 to July 2017, to identify studies reporting on the effects of OCT1 variants on metformin responses in T2DM individuals. The search strategy based on the combination of relevant terms was designed by a librarian. The main search strategy conducted in PubMed/Medline was as follows: "((((Diabetes [MeSH Terms]) OR type 2 diabetes)) AND (((((((Genetic [MeSH Terms]) OR genetic markers) OR genetic polymorphism) OR Single nucleotide polymorphism) OR Polymorphism) OR variant) OR gene) OR allele)) AND (((solute carrier family 22 organic cation transporter, member 1 [MeSH Terms]) OR Organic Cation Transporter 1) OR OCT 1)". This search strategy was adapted when searching other databases. The search was performed independently by 2 investigators (EPMM and MGF) who identified articles in sequential fashion (titles, abstracts, and then full texts). In addition, references cited in the selected articles and published reviews were manually searched in order to identify any additional relevant studies.

2.2. Study selection

We included genetic association studies that reported data on the genetic effects of OCT1 polymorphisms on levels of HbA1c, fasting plasma glucose (FPG) and post-prandial plasma glucose (PPG), and also on gastrointestinal side effects in T2DM individuals. Two review authors (EPMM and MGF) independently assessed eligibility for inclusion in the review based on the inclusion and exclusion criteria. Any disagreements between the 2 review authors were resolved by consensus or consulting a third review author if necessary. Here we excluded animal studies, review articles, meta-analyses, case reports, editorials, and comments. In addition, 2 articles written in Russian were also excluded. A PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis) flow diagram was used to document the process of literature selection and reasons for exclusion (Fig. 1).

2.3. Data extraction, assessment, and synthesis

Two reviewers (EPMM and MGF) independently extracted data using a preconceived data extraction sheet. Data were collected on the first author name, year of publication, geographical location (population where the study was performed), study design, sample size, participants' characteristics (mean or median age, age range, and proportion of males), duration of treatment with metformin monotherapy, relevant OCT1 polymorphisms, minor allelic frequencies in each population with Hardy Weinberg equilibrium if available, and primary outcome measurements (measure of metformin response after treatment with metformin). Disagreements were settled by consensus among the authors. The STREGA (Strengthening the Reporting of Genetic Association Studies) statement was used to assess the reporting quality of included studies.[16] Briefly, we assessed the reporting quality of all included studies in accordance with the following criteria: title and abstract, study design, selection criteria and basic characteristics of study participants, duration of metformin treatment, genotyping methods and its reliability, statistical method, accuracy and the outcome data on association between gene variants and the metformin responses. As the metrics used for assessment of genetic effects of OCT1 polymorphisms and study designs in the metformin response were not sufficiently similar, a narrative synthesis of the findings from the included studies was provided. This study is based on published data; therefore, ethical approval is not a requirement. This systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) on the December 7, 2017. Trial registration number: CRD42017079978.

3. Results

3.1. Study characteristics

Our initial search identified 4186 records: 1719 from PubMed/MEDLINE, 1907 from Embase, 546 from CINAHL, and 14 from

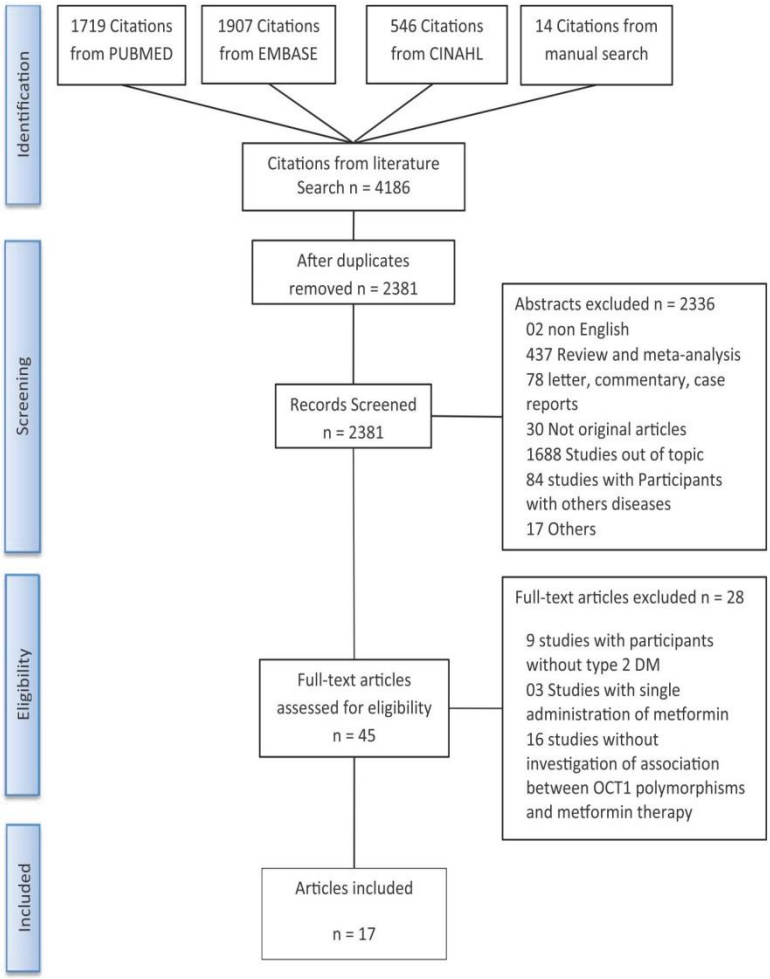

Figure 1. PRISMA flow diagram, describing the process of literature selection and reasons for exclusion. A total of 4186 records have been identified: 1719 from PubMed/MEDLINE, 1907 from Embase, 546 from CINAHL and 14 from manually searching the reference list of other articles. After screening of the titles and abstracts, 45 studies were found potentially eligible and their full texts were downloaded for further screenings. Of these 45 studies, we excluded 28 articles and the remaining 17 articles were included in the review.

manually searching the reference list of other articles. After screening of the titles and abstracts, 45 studies were found potentially eligible and their full texts were downloaded for further screenings. Of these 45 studies, we excluded 28 articles and the remaining 17 articles were included in the review (Fig. 1).

The majority of included studies were concentrated in Asia (47%), followed by Europe (29.4%). There were no study reported for the African continent. Of these eligible studies, 3 were conducted for each of the Caucasian and Indian population, 2 were conducted for each of the Chinese, Japanese and Scottish populations, while only 1 study was conducted for each of the Latvian, Danish, and Iranian populations. In 2 studies, the population was not specified. Most studies, but not all, reported sufficient details about selection criteria and basic characteristics for participants. The number of participants ranged from 33 to 2216, the proportion of men from 13.6% to 62.7% and the age of participants was above 29 years. Study participants were diagnosed with T2DM and treated with metformin monotherapy for at least 3 months and here the diagnosis was based on World Health Organization (WHO) criteria, HbA1c levels, FBG, and PPG. The response to metformin in individuals diagnosed with T2DM was categorized as responders and nonresponders, respectively. Nonresponders constituted patients whose HbA1c levels declined by <1% after 3 months treatment or who experienced gastrointestinal side effects, while responders were cases where HbA1c levels decreased by more than 1%. Measures of association were used to assess the effects of OCT1 polymorphisms in terms of the metformin response and included: differences in HbA1c, FPG, PPG levels among the various genotyping groups before and after metformin therapy, and odds ratios that were often adjusted for age, gender, and use of co-medications.

3.2. Genetic effects of OCT1 polymorphisms

Over 34 polymorphisms were investigated in 10 different populations with a minimum of 1 and a maximum of 12 per study. Significant differences in the frequencies of common variants have been observed among ethnic groups (Table 1).

3.2.1. rs628031 (1222A>G). The polymorphism *rs628031 (1222A>G)* was the most genotyped and its frequency has been found to range from 15% to 80% (median, 36%). Japanese population has the highest frequency (80%) while; *rs628031* is present only in 40% in Caucasians.[17,18] Association of *rs628031* with the glycemic response to metformin was assessed in 7 studies.[17–23] Here a study investigating *rs628031* in 277 Han Chinese participants found a significant reduction in HbA1c levels ($P<.02$) in individuals carrying the A/A genotype compared to those with the heterozygous genotype (A/G).[20] However, the A allele of A/A genotype was significantly associated with metformin side effects in a study conducted with 246 Latvian participants ($P=.02$).[22] By contrast, investigation of *rs628031* in Iranian,[19] Indian,[21] Caucasian,[18] and Japanese[17,23] populations showed that there were no association between this polymorphism and metformin responses.

3.2.2. rs122083571 (181C>T) and rs72552763 (1258_1260delATG). Polymorphisms *rs122083571 (181 C>T)* and *rs72552763 (1258_1260del ATG)* were assessed in 6 studies[13,22,24–28] and their frequencies varying from 67% to 89% and 18% to 28%, respectively. In 1 study with 92 participants, from unspecified population, the *rs122083571* and *rs72552763* polymorphisms were significantly associated with gastrointestinal side effects (OR=2.31, 95% CI [1.07–5.01], $P=.034$).[24] In addition, the investigation of *rs122083571* and *rs72552763* in 2216 participants from the GoDART Study found that such individuals (and receiving treatment with known OCT1 inhibitors) were over 4 times more likely to develop intolerance to metformin (OR=4.13, 95% CI 2.09–8.16, $P<.001$).[25] However, there was no association for such polymorphisms and metformin responses in Latvian and Danish populations.[22,27]

3.2.3. rs622342 (1386A>C). The association of *rs622342 (1386A>C)* with metformin responses was investigated in 5 studies that involved 3 populations (South Indian, Danish, and Caucasian)[18,27,29–31] with the frequency ranging from 5% to 37%. A study conducted in a Caucasian population found a positive significant association between *rs622342* and HbA1c levels ($P=.005$). This translated to an average of 0.28% lower decrease in HbA1c levels for each minor C allele.[31] By contrast, the major allele A of *rs622342* displayed a 5.6× greater chance of responding to metformin treatment in a South Indian population.[29] There was no significant relationship with HbA1c levels in a Danish population and also for 2 other studies involving a Caucasian population.[27,30]

3.2.4. rs2297374 (+43C>T). The polymorphism *rs2297374 (+43C>T)* and metformin response showed no significant association in Indian populations.[21] However, Han Chinese Shanghai individuals with the *rs 2297374 C/T* genotype exhibited significantly greater reductions in their FPG ($P=.002$) and HbA1c ($P=.039$) levels following metformin treatment versus homozygous *rs2297374* genotypes (C/C) and (T/T).[20] The frequency of *rs2297374* in these populations is close to 40%.

3.2.5. rs4646272 (−43T>G). The *rs4646272 (-43T>G)* polymorphism was assessed in 3 studies[17,21,31] with a frequency ranging from 20% to 67%. Here the one conducted with 66 Japanese subjects showed that *rs4646272* is a negative predictor of metformin efficacy.[17] However, the other studies showed no association.

3.2.6. rs34130495 (17857 G>A). Two studies assessed the association between *rs34130495 (17857 G>A)* and metformin responses.[25,27] Here a study investigating *rs34130495* in 371 Danish subjects found a significant association with absolute decreases in Hb1Ac levels.[27]

3.2.7. rs2282143 (1022C>T). *rs2282143 (1022C>T)* has been assessed in Indian population with a frequency of 20%. It has been demonstrated to impair OCT1 function in this population.[21]

3.2.8. rs1867351 (156T>C). The effect of *rs1867351 (156T>C)* on the glycemic response to metformin was investigated in Han Chinese and Indian populations with a frequency of 50% and 27%, respectively. No significant effect of the minor C allele has been shown while, the T/T genotype of *rs1867351* exhibited a greater reduction in PPG and HbA1c levels ($P=.020$) in Han Chinese population.[20]

3.2.9. rs594709 (597 A>G). The *rs594709 (597 A>G)* polymorphism was associated with the metformin treatment response in the Chinese population. Here GG genotype displayed a higher increase in FINS ($P=.015$) and a greater decrease in HOMA-IS ($P=.001$) and QUICKI ($P=.002$) than A allele carriers.[32]

3.2.10. rs200684404 (350C>T), 289C>A, and 616C>T. *rs200684404 (350C>T), 289C>A* and *616C>T* polymorphisms were investigated in the Japanese population and revealed a significant reduction in metformin uptake.[17]

3.2.11. Others polymorphisms. The association of other OCT1 polymorphisms, including *rs34104736*, *rs2297373*, *rs622591*, *rs2197296*, *rs4709400*, *rs461473*, *rs1443844*, *rs9457843*, and *rs6937722* was also investigated. However, no significant relationships were found between such variants and the metformin response.[18,23]

4. Discussion

Metformin is the most widely used first-line pharmacotherapy for T2DM. However, the interindividual variations in metformin efficacy ranging from improvement in HbA1c levels (by up to 4%) to a worse outcome with, estimates of ~35% failure rate with treatment clearly suggest that this treatment modality is affected by individual genetic imprints.[33–35] Although several studies have now identified a plethora of OCT1 genetic variants that underlie such interindividual differences,[36,37] no systematic review has thus far been conducted (as far as we are aware) to assess its impact. Most studies investigating the effects of OCT1 polymorphisms in the context of therapeutic responses to metformin for T2DM, essentially focused on European, Asian and Caucasian populations without consideration for other population group. Of note, Seitz et al[37] performed a global scale population analysis of OCT1 variants and identified 85 variants in 52 worldwide population groups that included sub-Saharan Africa, the Middle East and North Africa, Central Asia, East Asia and Oceania, Europe, and America. Although OCT1 polymorphisms have also been identified in Africans Americans and African populations such as the Xhosas (South Africa), Luhyas

Table 1

Characteristics of genetic association studies conducted.

Population	Reference	Study design	Total sample (Case/Control)	Age (Mean/ range), years	%Men	Duration of treatment	Polymorphism	MAF: (case/control) or population group	(*P*)* with HWE (i.a)	Reported outcome
Chinese	Di Xiao et al[32]	Case control	449 (53/214/182)	29–73	51	3 months	*rs 594709*	0.268 0.286	*P* = .555	Minor allele G is associated with higher increase of FINS (*P* = .015), decrease in HOMA-IS (*P* = .001) and QUICKI (*P* = .002).
Iranian	Shokri et al[19]	Cross-sectional	140 (77/63)	35–71	13.57	6 months	*rs628031*	0.331 0.317	–	No significant effect in metformin response (OR = 0.45, [95% CI 0.64–1.76], *P* = .45)
Not specify	Dujic et al [24]	Prospective observational cohort	92 (43/49)	51.48 ± 8.91	–	6 months	*rs12208357*	–	–	Significant association with common metformin induced gastrointestinal side effect. *P* = .034
							rs72552763	–	–	
GoDARTs Scotland	Dujic et al[25]	Observational cohort	2216 (251/1915)	57.80 ± 10.52	56.72	6 months	*rs12208357* *rs 55918055* *rs34130495* *rs72552763* *rs34059508*	–	–	Significant associated between intolerance to metformin and the presence of 2 reduced-function allele (*P* < .001)
South Indian	Umamaheswaran et al[29]	Observational cohort	122 (29/93)	31–60	39	3 months	*rs 622342*	0.362 0.156	–	Association of A allele with therapeutic efficacy of metformin and beneficial effects on HbA1c, FPG and PPG.
Han Chinese	Zhou et al[20]	Single-center prospective cohort	277 (153/124)	45–68	–	3 months	*rs1867351*	0.50 0.62	*P* = .44 *P* = .53	Association of T/T genotype with greater reductions in the HbA1c levels (*P* = .02).
							rs4709400	0.70 0.532	*P* = .45 *P* = .88	Association of G/G genotype with greater reductions in FPG levels (*P* = .046)
							rs628031	0.10 0.262	*P* = .49 P<0.01	Association of G/G genotype with greater reductions in FPG level (*P*<.01), and A/A genotype exhibited significantly greater reductions in HbA1c level (*P*<.02)
							rs2297374	0.40 0.343	*P* = .53 *P* = .43	Association of C/T genotype with greater reductions in FPG (*P* = .002) and HbA1c (*P* = .039) level.
Not specified	Maahroz et al[13]	Cross-sectional	108 (59/49)	43–63	19.4	3 months	*rs 72552763*	0.288 0.188	*P* = .088	No significant change in the ability of metformin to lower HbA1c (OR: 0.57, [95% CI 0.298–1.09], *P* = .088). More effective in reducing FPG.
Indian	D Sur et al[21]	Cross-sectional	50	46 ± 12.72	48	–	*rs 683369*	0.63	*P* = .0002	Unchanged function of OCT1
							rs 2282143	0.20	*P* = .008	Probably damaging function of OCT1
							rs628031	0.43	–	Unchanged function of OCT1
							rs1867351	0.27	*P* = .091	Not provided
							rs4646272	0.2	–	Not provided
							rs3737088	0.17	–	Not provided
							rs2282142	0.18	–	Not provided
							rs2297374	0.37	–	Not provided
South Indian Tamalian	Koshy et al[26]	Cross-sectional	60	35–55	–	–	*rs1220833571*	0.89	–	C allele is associated with poor glycemic control and significantly higher FPG, insulin and blood metformin level ($p < 0.05$)

(*continued*)

Table 1
(continued).

Population	Reference	Study design	Total sample (Case/Control)	Age (Mean/ range), years	%Men	Duration of treatment	Polymorphism	MAF: (case/control) or population group	(*P*)* with HWE (i.a)	Reported outcome
Caucasian	Tkáč et al[30]	Cross-sectional	148	57.5 ± 0.9	49	6 months	*rs622342*	0.05	*P* = .95	No significant relationship with HbA1c (*P* = .95)
Latvian	Tarasova et al[22]	Case control	246 (53/193)	50–69	30.1	≥3 months	*rs628031*	0.39	*P* = .785	Significant association with gastrointestinal side effects of metformin (OR = 0.389, [95% CI 0.186–0.815], *P* = .012)
							rs 34059508	0.04	P = 1	Not provided
							rs 72552763	0.18	P = 1	Not provided
							rs 36056065	0.39	*P* = .686	Significant association with side effects of metformin (OR = 0.405, [95% CI 0.226–0.724], *P* = .002).
Danish	Christensen et al[27]	Randomized controlled trial	371	52–62	62.7	9 months	*rs 122083571*	0.082	–	No significant effect in metformin response
							rs 34104736	0.0	–	No significant effect in metformin response
							rs34130495	0.044	–	Significant association with absolute decrease in Hb1Ac
							rs72552763	0.175	–	No significant effect in metformin response
							rs 461473	0.114	–	Significant association with initial decrease in Hb1Ac
							rs 622342	0.38	–	No significant effect in metformin response
Caucasian	Becker et al[31]	Prospective cohort	98	70–83	39	–	*rs 622342*	0.37	*P* = .40	A allele associated with decrease of Hb1Ac
Japanese	Chen et al[17]	Cross-sectional	66	46–71	48.5	–	*rs4646272*	0.34		Negative predictor of efficacy of metformin
							rs 200684404	0.023	–	Significant association with reduced uptake of metformin
							616C > T	0.008	–	Significant association with reduced uptake of metformin
							rs 628031	0.15	–	No significant effect in metformin response
							289C > A	0.017	–	Significant Association with reduced uptake of metformin
Scotland	Zhou et al[28]	Cross-sectional	1531	46–68	56.14	At least 6 months	*rs 122083571*	0.67	*P* > .05	No significant reduction in the ability of metformin to lower HbA1c (*P* = .47)
							rs 72552763	0.198	*P* > .05	No significant reduction in the ability of metformin to lower HbA1c (*P* = .92)
Caucasian	Becker et al[18]	Cross-sectional	102	70–83	39	–	*rs3798174*	0.05	*P* = .56	No significant effect in metformin response (*P* = .49)
							rs6937722	0.06	*P* = .49	No significant effect in metformin response (*P* = .40)
							rs3798168	0.02	*P* = .80	No significant effect in metformin response
							rs628031	0.40	*P* = .39	No significant effect in metformin response (*P* = .47)
							rs9457843	0.16	*P* = .63	No significant effect in metformin response (*P* = .40)
							rs3798167	0.19	*P* = .31	No significant effect in metformin response (*P* = .20)

(*continued*)

Table 1
(continued).

Population	Reference	Study design	Total sample (Case/Control)	Age (Mean/ range), years	%Men	Duration of treatment	Polymorphism	MAF: (case/control) or population group	(*P*)* with HWE (i.a)	Reported outcome
							rs2197296	0.26	*P*=.11	No significant effect in metformin response (*P*=.61)
							rs622342	0.37	*P*=.72	Association with glucose-lowering effect of metformin (*P*<.05)
							rs1443844	0.45	*P*=.60	No significant effect in metformin response (*P*=.18)
							rs2297374	0.41	*P*=.99	No significant effect in metformin response (*P*=.15)
							rs1564348	0.17	*P*=.55	No significant effect in metformin response (*P*=.71)
							rs622591	0.18	*P*=.81	No significant effect in metformin response (*P*=.19)
Japanese	Shikata et al[23]	Cross-sectional	33 (9/24)	29–73	27.3	>1 month	*rs2297373*	0.00	–	Not provided
							rs 200684404	0.02 0.06	–	Not provided
							rs683369	0.00 0.17	–	Not provided
							rs2282143	0.13 0.11	–	Not provided
							rs628031	0.19 0.72	–	Positive predictor for metformin efficacy
							rs1867351	0.81 0.56	–	Not provided
							243C>T	0.42 0.06	–	Not provided
							rs4646272	0.00 0.67	–	Negative predictor for metformin efficacy
							+26C>T	0.58 0.00	–	Not provided
							rs622591	0.02 0.61	–	Not provided
								0.54		

Reported outcome: genetic effect of polymorphism in the study population
FINS = fasting insulin, FPG = fasting plasma glucose, HWE (i.a) = Hardy Weinberg Equilibrium if applicable, HOMA IS = homeostatic model assessment of insulin sensitivity, MAF = minor allele frequency, PPG = postprandrial plasma glucose, QUICKI = quantitative insulin sensitivity check index.
(*P*)*: *P* value

(Kenya), and the Yorubas (Nigeria), their effects on metformin responses remain unknown.[38,39] In light of this, there is a robust need to complete association studies between OCT1 polymorphisms and therapeutic responses to metformin, by evaluating a comprehensive and representative dataset.

The common polymorphism *rs628031 (A>G)* causes a missense mutation in exon 7 that consists of an amino acid substitution of methionine to valine at position 408 *(Met408Val)* in the OCT1 protein.[18] Its frequency varies across ethnic population. *Met408Val* tends to lower OCT1 mRNA expression in enterocytes leading to decreased intestinal metformin uptake and hence its accumulation.[40] Here the local increase of metformin concentrations within intestinal tissues is proposed as a putative mechanism for gastrointestinal side effects.[41] This deleterious effect of *Met408Val* has been reported in Latvian population.[25] However, *Met408Val* has been also characterized as a variant that lacks strong effects (did not cause >50% decrease in OCT1 activity).[42,43] In agreement with this, no significant effects of *Met408Val* against metformin response have been found in Iranian,[19] Indian,[21] Caucasian,[18] and Japanese[17,23] populations. Surprisingly, G/G and A/A genotypes of *Met408Val* exhibited significant reduction of FPG and HbA1c in Han Chinese,[20] and have been revealed to be a positive predictor for metformin efficacy in Japanese population.[23]

Numerous studies reported the deleterious effect of *rs122083571* polymorphism.[24–26] The *181C>T* polymorphism at *rs122083571* consisting of an amino acid substitution (arginine to cysteine at position 61 *(Arg61Cys)*, is known to induce a robust substrate-wide loss of OCT1 activity, leading to decrease in OCT1-mediated uptake by more than 70% for all substrates tested (including metformin). Indeed, the *rs122083571* polymorphism is responsible for the retention of OCT1 proteins in the endoplasmic reticulum thus leading decreased sarcolemma protein expression.[37]

The *rs72552763* polymorphism constitutes a 3 bp deletion at position 420 *(Met420del)* and is the most common functional OCT1 variant. *Met420del* does not change OCT1 membrane localization and the exact mechanism how it affects OCT1 function remains unknown.[44] Although it is associated with gastrointestinal side effects in Asian and Caucasian populations, no significant effects were reported for European populations.[22,25,27] Functional modifications by *Met420del* appear substrate dependent and in combination with other OCT1 variants. For example, *Met420del* does not affect the uptake of MPP^+ (1-methyl-4-phenyl pyridinium). By contrast, it causes a robust decrease in metformin uptake (>60%) together with more than 80% reduction in tropisetron uptake.[37] If the *Met420del* manifests in combination with *Cys88Arg* or *Gly465Arg*, the encoded OCT1 will be inactive regardless of the substrate used.[45,46]

The *rs622342 (1386 A>C)* variant (in intron between exons 8 and 9) does not elicit strong effects on OCT1 function.[18] The A allele of *rs622342* variant has rather been associated beneficial effects on HbA1c in Caucasian population.[31] The study performed by Umamaheswaran et al[29] strengthened this positive effect of A allele on HbA1c in Indian population.

The *32870 G>A* polymorphism at *rs34059508* consisting of the amino acid substitution glycine to arginine at codon 465 *(Gly465Arg)*, leads to the impairment of OCT1 localization and complete inactivation of OCT1. The exact mechanisms leading to this impairment remain unclear.[44] Dujic et al[25] observed metformin intolerance in carriers of both *rs34059508* and another OCT1 reduced function.

The amino acid substitution of glycine to serine at codon 401 *(Gly401Ser)*, resulting from the *17857 G>A* polymorphism at *rs34130495*, causes a strong substrate-independent loss of OCT1 activity. The *Gly401Ser* variant apparently causes a general impairment of the transport process without affecting OCT1 membrane localization.[37] Surprisingly, in a study conducted in a Danish population, *Gly401Ser* was associated with a significant, absolute decrease in HbA1c levels.[27]

Cys88Arg (rs55918055) in exon 1 is a rare loss of function polymorphism that causes improper membrane localization of OCT1 in the cytoplasmic membrane. It has been associated with intolerance to metformin in a Scottish population.[25] *Cys88Arg* is located in the large extracellular loop that contains the transporter regulatory and substrate recognition domains. It is generally observed in combination with the *Met420del* variant. Substitution of Cysteine 88 by arginine destroys a cysteine residue known to build disulfide bonds. Indeed, several cysteine residues within the large extracellular loop between transmembrane helices 1 and 2 are involved in building intramolecular disulfide bonds essential for the oligomerization and targeting of OCT1 to the plasma membrane.[45–47]

The *1022C>T* polymorphism at *rs2282143* consisting of the amino acid substitution proline to leucine at codon 341. Pro341Leu, can either elicit no effects or instead decrease OCT1 activity (<50%).[37] The replacement of a rigid proline with leucine (which contains a relatively flexible side chain) could possibly change the local structure of OCT1.[24] This is in agreement with findings of the Indian population in which *rs2282143* has been reported to probably damage OCT1 function.[21]

The true effect of the intronic variant *rs4646272 (-43T>G)* and *rs2297374 (+43C>T)* remains unknown. *rs4646272 (-43T>G)* was considered as negative predictor of metformin efficacy in a Japanese population[17] while *rs4646272 (-43T>G)* exhibited a greater reduction of FPG and HbA1c in Han Chinese population.[20]

Other rare variants known to cause OCT1 loss of function, that is, *Pro117Leu (rs200684404)*, *Gln97Lys (289C>A)*, *Arg206Cys (616C>T)*, and *Ser189Leu (rs34104736)*, were also reported and associated with significantly lowered metformin uptake.[17,21,23,27] For Gln97Lys, the replacement of the polar glutamate with a stronger positively charged lysine at 97 could increase the repulsion of cationic substrates. By contrast, the reduced activity of the *Arg206Cys* can be explained by decreased export of the OCT1 from the endoplasmic reticulum to the plasma membrane.[26]

This systematic review demonstrated that the potential role of OCT1 polymorphisms in metformin therapeutic responses is population specific and some of them exhibited positive effects on metformin efficacy. The controversial findings related to these polymorphisms may be attributable to differences in the frequency of associated genetic variants and/or population differences that could be genetic or environnemental.[48] Indeed, environmental factors like chemicals and radiation exposure, lifestyle factors like diet, drinking, smoking, exercises, and physiological factors like age, sex can also work alone or in combination to influence drug responses.[49,50] Advancing age for example is characterized by physiological changes affecting different organ systems and their implications for pharmacokinetics and pharmacodynamics of drugs. Although very few data exist in literature about the effect of biguanides particularly metformin in aging patients, no evidence indicated that metformin should be denied "a priori" to aging T2DM patients.[51,52] However, age-dependent downregulation of OCT1 has been shown in mice brain microvessels of mice.[53]

Moreover, the way a person responds to a drug (this includes both positive and negative reactions) is a complex trait that is influenced by many different genes. Knowing all of the genes involved in drug response and understanding an individual's genetic make-up, can help to develop genetic tests that could predict a person's response to a particular drug and create personalized drugs with greater efficacy and safety.[54,55]

All studies included in this systematic review were rigorously assessed for their reporting quality—a key criterion for inclusion for analyses here completed. Different study designs have been used in included studies but, most of them had a cross-sectional design. As such, a causal relationship between genetic variants of OCT1 and responses to metformin cannot be inferred from these studies. For instance, confounding variables such as duration of treatment, metformin dosing might affect metformin responses leading to inconsistent results among studies. In terms of limitations, our investigation was conducted on a relatively small number of primary studies and more research work should help to obtain a wider range of relevant data to generate more conclusive findings. In addition, all studies surveyed did not always report key methodological information, for example, testing of the Hardy Weinberg Equilibrium and the sample size/power calculation. Some studies also reported a relatively small sample size meaning that the study population may not cover the entire spectrum of OCT1 variants. Thus larger sample sizes will enable a firmer correlation between OCT1 genetic variants and metformin responses. For example, we found that 2 studies included more than 1500 participants, 4 studies more than 200 participants, while the rest included less than 200 participants. Discordance of the type of study population and methods used for assessing the genetic effects of OCT1 variants may all have contributed to less powerful conclusions being derived by the current study. Finally, we were not able to pool data collected for a meta-analysis due to the methodological heterogeneity observed.

Great efforts are made to understand the effects of OCT1 genetic polymorphisms on interindividual variability in relation to metformin's clinical efficacy. However, some questions remain unanswered, e.g. the relationship between OCT1 variants and lactic acidosis. This is a crucial issue as lactic acidosis is a rare but potentially fatal metabolic consequence of metformin therapy.[56] For example, metformin-induced lactic acidosis is associated with an elevation in plasma metformin concentrations in patients with severe renal impairments and is considered as a contraindication of this drug. However, such adverse effects also occur in patients without well-known risk factors.[57] Thus a hypothesis emerges that OCT1 polymorphisms that decrease metformin uptake and cause its accumulation in circulation may induce lactic acidosis. However, further studies are required to investigate this intriguing notion.

In summary, this systematic review focused on the genetic effects of OCT1 polymorphisms on metformin treatment in T2DM patients. Our study shows evidence for a contribution of some OCT1 polymorphisms to variability in response to metformin with T2DM. Thus such associations remain unresolved and we suggest that further association studies be completed on defined populations with relatively large sample sizes as this should reveal significant insights into this vital clinical issue.

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All authors had full access to the data

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CHAPTER THREE

Manuscript Title: Naringenin exacerbates metformin-associated metabolic/lactic acidosis by upregulating hepatic organic cation transporter 1 protein expression.

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NARINGENIN EXACERBATES METFORMIN-ASSOCIATED METABOLIC/LACTIC ACIDOSIS BY UPREGULATING THE HEPATIC ORGANIC CATION TRANSPORTER 1 PROTEIN EXPRESSION

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Abstract

Hepatic uptake of metformin is predominantly dependent on organic cation transporter 1 (OCT 1) protein, and is an essential step in the reduction of hepatic glucose production as well as the occurrence of life-threatening condition, lactic acidosis. Naringenin is a citrus flavonoid that influences the bioavailability of some drugs by modulating protein expression of their transporters. This study investigates the effects of naringenin on hepatic OCT1 expression and metformin-associated metabolic/lactic acidosis in streptozotocin-induced diabetic rats. Forty-nine male Sprague Dawley rats were randomly divided into 7 groups (n =7) and were orally treated on a daily basis with 3.0 ml/kg body weight (BW) of distilled water (group 1) or 250 mg/kg BW of metformin (groups 3, 6 and 7) or 60 mg/kg BW of naringenin (groups 2, 5 and 7). Groups 4, 5, 6 and 7 were given a single intraperitoneal injection of 60 mg/kg BW of streptozotocin to induce diabetes. Body weights and water consumption were recorded daily and glucose tolerance tests (GTT) were performed. Thereafter, the animals were sacrificed and liver OCT1 expression assessed for all experimental groups. Diabetic rats treated with naringenin and metformin, either alone or in combination, exhibited weight gain, reduced polydipsia and serum anion gap compared to untreated diabetic rats. Co-administration of naringenin and metformin in diabetic rats significantly ($p= 0.02$) increased hepatic glycogen levels and plasma lactate concentration versus diabetic rats treated with metformin only. Furthermore, metformin and naringenin upregulated liver OCT1 protein expression in both normal and diabetic rats, respectively. We concluded that, upregulation of hepatic OCT1 protein expression by naringenin could exacerbate metformin-associated lactic acidosis.

Keywords: Naringenin, OCT1, metformin, lactic acidosis, diabetes mellitus

Introduction

Metformin, a synthetic 3-(diaminomethylidene) −1,1-dimethylguanidine (figure 1A) is commonly used as a first-line agent to treat type 2 diabetes mellitus owing to its favorable safety profile. It is a biguanide developed from galegine, a guanidine derivative found in Galega officinalis or the "French lilac" or "Goat ruce" [1]. Metformin lowers blood glucose in multiple ways that include suppression of hepatic gluconeogenesis, increased peripheral insulin-mediated glucose uptake, decreased fatty acid oxidation, and increased intestinal glucose consumption [2]. However, it is thought to increase the risk of lactic acidosis, and has been considered to be contraindicated in many chronic hypoxemic conditions that may be associated with lactic acidosis, e.g. cardiovascular, renal, hepatic and pulmonary diseases, and aging [3]. Here metformin can inhibit the mitochondrial respiratory complex I chain, leading to an impairment of the main site of aerobic energy metabolism. This results in an intracellular shift in oxidation–reduction reactions from aerobic to anaerobic metabolism, of which lactate is a by-product [1, 4]. Metformin also decreases hepatic metabolism of lactate and has a negative ionotropic effect on the heart, both of which elevate plasma lactate levels [5].

Metformin is a good substrate of the polyspecific organic cation transporter 1 (OCT1; Slc22a1), which is strongly expressed on the sinusoidal membrane of hepatocytes. Organic cation transporter 1 plays a key role in the disposition and hepatic clearance of mostly cationic drugs and endogenous compounds [6, 7]. The relation between the OCT1 expression and lactic acidosis was reported by use of OCT1 knockout (Oct -/-) mice. It has been shown that the blood lactate concentrations in metformin-treated wild-type mice (Oct +/+) was 2.5-fold greater than that in metformin-treated Oct(-/-) mice [8]. In fact, the hepatic uptake of metformin could be accounted for by OCT1, therefore, the lack of OCT1-mediated hepatic uptake would reduce the inducibility of blood lactate.

There is robust experimental evidence supporting the concept that plant-derived compounds like flavonoids provide a wide range of pharmacological benefits. Here the flavonoid naringenin is the major flavanone in citrus fruits and especially in grapefruit (Figure 1B) [9]. Naringenin has received considerable attention for pharmaceutical and nutritional development due to its ability to scavenge oxygen-free radicals, quench transition metals and/or boost the endogenous antioxidant defense system [10–13]. Although not many studies investigated the effects of naringenin on drug disposition and interactions, some pharmacokinetic investigations demonstrated that grape fruit juice can alter the effects of some drugs and inhibit organic-anion-transporting polypeptide 1B1- and 1B3- (OATP1B1 and OATP1B3) mediated drug transport [14–16]. In addition, we previously demonstrated that grape fruit juice may exacerbate metformin-induced lactic acidosis in non-diabetic rats but, the mechanism behind was not elucidated [17]. Based on these results, we hypothesized that naringenin (major flavonoid in grape fruit juice) can upregulate/stimulate OCT1 activity to enhance hepatic uptake of metformin. The current study therefore aimed to investigate naringenin's effect on OCT1 hepatic expression and its associated impact on metabolic/lactic acidosis induced by metformin in streptozotocin-induced diabetic rats.

Material and Methods

Material

Naringenin, D-glucose, streptozotocin (STZ), Tris Base, hydrochloric acid, sulphuric acid, potassium hydroxide, ethanol, sodium sulfate, ammonium acetate HPLC grade, acetonitrile HPLC grade and phenol were all purchased from Merck Pty. Ltd, Johannesburg, South Africa. Metformin hydrochloride (Accord Healthcare, South Africa), portable glucometers and glucose test strips (OneTouch Select, Lifescan Inc., Milpitas California, USA) were purchased from a local pharmacy in Durban, South Africa. A primary antibody specific to OCT 1 was purchased from Alpha Diagnostic Intl. Inc, Texas, USA. Halothane and other

accessories were provided by the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

Animals

Forty-nine male Sprague Dawley rats (250–300 g) were provided by the BRU of the University of KwaZulu-Natal (Durban, South Africa). Rats were housed in transparent plastic cages, with free access to standard commercial chow and drinking tap water *ad libitum*. Animals were maintained on a 12 hours dark-to-light cycle (08h00 to 20h00 in light) in an air-controlled room (temperature 25 ± 2°C, humidity 55% ± 5%) and were handled humanely according to the guidelines of the Animal Ethics Committee of University of Kwa-Zulu-Natal which approved the study (Ethics reference number: AREC/056/016D).

Experimental procedure

The rats were randomly divided into 7 groups (n=7 animals/group). Rats in group 1 (control) were orally treated on a daily basis with 3.0 ml/kg body weight (BW) of distilled water. Group 3, 6 and 7 similarly received metformin (250 mg/kg BW) while groups 2, 5, 7 were treated with naringenin (60 mg/kg BW). Diabetes was induced in groups 4-7 by a single intraperitoneal STZ injection (60 mg/kg BW) dissolved in 0.2 mL of 0.1 M citrate buffer (pH 4.5) after an overnight fast [18]. Three days after STZ administration, the development of diabetes was confirmed by tail picking to analyze blood glucose levels. Rats with fasting blood glucose more than 6 mmol/L were considered diabetic and included in the study (Table 1). Rat weights and water consumption were recorded daily for 56 days. Furthermore, on day 54 of treatment the rats were placed in solitary metabolic cages to allow for the collection of 24-hour urine samples that were stored at -80°C for further analyses.

Glucose tolerance tests

Fasting blood glucose (FBG) concentrations were determined after tail pricking and analyzed by using a glucometer.

For the glucose tolerance tests (GTT) the rats were intraperitoneally injected with D-glucose (3 g/kg BW) in normal saline and blood glucose concentrations thereafter measured at 0, 30, 60 and 90 min, respectively. The area under the curve (AUC) was calculated from blood glucose-time plots (mmol/L) x time (min) in GTT and expressed as AUC units.

Animal sacrifice

On day 56 of treatment the rats were sacrificed by halothane overdose and blood samples collected via cardiac puncture. Serum and plasma samples were collected and stored at -80°C for biochemical analyses. Liver tissues were surgically excised, rinsed in 1.0 M phosphate buffered saline (PBS), weighed, snap-frozen in liquid nitrogen and then stored at -80°C for further biochemical analyses.

Determination of 24-h micro-albuminuria

Albumin urinary levels were measured by a sandwich ELISA method according to the manufacturer's instructions (Rat micro-albumin Elisa kit, Elabscience, Bethesda, MD, USA)**.** Standard or sample (100 µl) were added to the micro Elisa plate wells and combined with 100 µl of the specific antibody. After incubation for 1 hour at 37°C, 100 µl HRP conjugate were added to each well and incubated. Thereafter, free components were washed away and 90 µl of substrate solution were added and incubated. The enzyme substrate reaction was terminated by the addition of 50 µl stop solution. The optical density was proportional to the concentration of micro-albuminuria and measured spectrophotometrically at 450 ŋm.

Serum electrolytes determination

Serum sodium (Na^+), potassium (K^+), chloride (Cl^-) and bicarbonate ($HC0_3^-$) levels were analyzed using an automated chemistry analyzer (Beckman Coulter, Synchron LX20 Clinical Systems, California, USA). The anion gap (AG) was calculated using the following formula [19]:

$AG = \{[Na^+] + [K^+]\} - \{[Cl^-] + [HCO_3^-]\}$

Hepatic glycogen assay

The determination of hepatic glycogen was performed according to the modified method of Seifter et al.[20] Briefly, 100 mg liver tissue was homogenized in 1.0 mL of 30% KOH saturated with Na_2SO_4. The homogenate obtained was dissolved by boiling in a water bath (100°C) for 30 min, vortexed and cooled on ice. Glycogen was then precipitated with 2.0 ml of 95% ethanol, vortexed, incubated on ice for 30 min and later centrifuged at 550 g for 30 min. The glycogen pellets obtained were then re-dissolved in 1 ml of distilled water which was thereafter treated with 1 ml of 5% phenol and 5 ml of 96–98% sulphuric acid, respectively. This was incubated on an ice bath for 30 min and the absorbance measured at 490 nm using a spectrophotometer (Genesys 20, ThermoSpectronic 1, ThermoFischer Scientific, South Africa). Glycogen content was expressed as mg/g liver protein.

Plasma lactic acid levels

Plasma lactic acid concentrations were determined as described by Borshchevskaya et al [21]. The method is based on the spectrophotometric determination of the colored product of the reaction of lactate ions with iron (III) chloride at 390 nm. Here plasma (50 μL) containing lactic acid was added to 2 mL of a 0.2% solution of iron(III) chloride and stirred and absorbance was measured at 390 nm against the reference solution (2 mL of a 0.2% $FeCl_3$

solution), using a spectrophotometer (Genesys 20, ThermoSpectronic 1 ThermoFischer Scientific, South Africa).

Metformin quantification

Instruments and methods

An HPLC system (Shimadzu LCMS-2020 Kyoto, Japan) equipped with a Shim-Pack GIST-HP C18 column (4,6 x 150 mm, 3µm) was used for metformin analyses. The isocratic mobile phase which consisted of 60:40 (v/v) mixture of acetonitrile and 10 mM ammonium acetate (pH 3 ± 0.05), was delivered at 1.1 mL/min. Aliquots of the processed samples (10 µl) were injected into the column, which was kept at 30°C. The detection was done by using a UV detector set at 236 nm.

The stock solution of metformin (1mg/ml) was prepared in acetonitrile. Every sample contained 20 µmol/l of internal standard (glibenclamide). Working standards of metformin were obtained by diluting the stock solution in drug-free sample.

Sample preparation

Each sample (200 µl) was mixed with 20 µl of the internal standard working solution. To this, 50 µl of the ammonia solution (25%) and 1.0 ml acetonitrile was added. After vortex-mixing for 30 s and centrifugation at 4000 rpm for 10 min, the supernatant was transferred to another clean test tube and evaporated to dryness at 45°C under a gentle stream of nitrogen. The residue was reconstituted with 500 µl of the mobile phase and 10 µl was injected into HPLC system.

OCT1 protein expression analysis

Hepatic OCT1 expression was assessed by Western blotting. Here 100 mg liver samples were homogenized in 3 x 300 µl of ice cold radio-immunoprecipitation assay buffer (RIPA buffer). The homogenate was centrifuged at 12000g at 4°C for 20 min. The supernatant was

transferred into 1.5 mL microcentrifuge tubes, kept on ice and protein content determined by the Bradford method [22]. Sample protein was standardized to 35 µg and denatured in Laemli buffer at 95°C for 5 min. The samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4% stacking, 10% resolving) by using a Mini-PROTEAN tetra System (Bio-Rad, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane for 2 h at 100 V and at 4°C by using the transblot apparatus (Bio-Rad, Hercules, CA). Thereafter, the membrane were blocked in Tris-buffered saline containing 0.5% tween 20 (TBS-T) and 5% nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibody rat anti-OCT1 (Alpha Diagnostic Intl. Inc, Texas USA) or ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:3000. After three 10 min washings in TBS-T, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers MA) at a dilution of 1:3000 for 1 h at room temperature. After extensive washing with TBS-T buffer, immunoreactivity was detected by using the Clarity Western ECL substrate (Bio-Rad, Hercules, CA) and visualized on a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA). The bands obtained were analyzed with Image Lab Software 6.0.1(Bio-Rad, Hercules, CA) and the data were presented as relative band density and fold change.

Statistical analysis

The data are presented as mean ± standard deviation (SD) and analyzed by the statistical software GraphPad Prism Version 5.0 (San Diego, CA). The unpaired t-test was used to compare two groups while one-way analysis of variance (ANOVA) followed by a Bonferroni test were used to compare more than two groups. A p-value < 0.05 was considered as statistically significant.

Results

Body weight changes

The untreated diabetic rats exhibited attenuated weight gain compared to controls ($p < 0.0001$) (Fig 2). However, treatment with metformin or co-administration metformin/naringenin improved weight in diabetic rats ($p = 0.0038$).

Water intake

The average daily water consumption was significantly elevated in all diabetic groups, but treatment with metformin or naringenin - alone or in combination - significantly lowered water intake in diabetic rats (Fig.3).

Urine output

All diabetic groups produced more urine over 24 hours compared to controls ($p < 0.001$). Metformin (with/without naringenin) lowered urine output compared to untreated diabetic controls, although this was not statistically significant (Fig. 4).

Glucose intolerance

Fasting blood glucose (FBG) concentrations were elevated for all diabetic groups ($p < 0.0001$) (Fig 5). Treatment with metformin or naringenin did not significantly improve FBG compared to untreated diabetic rats. In agreement, the calculated AUC for GTT showed that the diabetic rats exhibited impaired glucose tolerance ($p < 0.0001$). However, co-administration of naringenin and metformin did not significantly ($p = 0.0748$) improve glucose tolerance (Fig 6A and 6B).

Microalbumin levels

Microalbuminuria has been used to describe an amount of albumin in the urine. It is an early predictor and marker of diabetic nephropathy.

Microalbuminuria (24 h urine samples) was elevated in untreated diabetic rats compared to controls ($p < 0.0001$) (Fig 7). However, treatment with naringenin or metformin - alone or in combination - significantly reduced micro-albuminuria in diabetic rats.

Serum electrolytes

Serum sodium and chloride levels were attenuated in untreated diabetic rats (*p=0.0236, $^{\$}p = 0.002$, respectively). Treatments with either naringenin - alone or with metformin - slightly improved sodium and chloride concentrations in diabetic rats versus the untreated diabetic group. Treatment with metformin increased bicarbonate concentrations in normal rats ($p < 0.001$). However, serum potassium concentrations were almost similar in all groups.

Anion gap

The Anion Gap (AG) is a derived variable primarily used for the evaluation of metabolic acidosis. The calculated anion was increased in the untreated diabetic group versus the control group ($P = 0.008$). However, treatment with naringenin decreased the anion gap in diabetic rats compared to the untreated group ($p = 0.03$) while metformin increased this parameter - but not significantly (Fig 8).

Hepatic glycogen levels

Diabetes acutely impairs the ability of the liver to synthesize glycogen. Hepatic glycogen concentrations were reduced in untreated diabetic rats compared to the control groups ($p < 0.05$). Treatment with metformin increased (but not significantly) hepatic glycogen concentrations in normal and diabetic rats. However, co-administration of metformin significantly elevated glycogen levels in diabetic rats (Fig 9).

Plasma lactate concentrations

Plasma concentrations of lactate represent equilibrium between its production and metabolism. Lactate can be metabolized by various cells and tissues including liver. Plasma

lactate concentrations were elevated in controls treated with metformin ($p < 0.001$). Diabetic rats treated with either metformin alone, or in combination with naringenin, exhibited a significant increase in lactate concentrations versus the untreated diabetic group. However, naringenin alone lowered lactate concentrations in diabetic rats ($p < 0.001$) (Fig 10).

Metformin concentration

We evaluated the concentrations of metformin after 2 hours post-oral administration (Figure 11). Metformin levels in liver were lower in diabetic rats compared to non-diabetic rats ($P < 0.0001$). At the same time, metformin concentrations in liver were significantly lower in diabetic rats treated with metformin alone compared to diabetic rats treated with naringenin and metformin ($P < 0.0001$). In contrast to liver, analysis of metformin levels in plasma showed higher levels of the drug in diabetic rats compared to non-diabetic rats. However, co-administration of naringenin attenuated significantly ($P < 0.05$) this concentration (Table 3).

Liver OCT1 protein expression levels

The expression of hepatic OCT1 proteins in normal rats treated with metformin was elevated compared to controls ($p < 0.0001$), while diabetic untreated rats exhibited attenuated expression levels versus controls ($p < 0.05$). However, treatment with naringenin significantly increased OCT1 expression in untreated diabetic rats and also in combination with metformin (Fig 12A and 12B).

Discussion

Despite its well-known beneficial effects in diabetes patients, metformin prescription is limited due to concerns regarding potential adverse effects associated with lactic acidosis [23, 24]. The present study therefore employed a diabetic rat model to assess the effects of the citrus flavonoid naringenin on metformin's hepatic transporter (OCT1) and subsequent implications in terms of metabolic/lactic acidosis.

As expected, well-known markers of diabetes were displayed in all groups where STZ was administrated, i.e. hyperglycemia (Fig 4), sudden weight loss (Fig 1), polyuria (Fig 3), polydipsia (Fig 2) and glucose intolerance (Fig 5). Streptozotocin is a glucosamine–nitrosurea compound that induces diabetes by destroying insulin-producing pancreatic β-cells by its alkylating properties [25, 26]. This leads to hyperglycemia and the symptoms found in the diabetic rats, e.g. weight loss. The treatment of diabetic rats with metformin significantly improved weight gain and polydipsia, while the co-administration of naringenin slightly improved glucose tolerance, polyuria and polydipsia versus sole metformin treatment. These findings are in agreement with previous work demonstrating that naringenin supplementation mitigated the severity of diabetes and its related complications [27, 28]. By contrast, naringenin did not ameliorate hyperglycemia in diabetic rats in our study and like metformin does not impact on glycemic status without the presence of insulin [29, 30].

As albuminuria is a well-known predictor of renal outcome in diabetes, we also assessed this parameter in the current study. Here albumin excretion (24 h urine sample) was significantly elevated in untreated diabetic rats indicating progression towards diabetic nephropathy and if left untreated to clinical proteinuria or severely increased albuminuria [31]. For the current study, metformin and naringenin treatment (alone or in combination) resulted in a significant renoprotective effect by reducing microalbuminuria in diabetic rats. It is possible that metformin attenuated oxidative stress, a causal factor and key promoter of urolithiasis that is associated with renal tubular epithelium cell injury. It may also exert its protective effects by ameliorating podocyte functioning through the activation of 5'-AMPK protein kinase (AMPK) and the inhibition of mTOR signaling. However, naringenin acts by altering oxidative stress, modulation of cytokines expression and apoptotic events [32, 33].

Insulin normally stimulates muscle and hepatic glycogen synthesis by attenuating phosphorylation of glycogen synthase, the rate-limiting enzyme for glycogen synthesis. Thus with insulin deficiency there is an impairment with glycogen synthesis [34]. In agreement,

our results showed that hepatic glycogen levels were significantly reduced in untreated diabetic rats compared to controls, while naringenin or metformin did not significantly increase it. This was surprising as metformin is known to improve insulin-mediated glycogen synthesis and to inhibit glycogenolysis and gluconeogenesis in diabetic patients [35]. However, it is likely that metformin failed to exert its anti-hyperglycemic effects due to the insulin deficiency in the diabetic rats. Interestingly, hepatic glycogen levels were significantly increased by co-administration of naringenin and metformin thus indicating a synergistic effect as suggested recently by Nyane et al (2017) [36].

Our findings showed decreased plasma lactate concentrations in diabetic rats treated with naringenin while treatment with metformin increased it in both non-diabetic and diabetic rats. Of note, co-administration of metformin and naringenin in diabetic rats significantly elevated plasma lactate concentrations compared to metformin only. This indicates that naringenin increases lactate accumulation in presence of metformin. As lactate is the end product of anaerobic glycolysis, its accumulation may be caused by increased production or decreased clearance, or a combination of both [37]. Metformin therapy can increase lactate production in the liver a key organ for both the anti-diabetic effects of metformin and the development of lactic acidosis. However, the exact pathophysiology of hyperlactatemia caused by metformin is still not completely understood. One suggested mechanism is an inhibition of gluconeogenesis resulting in the accumulation of gluconeogenic precursors such as alanine, pyruvate and lactate [3]. However, others propose that an interference of the drug with mitochondrial function is a key factor for both its glucose-lowering effects [38, 39] and the development of metformin-associated lactic acidosis [40, 41]. As a consequence of mitochondrial inhibition, the cell partly shifts from aerobic to anaerobic metabolism thereby promoting glycolysis that results in elevated lactate levels [39]. Therefore increased lactate levels found in rats treated with naringenin and metformin may be due the fact that naringenin could potentiate metformin-associated lactic acidosis. Similarly, we have previously reported

that grape fruit juice potentiates metformin- associated lactic acidosis in non-diabetic rats, although the underlying mechanism was not elucidated [17].

Although a raised anion gap was found in diabetic groups, it was decreased by naringenin treatment while co-administration with metformin did not elicit any significant effects. Electrolyte and acid-base disturbances are quite common in patients with diabetes and may be the result of altered distribution related to hyperglycemia-induced osmotic fluid shifts or total-body deficits brought about by osmotic diuresis. Complications from end-organ injury and the therapies used in the management of diabetes may also contribute to electrolyte disturbances [42].

Unlike diabetic rats, OCT1 protein expression was increased in non-diabetic rats treated with metformin. This suggests that under normal physiological conditions the presence of metformin can induce hepatic expression of OCT1 protein in order to facilitate its uptake by the liver. It has been demonstrated *in vivo* that, AMPK activation by metformin could reverse downregulation of glucocorticoid receptors which regulates OCT1 expression via hepatocyte nuclear factor 4 alpha (HNF4A) upregulation in primary human hepatocytes [43, 44]. By contrast, OCT1 expression was decreased in untreated diabetic rats. This is in agreement with Grover et al. (2004) that showed decreased OCT1 expression in diabetic rats [45], suggesting that it may result from post-transcriptional alterations. For example, hyperglycemia promotes non-enzymatic glycation of extracellular and intracellular proteins resulting in irreversible advanced glycation end products that can change the structure and/or functionality of the affected proteins [46, 47]. Thus naringenin could potentially inhibit the formation of advanced glycation end products and increase OCT1 expression in diabetic rats. Furthermore, some reports indicate that naringenin induces peroxisome proliferator activated receptors (PPAR)γ and PPARα protein expression that mediate transcriptional upregulation of OCT1 in mousse hepatocytes [48, 49]. However, elevated lactate concentrations in diabetic rats treated with

naringenin and metformin support the hypothesis that naringenin upregulates OCT1 expression. This in turn will enhance metformin uptake and lactic acidosis. In support, hepatic metformin uptake is primarily mediated by OCT1. Therefore, the upregulation of OCT1 enhance metformin uptake that will inhibit mitochondrial respiratory-chain complex 1, resulting in reduced ATP levels and increased AMP. Increased AMP levels activate Adenosine Monophosphate-Activated Protein Kinase (AMPK), which contributes to inhibit mitochondrial glycerol-3-phosphate dehydrogenase (mGPD), leading to an increase in cytosolic NADH, which both stimulates the conversion of pyruvate to lactate [50]. Thus we hypothesized that, naringenin potentiates metformin action and thereby lead to elevated lactate production as shown here. This hypothesis was confirmed by the results of metformin levels analyses in liver and plasma showing higher concentration of the drug in diabetic rats treated with naringenin and metformin compared to diabetic rats treated with metformin alone. This study therefore strengthens the notion that concomitant administration of plant bioactive compounds can increase the plasma concentrations of various drugs within the clinical context. Moreover, such increases of drug plasma concentrations may result in adverse clinical effects in certain instances [51].

Conclusion

Although naringenin improves classic symptoms associated with the diabetic phenotype (e.g. weight gain, polydipsia, metabolic acidosis), it upregulates liver OCT1 expression that is associated with increased lactate production when co-administered with metformin. Thus our study alerts to side-effects associated with concomitant administration of metformin and dietary supplementation (e.g. naringenin) and such regimens are therefore not recommended for diabetic patients.

Acknowledgements

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Abbreviations

AMPK: 5' adenosine monophosphate-activated protein kinase, ANOVA: analysis of variance, AUC: area under curve, BW: Body weight, CONT: control, ELISA: Enzyme-Linked Immuno Assay, FBG: fasting blood glucose, GTT: glucose tolerance test, HPLC: High-performance liquid chromatography, MET: metformin, mTOR: mammalian target of rapamycin, NAR: naringenin, OCT: organic cation transporter, STZ: streptozotocin

Author contributions

MFE and PMOO: designed and conceived the study

EPMM: performed the main part of research study, acquired date and analysed

MFE and PMOO: revised the manuscript critically for important and intellectual content

EPMM: wrote the manuscript

Conflicts of interest

The authors declare that they have no conflicts of interest

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List of tables

Table 1: Animal treatment protocol. Metformin (MET) and naringenin (NAR) were dissolved in distilled water and 1.0ml containing relevant dose administrated orally. Streptozotocin (STZ) was dissolved in 0.1 M citrate buffer and 0.2 ml containing the relevant dose was intraperitoneally administrated.

Groups	Designation	Treatment			
		Distilled H_2O (mL)	MET (mg/Kg)	NAR (mg/Kg)	STZ (mg/Kg)
1	Control	3.0	-	-	-
2	NAR	-	-	60.0	-
3	MET	-	250	-	-
4	STZ	-	-	-	60.0
5	STZ+NAR	-	-	60.0	60.0
6	STZ+MET	-	250	-	60.0
7	STZ+NAR+MET	-	250	60.0	60.0

Table 2: Serum electrolyte levels. Sodium, potassium, chloride and bicarbonate concentration were determined in serum. Data were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p = 0.0236, $p = 0.002, #p < 0.001 vs.controls; ##p < 0.001 vs. STZ.

	Control	Control NAR	Control MET	STZ	STZ + NAR	STZ + MET	STZ + NAR + MET
Sodium (mM)	140,7±1,15	139,3±1,15	140,0±3,60	130,7±4,72*****	135,3±3,79	133,7±2,52	133,3±1,5
Potassium (mM)	4,17±0,19	4,14± 0,1	4,1±0,13	4,62±0,51	4,16±0,53	4,05±0,78	4,2±0,15
Chloride (mM)	107,0±1,0	107,3±1,16	104,3±0,58	93,33±1,52$^{\$}$	96,33±3,22	93,0±4,0	93,67±4,04
Bicarbonate (mM)	23,67±0,58	24,67±1,15	26,33±0,58 **#**	23,00±3,46	26,67±2,89##	24,00±1,73	25,33±0,58

Table 3: Metformin concentration in plasma and liver, 2 hours post-oral administration of 250 mg/kg metformin body weight. #P < 0.05 vs MET, @P < 0.05 vs STZ+MET *P < 0.0001 vs MET and ^P < 0.0001 vs STZ+MET.

	Plasma metformin (µg/ml)	Liver metformin (µg/ml)
MET	1.076 ± 0.1	1.53 ± 0.03
STZ+MET	1.69 ± 0.1$^{\#}$	0.22 ± 0.03*
STZ+ MET+NAR	1.34 ± 0.09$^{@}$	1.4 ± 0.05^

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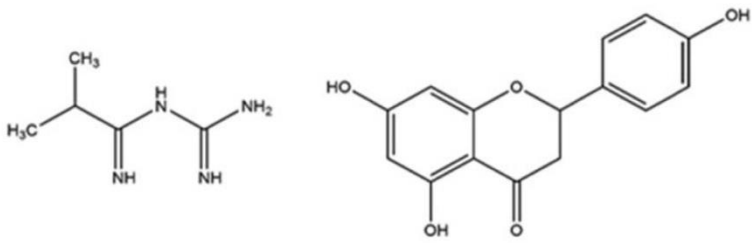


A B

Figure 1: Chemical structure of A) metformin and B) naringenin. ChemDraw, PerkinElmer®

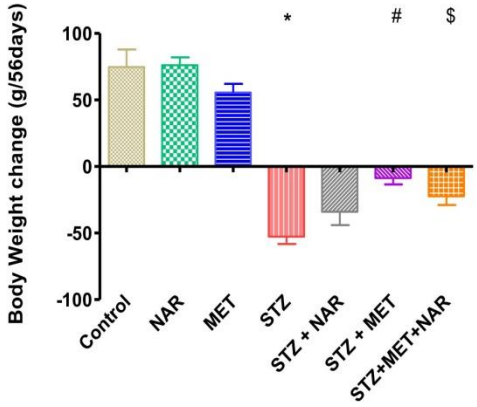


Figure 2: Body weight changes in response to various treatments. This value was obtained for each rat by doing the difference of body weight between the day 56 and day 0. All values were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *$p < 0.0001$ vs. control, #$p < 0.0001$ vs. STZ, \$$p = 0.0038$ vs. STZ.

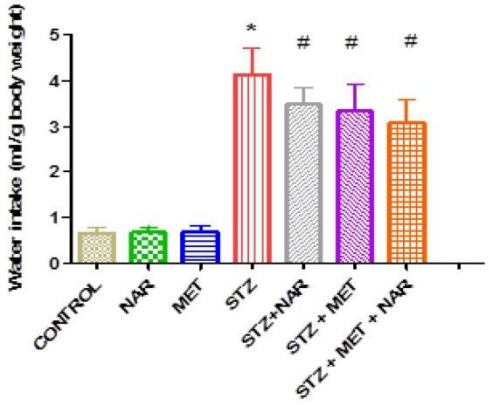


Figure 3: Average daily water intake (per gram body weight) in designated treatment groups. All values were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p < 0.0001 vs. controls, and #p < 0.0001 vs. STZ group.

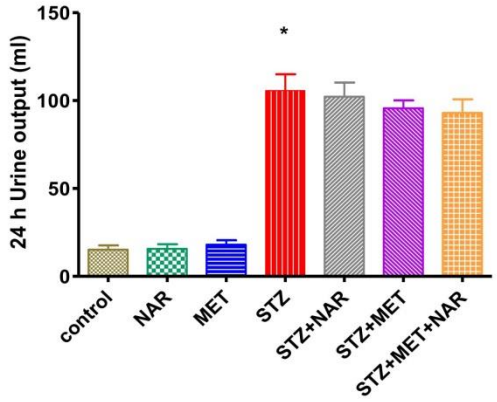


Figure 4: Average 24 hours urine output. Animals were placed in solitary metabolic cages to allow for the collection of 24-hour urine. All values were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p < 0.0001 vs. controls.

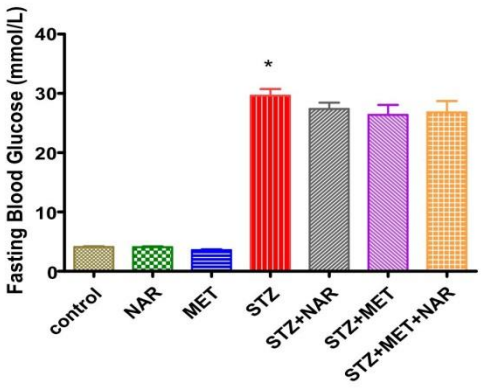
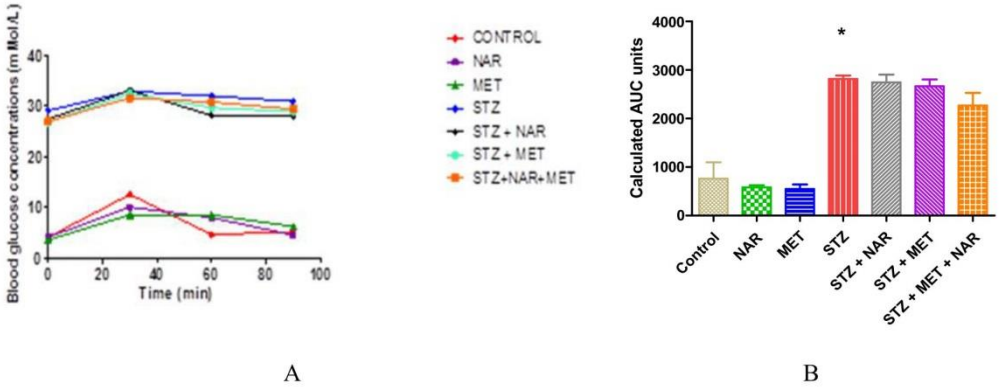


Figure 5: Fasting blood glucose levels. Blood was collected by tail pricking after an overnight fast (8 hours) and analysed by a glucometer. Data were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p < 0.0001 compared to the control group



A B

Figure 6: Glucose tolerance tests in various treatments groups. Rats were intraperitoneally injected with D-glucose (3.0g/kg BW) in normal saline and blood glucose concentrations measured at 0, 30, 60 and 90 mins in all groups. A) GTT curves, B) Calculated Area Under the Curve (AUC) from GTT plots. Superscript symbols indicate significance at a confidence levels *p < 0.0001 vs. control group.

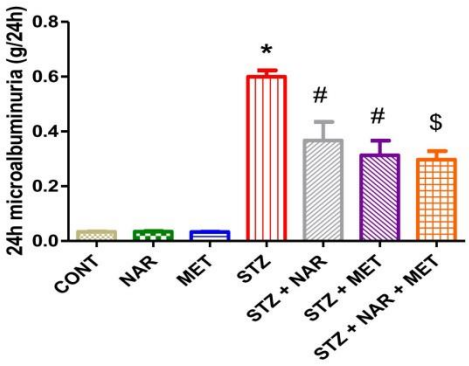


Figure 7: Micro-albuminuria concentration in 24 h urine sample. Data were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p < 0.0001 vs. control; #p < 0.05 vs. STZ; $p < 0.001 vs. STZ.

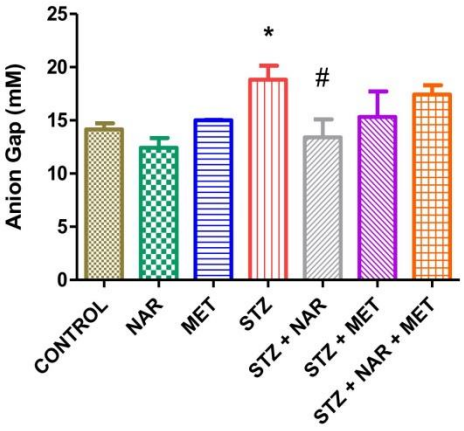


Figure 8: Anion gap calculated from serum electrolytes. This value is the difference between measured cations (sodium and potassium) and the measured anions (chloride and bicarbonate) in serum. Data were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *P = 0.008 vs. control; $^{\#}$ p = 0.03 vs. STZ.

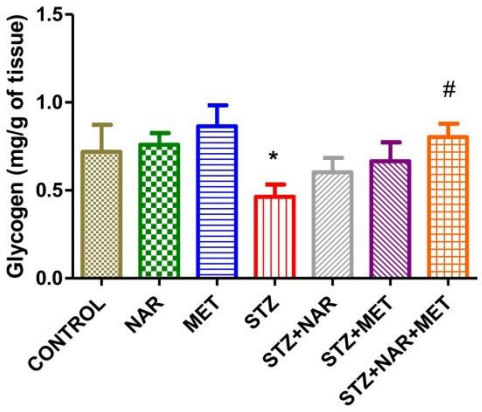


Figure 9: Hepatic glycogen levels in homogenized tissue. Liver sample was digested with 30% KOH and then precipitated with ethanol. The precipitate was treated with phenol and sulfuric acid. Glucose in the hydrolysate was determined by spectrophotometry. Data were expressed as mean ± SD. Superscript symbols indicate significance at a confidence levels *p < 0.05 vs. control; #p < 0.05 vs. STZ.

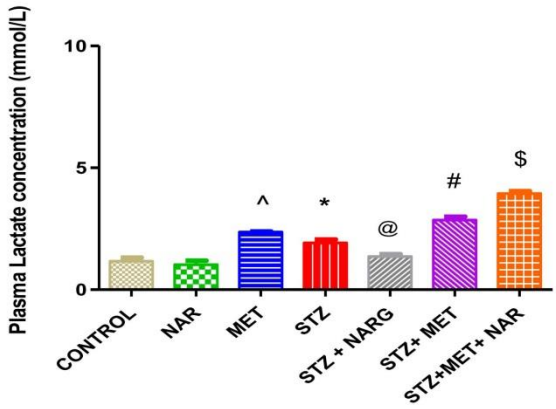
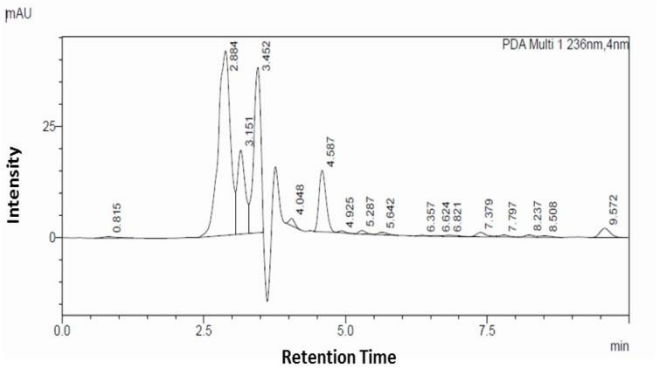


Figure 10: Plasma lactate concentrations. Data were expressed as mean ± SD. Lactate measurement was based on the spectrophotometric determination of the colored product of the reaction of lactate ions with iron (III) chloride at 390 nm. Superscript symbols indicate significance at a confidence levels ^p < 0.0001 vs. controls; @p < 0.001 vs. STZ group; *,#,$p < 0.05 vs. controls, STZ, STZ+MET group respectively.

A



B

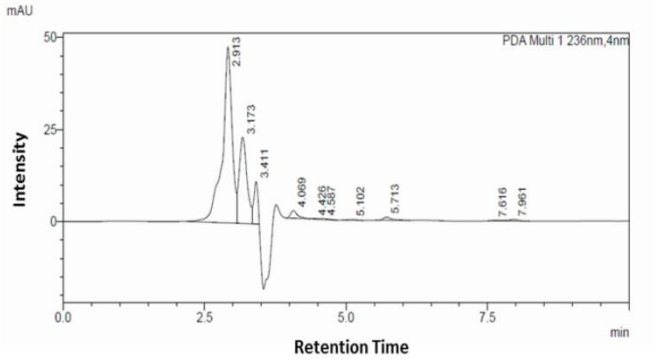


Figure 11: Representative chromatograms obtained from A) liver and B) plasma sample after metformin administration. Separation was performed using a Shim-Pack GIST-HP C18 column (4,6 x 150 mm, 3µm). The mobile phase included 60:40 (v/v) mixture of acetonitrile and 10 mM ammonium acetate. The retention time was 2.884 and 2.913 respectively.

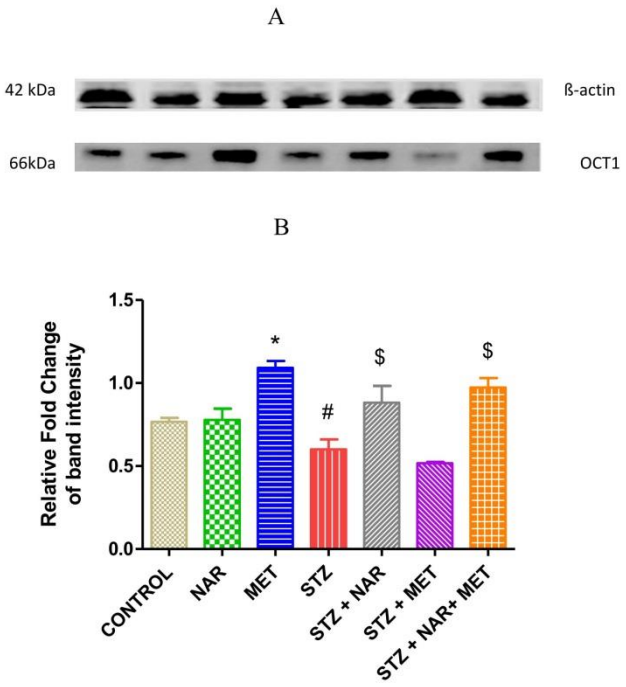
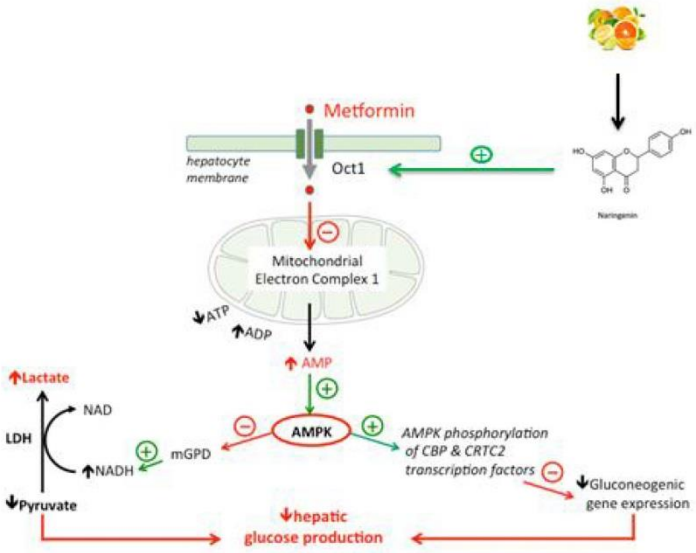


Figure 12: Immunoblots of A) OCT1 protein expression analyzed by Western blotting against ß-actin bands for equal loading and B) corresponding densitometry scans. Experiment was done in triplicate. Superscript symbols indicate significance at a confidence levels *$p < 0.0001$, #$p < 0.05$ vs. controls; and \$$p < 0.0001$ vs. STZ.

Graphical Abstract (for review)



Graphical illustration: The major flavonoid of citrus fruits naringenin, upregulates organic cation transporter 1 (OCT1) which is the main membrane transporter of metformin in hepatocytes. This in turn will enhance metformin uptake that will inhibit mitochondrial respiratory-chain complex 1, resulting in reduced ATP levels and increased AMP. Increased AMP levels activate Adenosine Monophosphate-Activated Protein Kinase (AMPK), which contributes to inhibit mitochondrial glycerol-3-phosphate dehydrogenase (mGPD), leading to an increase in cytosolic NADH, which both stimulates the conversion of pyruvate to lactate. Lactic acidosis can occur when lactate accumulates in plasma

(Adapted from He & Wondisford, 2015 and Nolte Kennedy & Masharani, 2015).

He L, Wondisford FE. Metformin action: concentration matters. Cell Metab. 2015; 21(2):159-62. doi: 10.1016/j.cmet.2015.01.003.

Nolte Kennedy MS, Masharani U (2015): Pancreatic Hormones & Antidiabetic Drugs (Chapter 41). In: Basic & Clinical Pharmacology. 15th Ed. Katzung BG, Trevor AJ (Editors). McGraw-Hill / Lange

CHAPTER FOUR

Edith Pascale Mofo Mato, M. Faadiel Essop, Peter Mark Oroma Owira (2019). Effects of naringenin on renal expression of organic cation transporter 1 and 2 proteins and metformin disposition in diabetic rats. Journal of Functional Foods, 59:1-7. https://doi.org/10.1016/j.jff.2019.05.021.

Effects of naringenin on renal expression of organic cation transporter 1 and 2 proteins and metformin disposition in diabetic rats

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Abstract

Background

Metformin, a commonly prescribed anti-hyperglycemic drug is eliminated by renal tubular secretion that involves a variety of transporters including organic cation transporters (OCT1 and OCT2). Modulation of these transporters could affect metformin pharmacokinetics/pharmacodynamics. The effects of citrus fruit flavonone, naringenin on the renal tubular OCT1 and OCT2 protein expressions and subsequently the metformin clearance in diabetic rats are hereby investigated.

Methods

Forty-nine Male Sprague Dawley rats [250 – 300 g body weight (BW)] were divided into 7 groups (n = 7). Diabetes was induced by single intraperitoneal injection of streptozotocin (60 mg/kg BW). Diabetic and non-diabetic rats were daily treated orally with metformin (250 mg/kg BW) or naringenin (60 mg/kg BW) alone or both for 56 days. Fasting blood glucose (FBG) and glucose tolerance tests were done. Animals were placed in metabolic cages to allow the collection of 24-hours urine samples. Thereafter, the rats were sacrificed by halothane overdose. Serum and kidney samples were collected and stored at -80°C for further analysis.

Results

FBG was significantly higher in diabetic rats compared to controls. Naringenin alone or in combination with metformin significantly increased creatinine clearance in diabetic rats. Naringenin treatment significantly lowered plasma but not urine metformin concentrations in diabetic rats compared to controls. OCT1 and OCT2 protein expressions in renal tissues were unchanged in diabetic rats treated with either naringenin or metformin, respectively. However, the co-administration of metformin and naringenin upregulated renal OCT2 expression in diabetic rats.

Conclusion

Naringenin might stimulate renal uptake/excretion of metformin by increasing OCT2 expression thus ameliorating metformin excretion in diabetic rats.

Keywords: renal tubular secretion, metformin, naringenin, diabetes, OCT1, OCT2.

1. Introduction

Renal clearance is a major route for the excretion of drugs or their metabolites (Morrissey et al. 2012). It involves glomerular filtration, tubular secretion and/or tubular reabsorption (Bendayan, 1996). Glomerular filtration is a passive and unidirectional process while tubular secretion and reabsorption are bidirectional processes which involve a variety of protein transporters (Yin, 2016). These transporters are mainly expressed in the basolateral or apical side of the renal proximal tubule and they work in tandem to excrete anionic and cationic drugs from blood circulation into the tubular lumen (International Transporter Consortium et al. 2010). Here organic cation transporter 2 (OCT2) is the major isoform that is localized to the basolateral side of the proximal tubule, while in rodents OCT1 is also strongly expressed in this tubule (Karbach et al. 2000).

OCT1 and 2 are members of the solute carrier family 22 (SLC22) - which are polyspecific transporters and located at the basolateral membrane of renal proximal tubules cells (Koepsell et al. 2007) and are responsible for the first step in cation tubular secretion. The anti-diabetic drug metformin is a common substrate of both OCT1 and OCT2, which facilitate its clearance by active proximal tubular secretion (Graham et al. 2011). In OCT1/2 double knockout mice, metformin clearance is substantially diminished thereby emphasizing the important role of OCT transport in this process (Higgins et al. 2012). This in turn can lead to plasma metformin accumulation and increase the risk of metformin-induced lactic acidosis (Lalau and Race, 2001). Moreover, in experimental diabetes, basolateral tubular transport is disrupted

and tubular cation clearance lowered due to downregulated OCT expression (Grover et al. 2004).

Diabetes is a condition characterized by hyperglycemia and increased oxidative stress that can result in the formation and accumulation of advanced glycation end-products (AGEs). Proximal tubular cells represent the principal site of uptake of filtered AGEs and may therefore be uniquely vulnerable to AGE-mediated injury (Asano et al. 2002). Daily nutritional intake of various plant-derived natural compounds could exert significant protective effects on renal physiology. For example, the flavonoid naringenin (derived from citrus fruits) possesses numerous biological and pharmacological activities and is able to modify cell membrane transporter activity and thus affect drug pharmacokinetics (Alam et al. 2014). We have previously suggested that naringin/naringenin could be the bioactive chemical constituent in the grapefruit juice and that it is associated with upregulation of hepatic OCT1 expression and hence metformin accumulation in plasma (Owira and Ojewole, 2009). In light of this, we here hypothesize that naringenin increases renal tubular OCT1/2 protein expression and hence facilitate renal clearance of metformin in diabetic rats.

2. Material and methods

2.1 Material

Naringenin, streptozotocin (STZ), methanol and Tris base were all purchased from Merck (Johannesburg, South Africa.). Serum and urinary creatinine and urea concentrations were determined using a diagnostic kit (Beckman Coulter, Indianapolis IN). Metformin hydrochloride (Accord Healthcare, South Africa), and portable glucometers and glucose test strips (OneTouch Select, Lifescan Inc., Milpitas CA) were purchased from a local pharmacy. Primary antibodies specific to Oct1 and Oct 2 were purchased from Alpha Diagnostic International Inc. (San Antonio TX) and Santa Cruz Biotechnology (Dallas TX), respectively.

2.2 Animals

Forty-nine Male Sprague Dawley rats (250–300 g) were provided by the Biomedical Resource Unit of the University of KwaZulu-Natal (Durban, South Africa). The rats were housed in transparent plastic cages, with free access to standard commercial chow and drinking tap water *ad libitum*. They were maintained on a 12 hours dark-to-light cycle (08h00 to 20h00 in light) in an air-conditioned room (temperature 25±2°C, humidity 55% ± 5%) and were handled humanely according to the guidelines of the Animal Ethics Committee of University of Kwa-Zulu-Natal which approved the study (Ethics reference number: AREC/056/016D).

2.3 Experimental procedures

The rats were randomly divided into 7 groups (n=7). Rats in group 1 (control) were orally treated daily with 3.0 ml/kg body weight (BW) of distilled water. Group 3, 6 and 7 similarly received metformin (250 mg/kg BW) dissolved in distilled water while groups 2, 5, 7 were treated with naringenin (60 mg/kg BW). Diabetes was induced in groups 4-7 by a single intraperitoneal STZ injection (60 mg/kg BW) dissolved in 0.2 ml of 0.1 M citrate buffer (pH 4.5) after an overnight fast (Mensah-Brown et al. 2005) (Table 1). Three days after STZ administration, development of diabetes was confirmed by tail pricking to analyze blood glucose concentrations in droplets on strips by a portable glucometer. Rats with fasting blood glucose more than 6 mmol/l were considered diabetic and included in the study. Furthermore, on day 54 of treatment the rats were placed in solitary metabolic cages to allow for the collection of 24-hour urine samples.

For GTT, the rats were intraperitoneally injected with D-glucose (3 g/kg BW) in normal saline and blood glucose concentrations thereafter measured at 0, 30, 60 and 90 min, respectively. The area under the curve (AUC) was calculated from blood glucose-time plots (mmol/L) x time (min) in Glucose Tolerance Test and expressed as AUC units.

On day 56 of treatment, the animals were sacrificed by halothane overdose and blood samples collected by cardiac puncture. Kidneys were surgically excised, rinsed in 1.0 M phosphate buffered saline (PBS), weighed, snap-frozen in liquid nitrogen and then stored at -80°C for further biochemical analyses.

2.4 Serum and urinary creatinine determination

Creatinine concentrations in urine and serum were determined by the modified Jaffe method (Heinegård and Tiderström, 1973). In summary, 220 µl and 216 µl of reagent solutions were added to 20 µl of serum or 3 µl of urine, respectively. The absorbance of the deep red creatinine–picrate complex formed in the alkaline solution was measured at 520 nm.

- **Creatinine clearance**

Creatinine clearance (CrCl) was calculated using the following formula (Heinegård and Tiderström, 1973):

$$CrCl = \frac{Creatinine\ urine\ (umol/l)}{Creatinine\ serum\ (umol/l)}\ x\ \frac{urine\ volume\ (ml)}{time\ (\text{min})}$$

2.5 Urea/blood urea nitrogen

Urea concentrations in serum were determined by the modified enzymatic method of Talke and Shubert (Talke and Schubert, 1965). Three microliters of serum were added to 300 µl of the reagent solution. The change of absorbance was proportional to the concentration of urea in the sample was measured at 340 nm.

2.6 Urine electrolyte

Serum sodium (Na^+), potassium (K^+) and chloride (Cl^-) levels were analyzed using an automated chemistry analyzer (Synchron LX20; Beckman Coulter, Indianapolis IN).

The urinary anion gap (AG) was calculated using the following formula (Goldstein et al. 1986) :

UAG = {[Na+] + [K+]} – {[Cl-]

2.7 Plasma metformin concentrations

Plasma metformin concentrations were analyzed by a High Performance Liquid Chromatography (HPLC) system (Shimadzu LCMS-2020 Kyoto, Japan) equipped with a Shim-Pack GIST-HP C18 column (4,6 x 150 mm, 3µm). The isocratic mobile phase 60:40 (v/v) comprised a mixture of acetonitrile and 10 mM ammonium acetate (pH 3 ± 0.05), and was delivered at 1.1 ml/min. Aliquots of the processed samples (10 µl) were injected into the column, which was kept at 30°C. The detection wavelength was 236 nm.

- **Preparation of standard solutions**

The stock solution of metformin (1.0 mg/ml) was prepared in acetonitrile. Every sample contained 20 µmol/l of internal standard (glibenclamide). Working standards of metformin were obtained by diluting the stock solution in drug-free sample. The calibration curves for metformin were constructed by plotting the area of the peaks versus the concentrations.

- **Samples preparation**

Each sample (200 µl) was mixed with 20 µl of the internal standard working solution. Thereafter, 50 µl of ammonia solution (25%) and 1.0 ml acetonitrile were added. After vortex-mixing for 30 s and centrifugation at 4, 000 rpm for 10 min, the supernatant was then transferred to another clean test tube and evaporated to dryness at 45°C under a gentle stream of nitrogen. The residue was reconstituted with 500 µl of the mobile phase and 10 µl was injected into the HPLC system.

2.8 OCT1 and OCT2 protein expression analysis

Renal OCT1 and OCT2 protein expression were analyzed by Western blotting. Here, 100 mg of kidney samples were homogenized in 3 x 300 µl of ice-cold radio-immunoprecipitation assay buffer (RIPA buffer). The homogenate was centrifuged at 12, 000 g at 4°C for 20 min. The supernatant was then transferred into 1.5 ml microcentrifuge tubes, kept on ice and protein content determined by the Bradford method (Bradford, 1976). The protein content of samples was standardized to 35 µg and denatured in Laemmli buffer at 95°C for 5 min. The samples were then separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (4% stacking, 10% resolving) in a Mini-PROTEAN tetra System (Bio-Rad, Hercules CA). Proteins were transferred onto a nitrocellulose membrane for 2 h at 100 V and at 4°C using the transblot apparatus (Bio-Rad, Hercules CA). Thereafter, the membranes were blocked in Tris-buffered saline containing 0.5% tween 20 (TBS-T) and 5% non-fat dried milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibody (rat anti-OCT1) (Alpha Diagnostic International Inc., San Antonio TX), or rat anti-OCT2 (Santa Cruz Biotechnology, Dallas TX), or β-actin (Santa Cruz Biotechnology, Dallas TX) at a dilution of 1:3, 000. After three 10 min washings in TBS-T, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers MA) at a dilution of 1:3, 000 for 1 h at room temperature. After extensive washing with TBS-T buffer, immunoreactivity was detected by using the Clarity Western ECL substrate (Bio-Rad, Hercules CA) and visualized on a ChemiDoc Touch Imaging System (Bio-Rad, Hercules CA). The bands obtained were analyzed with Image Lab Software 6.0.1 (Bio-Rad, Hercules CA) and the data were presented as relative band density against ß-actin bands for equal loading.

2.9 Statistical analysis

The data are presented as mean ± standard deviation (SD) and analyzed by the statistical software GraphPad Prism Version 5.0 (San Diego CA). The unpaired Students't-test or one way analysis of variance (ANOVA) followed by a Bonferroni test were used where appropriate. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1 STZ-diabetes model

Fasting blood glucose (FBG) levels were significantly ($p < 0.0001$) elevated in all diabetic groups compared to controls. Treatment with metformin or naringenin, or both, did not significantly improve FBG (Fig 1). Similarly, calculated AUC for GTT showed that the diabetic rats displayed impaired glucose tolerance ($p < 0.0001$). However, co-administration of naringenin and metformin did not significantly improve glucose tolerance (Fig 2A and 2B).

3.2 Serum and urinary creatinine

Serum and urine creatinine concentrations were standardized by dividing values obtained with the final live total bodyweights recorded. Here the serum creatinine to body weight ratio was significantly higher in untreated diabetic rats compared to the control group ($P < 0.0001$). However, metformin (with/without naringenin) significantly ($P < 0.001$) lowered this ratio in diabetic compared untreated diabetic rats. Naringenin treatment lowered ($p < 0.05$) serum creatinine body weight ratio in non-diabetic but not diabetic rats compared to controls or non-treated diabetic rats, respectively (Fig 3 A).

By contrast, urinary creatinine to body weight ratio in untreated diabetic rats was significantly reduced compared to the control group ($P < 0.0001$) but treatment with either metformin -

alone or with naringenin – significantly ($P < 0.05$) increased urinary creatinine excretion in diabetic rats (Figure 3 A and B).

3.3 Calculated creatinine clearance

Creatinine clearance significantly ($p < 0.0001$) reduced in untreated diabetic rats compared to controls but treatment with metformin or naringenin, or both, significantly ($p < 0.05$) improved creatinine clearance in diabetic compared to non-treated diabetic rats (Figure 4). Creatinine clearance was non-significantly increased in naringenin- or metformin-treated non-diabetic rats compared to controls, respectively (Fig 4).

3.4 Blood urea nitrogen

Untreated diabetic rats exhibited significantly ($p < 0.0001$) elevated blood urea nitrogen (BUN) concentrations compared to the control group ($p < 0.0001$). However, treatment with either metformin or naringenin, or both, significantly ($p < 0.05$) reduced BUN in diabetic compared to non-treated diabetic rats (Figure 5).

3.5 Urine electrolytes

Urine sodium concentrations were significantly ($p < 0.0001$) higher in untreated diabetic rats compared to the control group. Treatment with metformin significantly ($p < 0.05$) increased urine sodium concentrations in diabetic and non-diabetic rats compared to untreated diabetic group and controls, respectively. Similarly potassium concentrations in urine were significantly ($p < 0.05$) elevated in untreated diabetic rats compared controls, but were significantly ($p < 0.05$) diminished in rats that were treated with both naringenin and metformin compared to the untreated diabetic group. Chloride concentrations in urine were significantly ($p < 0.0001$) lowered in untreated diabetic rats compared to controls (Table 2).

3.6 Calculated urinary anion gap

Urinary anion gap (UAG) levels were significantly ($p < 0.0001$) elevated in non-treated diabetic rats compared to controls (Figure 6). Treatment with metformin significantly ($p < 0.05$) increases UAG in diabetic and non-diabetic rats while naringenin did not show significant effects on diabetic and non-diabetic rats.

3.7 Plasma and urinary metformin concentrations

Plasma metformin concentrations were significantly ($p < 0.05$) elevated in diabetic compared to non-diabetic controls. Treatment with naringenin significantly ($p < 0.05$) lowered plasma metformin concentrations in diabetic rats (Table 3). Urinary metformin concentrations were significantly ($p < 0.05$) elevated in non-diabetic compared to diabetic rats. However, naringenin treatment non-significantly increased metformin concentrations in the urine of diabetic rats compared to metformin-only treated diabetic rats (Table 3).

3.8 Renal OCT1 and OCT2 protein expression levels

The expression of renal OCT1 protein was significantly reduced in untreated diabetic rats ($P < 0.0001$) versus controls. Treatment of non-diabetic rats with metformin or naringenin did not significantly improve renal OCT1 protein expression compared to controls (Figure 7A and B).

Renal expression of OCT2 protein was significantly ($p < 0.0001$) attenuated in untreated diabetic rats compared to the control group. Treatment with naringenin significantly ($p < 0.05$) improved renal OCT2 protein expression in diabetic compared to non-treated diabetic rats (Figure 8A and B).

4. Discussion

Natural polyphenolic compounds such as naringenin that possess numerous health benefits are common components in our daily diet. Therefore the concurrent administration of such

compounds together with conventional medications is unavoidable. Most importantly, they can interact with standard medications through drug transporters and thus change the nature of drug exposure and potentially its pharmacokinetics and efficacy. The present study therefore investigated the effects of naringenin on metformin disposition by renal OCT1 and OCT2 protein expression.

It is well known that creatinine clearance is widely used as a marker of renal function and to also estimate the glomerular filtration rate in numerous pathological conditions such as diabetes (Nankivell, 2001). Low creatinine clearance, elevated BUN and serum creatinine are pivotal indicators of kidney disease progression in diabetes. This is in agreement with our results showing that untreated diabetic rats displayed a higher BUN, increased serum creatinine levels and reduced creatinine clearance compared to controls (Fig 4). Here hyperglycemia-mediated formation of non-enzymatic glycosylation can result in a loss of functional nephrons and a reduced glomerular filtration rate (Remuzzi et al. 2002). BUN and serum creatinine levels rise when the renal dysfunction diminishes the ability to clear creatinine and urea (Mishra et al. 2015). In parallel with this increase, the concentration of these metabolites in urine is reduced. This is consistent with our results that showed lower urine creatinine excretion in untreated diabetic rats. However, treatment with metformin (with or without naringenin) lowered BUN/serum creatinine and improved the creatinine clearance in diabetic rats. Of note, metformin treatment can inhibit the deleterious effects of AGEs by down-regulating the expression of AGEs receptors (RAGE) and can subsequently suppress reactive oxygen species (ROS) production (Ishibashi et al. 2012). Activation of AMP-activated protein kinase (AMPK) by metformin, also prevents hypoxia and protects renal cells from ROS-induced functional and structural damages (Taheri et al. 2012). Our data showed that the co-administration of metformin and naringenin was more efficient at reducing urea and creatinine serum and improving creatinine clearance than sole treatment with either metformin or naringenin. Here previous work highlighted some mechanistic insights as

naringenin ameliorated STZ-induced diabetic rat renal impairment by downregulating transforming growth factor- β1 (TGF-β1) and interleukin-1 (IL-1) through the modulation of oxidative stress (Roy et al. 2016). Together these data indicate that naringenin may lead to additive effects in terms of metformin's renoprotective properties.

Urinary sodium and potassium were significantly higher in untreated diabetic rats compared to controls. Hyperglycemia increases osmotic diuresis and the subsequent loss of sodium and potassium in urine (Hillier et al. 1999). However, metformin treatment elicited a significant increase of urinary sodium in diabetic and non-diabetic rats. This is in agreement with the results of Hashimoto et al. (2018) demonstrating that metformin increases urinary sodium excretion by reducing phosphorylation of the sodium-chloride co-transporter (Hashimoto et al. 2018). Urinary anion gap is used as a surrogate marker of ammonium (NH4+) excretion and is important in the evaluation of renal tubular acidosis (Yaxley and Pirrone, 2016). Under normal circumstances, urinary anion gap ranges from 20-90 mEq/L (Bagga and Sinha, 2007). Our results showed that, the urinary anion gap appears normal in all groups. However, a positive urinary anion gap as found in diabetic groups also indicates inappropriately low renal $NH4^+$ excretion, as in renal tubular acidosis. In this case only urinary anion gap may not be sufficient to confirm the diagnostic of renal tubular acidosis in diabetic groups. Additional test like urinary osmolal gap is needed to clarify the diagnostic.

Metformin is not metabolized and is excreted unchanged in the urine. It has a mean plasma elimination half-life after oral administration of between 4.0 and 8.7 hours (Scheen, 1996). Two hours after its administration, plasma metformin concentration was significantly higher in diabetic rats compared to non-diabetic. In line with that finding, the renal excretion of metformin was significantly reduced in diabetic rats compared to non-diabetic. This might be due by renal impairment occurring in diabetes and that delays excretion of metformin which could result in a higher plasma level (Scheen, 1996). However, naringenin slightly improved

metformin clearance in diabetic rats. This could be due by the renoprotective properties of naringenin that ameliorates STZ-induced diabetic rat renal impairment and prevents renal tubular cells apoptosis (Roy et al. 2016).

Various membrane transporters including OCT1 and OCT2 mediate the secretion of organic cations such as metformin in the renal proximal tubule. We assessed the protein expression of these transporters in all the study groups and found that OCT1 and OCT2 were down-regulated in all diabetic groups compared to controls. Indeed, prolonged exposure to hyperglycemia in diabetes can result in progressive tubular dysfunctions that potentially contribute to reduce renal expression of organic cation transporters. The mechanism of altered expression of renal transporters remains to be elucidated. However, it has been suggested that renal OCT1 deficit may be due to post-transcriptional changes while alteration of OCT2 expression may result from transcriptional and/or translational changes associated to the formation and accumulation of AGEs (Grover et al. 2004). Both OCT1 and OCT2 expression were unchanged in diabetic rats treated with only naringenin. Contrarily, previous *in vitro* investigations showed that, pummelo, oranges and lime juices upregulate the expression of OCT2 in porcine cells after 24 hours exposure (Lim and Lim, 2008). The mechanisms behind these effects were not established. To explain the findings of our study, it has been suggested that, naringenin may not be the sole flavonoid component responsible for the modulation of OCT2 expression in pummelo, oranges and lime juices. In agreement, the same study showed that grapefruit juice which contains naringenin as a major flavonoid, did not modulate OCT2 in porcine cells.

Despite its known renoprotective effect, metformin did not improve significantly OCT1/2 expression in diabetic rats. This finding supports results of a previous study testing the dependency of the renoprotective effects of metformin on the renal expression of OCT1, OCT2 and AMPK in 3 day unilateral uretral obstruction (3dUUO) mouse model. The authors

found that, the protective effects of metformin were independent of the expression of OCT1/2. demonstrating that metformin can attenuate renal tubular damages but independently of the expression of OCT1, OCT2 and AMPK (Christensen et al. 2016). They have speculated that, a compensatory upregulation of OCT3 and potentially other unknown transporters of metformin might be involved (Ross and Holohan, 1983). In contrast to the level of OCT1, the amount of OCT2 was significantly improved when metformin and naringenin were co-administrated in diabetic rats. These observations indicate that naringenin appear to increase renal expression of OCT2 only in the presence of metformin. Here, naringenin might act as a regulator that stimulates metformin uptake in kidneys, by upregulation of OCT2 expression which improves metformin clearance in diabetic rats.

5. Conclusion

Based on the results of this study, concurrent administration of naringenin and metformin could ameliorate metformin clearance thus avoiding its accumulation in plasma in diabetic rats with reduced renal function.

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Abbreviations

AGEs: Advances glycation end-products, AMPK: 5' adenosine monophosphate-activated protein kinase, ANOVA: analysis of variance, AUC: area under curve, BUN: blood urea nitrogen, BW: Body weight, CONT: control, CrCl: creatinine clairance, FBG: fasting blood glucose, GTT: glucose tolerance test, HPLC: High-performance liquid chromatography, MET: metformin, NAR: naringenin, OCT: organic cation transporter, ROS: reactive oxygen species STZ: streptozotocin, UAG: urinary anion gap.

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Author contributions

Edith Pascale MOFO MATO contributed to the design of the study, acquisition of data, analysis, interpretation and drafting of the article.

M. Faadiel ESSOP contributed to the design of the study, validation, supervision and revised the article for important intellectual content

OWIRA PMO contributed to the conception and design of the study, validation, supervision and revised the article for important intellectual content

Conflicts of interest

The authors declare that they have no conflicts of interest

Data Statement

All data are available from the corresponding authors on request

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Figures

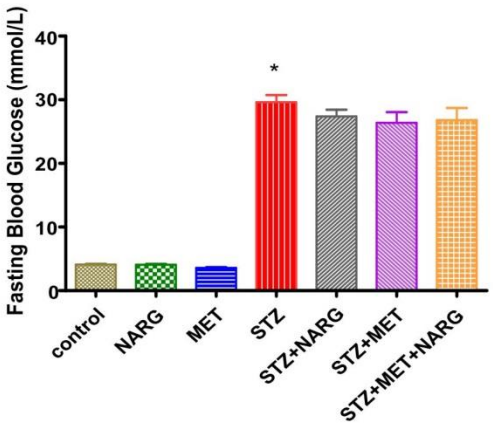

Figure 1: Fasting blood glucose levels (*p < 0.0001 compared to the control group).

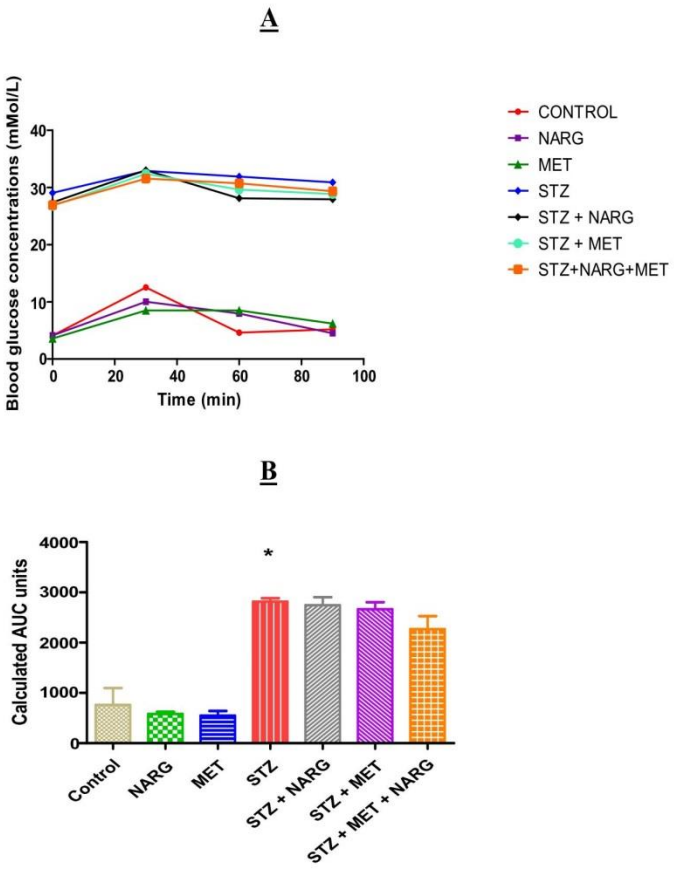

Figure 2: Glucose tolerance tests in treatment groups. Rats were intraperitoneally injected with D-glucose (3.0g/kg BW) in normal saline and blood glucose. A) GTT curves, B) Calculated area under the curve (AUC) derived from GTT plots. (*p < 0.0001 vs. controls).

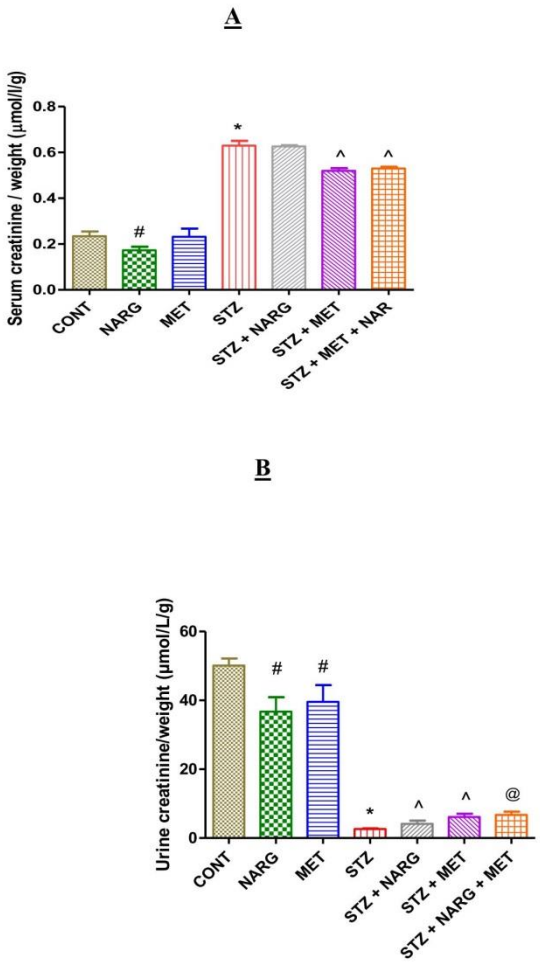

Figure 3: A) Serum creatinine to live body weights ratio (*,$^{\#}$P < 0.05) vs controls; ^P < 0.001 vs STZ. B) Urine creatinine (*,$^{\#}$P < 0.05), vs controls; ($^{@}$,^P < 0.05) vs STZ.

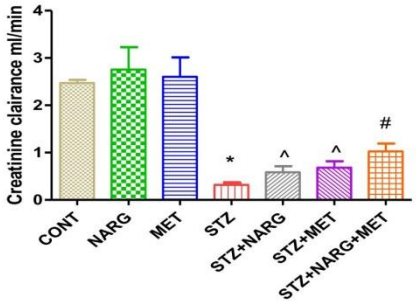

Figure 4: Calculated creatinine clearance. (*P < 0.0001 vs controls; #,^P < 0.05 vs STZ).

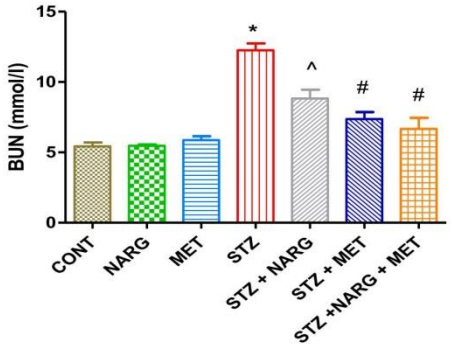

Figure 5: Blood urea nitrogen concentrations. (*P < 0.0001 vs controls, #,^P < 0.001 vs STZ).

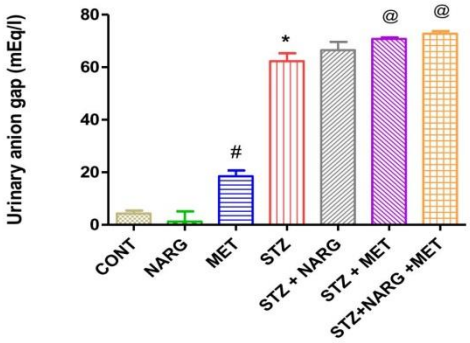

Figure 6: Urinary anion gap calculated as previously described [17]. (*p < 0.0001 vs Controls; $^{\#,@}$ p < 0.05).

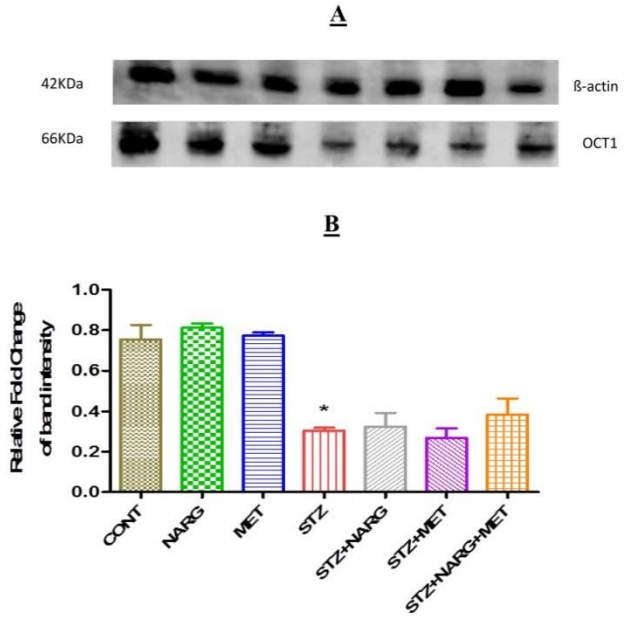

Figure 7: (**A**) Immunoblots of renal OCT1 protein expression analyzed by Western blotting standardized with ß-actin bands for equal loading and (**B**) Corresponding densitometry scans. (*P < 0.0001 vs controls).

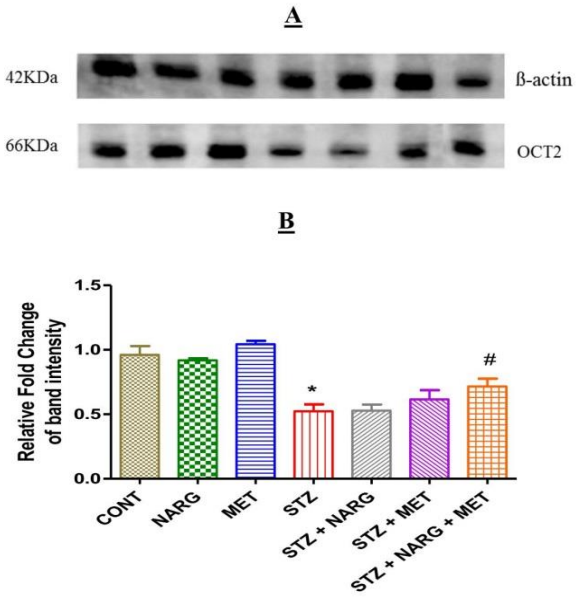

Figure 8: (**A**) Immunoblots of renal OCT2 protein expression analyzed by Western blotting standardized with β-actin bands for equal loading and (**B**) corresponding densitometry scans. (*P < 0.0001 vs controls, $^{\#}$P < 0.05 vs STZ).

List of Tables

Table 1: Animal treatment protocol. Metformin (MET), naringenin (NARG) and streptozotocin (STZ)

Groups	Designation	Treatment			
		Distilled H_2O (mL)	MET (mg/kg)	NARG (mg/kg)	STZ (mg/kg)
1	Control	3.0	-	-	-
2	NARG	-	-	60.0	-
3	MET	-	250	-	-
4	STZ	-	-	-	60.0
5	STZ+NARG	-	-	60.0	60.0
6	STZ+MET	-	250	-	60.0
7	STZ+NARG+MET	-	250	60.0	60.0

Table 2: Urine electrolyte levels. Sodium (*, $^{\#}P < 0.0001$ vs controls; $^{@}P < 0.005$ vs STZ), Potassium (*,$^{@}P < 0.05$ vs controls and STZ respectively), ($^{@,*}P < 0.05$ vs controls and STZ).

	Control	Control NARG	Control MET	STZ	STZ + NARG	STZ + MET	STZ + NARG + MET
Sodium (mM)	105,3±2,73	101,3±2,25	121,3±5,75 $^{\#}$	128,3±1,86*	129,7±4,03	137,0±6,26$^{@}$	128,0±9,08
Potassium (mM)	4,17±0,17	4,142±0,09	4,10±0,12	4,62±0,46*	4,16±0,47	4,05±0,7	4,20±0,134$^{@}$
Chloride (mM)	104,7±3,72	103,0±7,0	107,3±1,9	69,67±4,9*	66,67±3,39	70,33±4,9	59,33±11,91$^{@}$

Table 3: Metformin concentrations in plasma and 24 h urine after post-oral administration of 250 mg/kg metformin body weight. ($^{\#}P < 0.05$ vs MET, $^{@}P < 0.05$ vs STZ+MET *$P < 0.0001$ vs MET.(ND= Non diabetic)

	Plasma metformin (µg/ml)	Urine metformin (µg/ml/24 hrs?)
MET (ND)	1.076 ± 0.1	28.64 ± 0.64
STZ+MET	1.69 ± 0.1$^{\#}$	2.8 ± 0.4*
STZ+ MET+NARG	1.34 ± 0.09$^{@}$	3.6 ± 0.5*

Graphical abstract

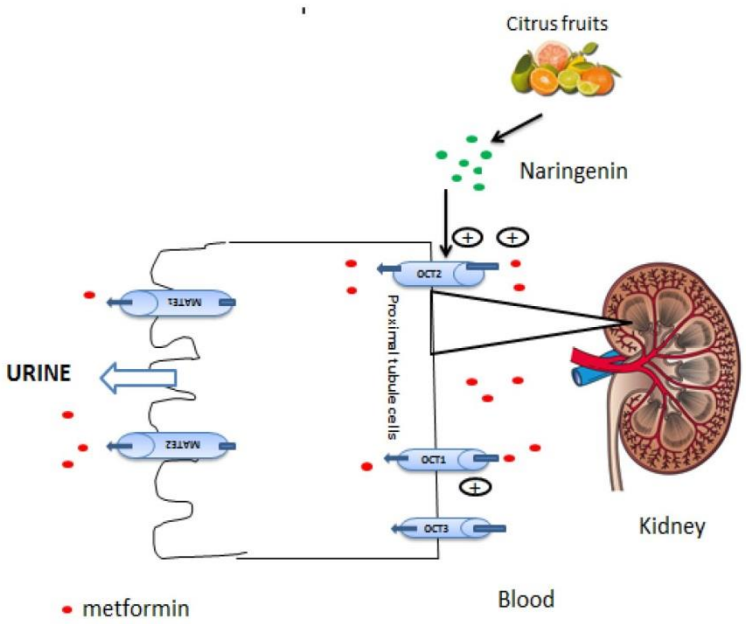

Illustration of the effect of naringenin on renal tubular cation transporters OCT1 and OCT2

Naringenin is the major flavonone in citrus fruits. It might increase renal organic cations transporters expression in the presence of metformin. This in turn could reduce metformin concentration in the blood and facilitate its excretion in the urine.

CHAPTER FIVE

General discussion and Conclusion

GENERAL DISCUSSION AND CONCLUSION

Organic cation transporters mediate the hepatic uptake and renal transport of the organic cation drug metformin. The present study used STZ-induced diabetic rats to investigate the effects of the citrus fruit-derived flavonoid, naringenin on metformin transporters OCT1 and OCT2. Firstly, we conducted a systematic review of relevant studies reporting the effects of OCT1 polymorphisms on metformin therapy in T2DM individuals. This review demonstrates the crucial role of OCT1 on metformin therapeutic responses and we provided proof of concept that genetic variations of SLC22A1 gene, that encoding for OCT1 may be associated with inter-individual variations in metformin's glucose-lowering efficacy. Although genetic studies reporting the effects of OCT1 polymorphisms on metformin responses in diabetic patients were limited, striking differences were observed in the frequency of such genetic variations in the study populations. We observed that, 7 of the 34 OCT polymorphisms identified including Met408Val, Gly465Arg, Arg61Cys, 32870G>A, -43T>G, Gly401Ser and Pro341Leu exhibited reduced transport of metformin while, other polymorphisms such as -43T>G and 1386A>C have been associated with beneficial effects on Hb1Ac [1]. The mechanisms by which these genetic variants of OCT1 affect the response to metformin could be inactivation of OCT1, lowering OCT1 mRNA expression and decreasing OCT1 protein expression by its retention in the endoplasmic reticulum.

Secondly, we performed experimental investigations using STZ-induced diabetic rats. As expected, surrogate diagnostic markers for diabetes such as weight loss, polydipsia, polyuria, increased fasting blood glucose and glucose intolerance were evident in diabetic rats. Electrolytes and acid/base balance were also altered. Treatment of diabetic rats with metformin, naringenin or both significantly reduced weight loss and polydipsia. However, naringenin and metformin did not improve fasting blood glucose or glucose intolerance. In fact, like metformin, naringenin has antihyperglycemic and not hypoglycemic effects.

Furthermore, the antihyperglycemic effects of metformin or naringenin could not be realized in diabetic animals due to insulin deficiency. Similarly, hepatic glycogen levels were not significantly improved in diabetic rats treated with metformin compared to untreated diabetic rats. In a normal state, insulin stimulates hepatic glycogen synthesis. With a deficiency of insulin, there is increased hepatic glycogenesis [2]. This study also provides convincing experimental evidence on the renoprotective effects of metformin and naringenin in diabetic rats. Our results showed that untreated diabetic rats were exhibited an elevated microalbuminuria and blood urea nitrogen and altered creatinine clearance compared to controls but naringenin or metformin or both significantly reversed these in diabetic animals. The protein expression of organic cations transporters (OCT 1 and OCT 2) was depleted in diabetic rats. Metformin increased OCT 1 protein expression in non-diabetic rats but had no significant effects on OCT1 protein expression in diabetic rats compared to untreated diabetic rats. Due to its antioxidative effects naringenin with or without metformin upregulated hepatic expression of OCT1 in diabetic rats. Our results show that, this upregulation of OCT1 protein expression lead to increased hepatic uptake of metformin. As a consequence, metformin will accumulate in hepatocytes and inhibit complex I of the mitochondrial respiration chain causing cellular energetic stress and elevation of the AMP: ATP ratio. Increases in AMP: ATP ratio will inhibit fructose-1,6-biphosphatase, leading to the acute inhibition of gluconeogenesis [3]. This in turn results in both accelerated lactate production and reduced lactate metabolism. Naringenin could be therefore implicated in the hepatic accumulation of metformin and concomitant increase in plasma lactate concentrations. Here is the possible mechanism whereby naringenin potentiates metformin – induced lactic acidosis as we have previously reported [4]. Unlike hepatic OCT 1, naringenin did not show the potency to modulate renal OCT1 in both diabetic and non-diabetic rats. Indeed, OCT1 is expressed to an extremely low level in kidneys [4] and may therefore not play an influential role in the renal excretion of metformin.

Furthermore, our results show that, naringenin significantly increased renal OCT2 protein expression in diabetic rats only in the presence of metformin. Urinary metformin concentration was higher in untreated diabetic rats. However, naringenin increased urinary metformin concentration in diabetic rats treated with metformin. We have suggested that, the upregulation of renal OCT 2 protein expression by naringenin stimulate uptake of metformin from the blood into the renal tubule and thereby facilitate its excretion in the urine.

Despite the increase excretion of metformin, the plasma lactate concentration was still elevated in diabetic rats treated with metformin and naringenin compared to diabetic rats treated with metformin only. This suggests that, increased renal OCT2 protein expression and excretion of metformin by naringenin may not be sufficient to avert metformin-induced lactic acidosis in diabetic rats. Taken together, these findings suggest that naringenin is a natural compound to be considered for its anti-oxidant capacity, anti-hyperglycemic properties and renoprotective actions. In addition, naringenin may be an effective therapeutic agent to help in attenuate diabetes-related complications. However, the concurrent administration of metformin and naringenin supplements is not recommended in diabetic patients.

Future studies are required to investigate the effects of naringenin on other membrane transporters involved in the disposition of metformin such as PMAT, OCT3, MATE1 and MATE2-K. Furthermore, it would be also interesting to evaluate the effects of naringenin on the gene expression of such transporters.

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APPENDIX A: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

19 September 2016

Ms Edit Pascale Mofo Mata (216056711)
School of Health Sciences
Westville Campus

Dear Ms Mofo Mata,

Protocol reference number: AREC/056/016D
Project title: Effects of Naringenin on Metformin disposition in a diabetic rat model

Full Approval – Research Application

With regards to your revised application received on 13 September 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

1. Oral Glucose Tolerance Test (OGTT) must be conducted at least 3 days before the sample collection (sacrifice).
2. Animals must be fasted at least for 12 hours before sacrifice in order to avoid vomiting and other gastrointestinal problem during sacrifice or sample collection

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 19 September 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Peter Owira
Cc Acting Academic Leader Research: Professor M Soliman
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

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