School of Pharmacy

Factors Affecting Metformin Plasma Concentrations

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"And say: "O my Lord! Increase me in knowledge".

The Holy Quran, Taha (20-114).

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Abbreviation

ADA	American Diabetes Association
AICAR	4-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine Mon-Phosphate
AMPK	Adenosine Monophosphate activated Protein
ATP	Adenosine Tri-Phosphate
ATP III	Adult Treatment Panel III
AUC	Area Under Plasma Concentration Curve
BMI	Body Mass Index
BNF	British National Formulary
BP	Blood Pressure
CEU	European population
CHB	Chinese population
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
Clcr	Creatinine Clearance
Cmax	Maximum Concentration
COSMIC	Comparative Outcome Study of Metformin Intervention versus Conventional approach
CVD	Cardiovascular Disease
DDD	Defined daily doses
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
DPP	Diabetes Prevention Program
DPS	Diabetes Prevention Study
FDA	Food and Drug Administration
FIS	Inbreeding coefficient
FPG	Fasting Plasma Glucose
GFR	Glomerular Filtration Rate
GVS	Genome Variation Server
HbA1c	Glycated Haemoglobin
HDL	High- Density Lipoprotein
HWE	Hardy Weinberg Equilibrium
IBW	Ideal Body Weight
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
JPT	Japanese population
LDL	Low Density Lipoprotein
LKB1/STK11	Serin Threonine Kinase
MAF	Minor Allele Frequency
MALA	Metformin Associated Lactic Acidosis
MATE	multidrug and toxic extrusion
MDRD	Modification of Diet in Renal Disease study
MetS	Metabolic Syndrome
MI	Myocardial Infarction

MPP^+	[^{3}H]-1-metheyl-4-phenylpyridinium
NCEP	National Cholesterol Education Program
NHLBI/AHA	National Heart Lung and Blood Institute and the American Heart Association
NHMRC	National Health and Medical Research Council
OCT	Organic Cation Transporter
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
PVD	Peripheral Vascular Disease
SD	Standard Deviation
SLC	Solute carrier transporter
SLC22A	Solute carrier transporter, subfamily 22
SNPs	Single Nucleotide Polymorphisms
Srcr	Serum Creatinine
STZ	Streptozotocin
TEA	Tetraethylammonium
UAE	United Arab Emirates
UKPDS	United Kingdom Prospective Diabetes Study
V_d	Volume of Distribution
WHO	World Health Organization
YRI	African population

Abstract

Background: According to the United Kingdom prospective diabetes study, metformin is considered the drug of choice in overweight newly diagnosed type 2 diabetic patients. Metformin is well characterized as a substrate of the organic cation transporter (OCT) 1 and OCT 2. In *vitro* and in *vivo* studies have revealed that OCT1 and OCT2 genes exhibited polymorphic variations in different ethnic groups, and metformin uptake was impaired in individuals who carried polymorphic single nucleotide polymorphisms (SNPs). It was observed that in clinical practice there was inter-individual variation to metformin therapy; it could be due to a variety of reasons including genetic variations of the OCT1 and OCT2 genes. To date no genetic studies have been conducted on Arab populations to investigate the clinical effects of genetic polymorphisms of OCT1 and OCT2 genes on metformin plasma concentrations in diabetic populations.

Objective: To investigate factors affecting metformin plasma concentrations and to analyse genetic variations of OCT1, OCT2, and serine threonine kinase (LKB1) genes in the United Arab Emirates (UAE) population. Also, to investigate the possible effects of genetic variations of these genes on metformin plasma concentrations among a diabetic group of patients in the UAE population.

Study design: A cross sectional prospective study recruited control non-diabetic and diabetic (test) individuals from four different hospitals and health centres in the UAE. Diabetic patients had been prescribed metformin therapy for at least seven days. One fasting blood sample was taken to investigate metformin plasma

concentrations, fasting plasma glucose, glycated haemoglobin, serum creatinine, and DNA genotypes.

Results: Overall, 170 control non-diabetic and 292 diabetic subjects completed the study. The mean age for the diabetic patients was 54± 0.7 years, mean glomerular filtration rate (GFR) was 88 ±1.6 mL/min/1.73m^2, mean metformin dose was 1622 ±28 mg/day, and the mean metformin plasma concentration was 0.62 ±0.03 mg/L. Among the reported OCT1 SNPs in the database 12 were polymorphic in the UAE population, while five polymorphisms were reported in the OCT2 gene, and two were polymorphic in the LKB1 gene. One novel synonymous Ser 279 (A/G) SNP was found in exon 1 in the OCT2 gene that has not been reported in any other population with minor allele frequency (MAF) of 1.03%.

The result of the current study showed that among the investigated variables, metformin dose, GFR, OCT1-E1 synonymous SNP rs1867351 and OCT1-E9 non-synonymous 488Arg>Met SNP were found to be associated with metformin plasma concentrations with p = 0.002, p = 0.008, p = 0.005, and p = 0.007 respectively.

Conclusion: This is the first large study to investigate the allele frequencies of OCT1, OCT2, and LKB1 genes in the Eastern Mediterranean population, and to report the effect of genetic variations of drug transporters on metformin plasma concentrations. These findings emphasise that each ethnic group carries a unique genetic background that should be genotyped and investigated independently. It also highlights the important role played by the synonymous SNPs on the drug

transporters function. In order to implement these findings in clinical practice, further pharmacoeconomic studies are recommended to evaluate the cost effectiveness of genotyping selected sub-groups of patients for genetic variations in OCT genes. It is also recommended that further pharmacogenomic studies should be carried out to investigate the impact of genetic variations in other candidate genes that might affect metformin efficacy and disposition, such as plasma membrane monoamine transporter (PMAT) and multidrug and toxic extrusion (MATE) genes.

1 Background

Diabetes is one of the most common non-communicable diseases worldwide and is a leading cause of death in many countries; the global epidemic of diabetes will impact on both the developed and the developing world (1-3). An estimated 336 million people worldwide are expected to have diabetes by the year 2030, of which 1,673,000 in Australia and 684,000 in the United Arab Emirates (UAE) (4). The prevalence of diabetes is increasing due to population growth, ageing, urbanization, and increasing levels of obesity and physical inactivity (5-7). It is of note that the rising incidence of obesity may cause these figures to be an underestimate of future diabetes prevalence.

Diabetes mellitus (DM) is a public health issue of significant economic importance because of the chronic nature of the disease, its high and globally increasing prevalence (4, 8-9), the demand for multi-modal treatment (10-11), and the serious complications associated with long disease duration (12-14).

According to the guidelines, metformin is considered the first choice of mono-therapy in patients with type 2 DM (8, 15-16), and it can be safely combined with other classes of oral hypoglycaemic agents (11). However, a total of 38% of type 2 diabetes patients receiving metformin therapy failed to reach the target glycated haemoglobin (HbA1c) level (15, 17). Metformin is well characterized as a substrate of the organic cation transporter (OCT)1 and OCT2 genes (18-20). Genetic polymorphisms in the drug transporters gene contribute to variability in

the pharmacokinetic properties of metformin (21-23). Genetic and molecular studies have demonstrated that OCT genes exhibit polymorphic variation in the coding region (24-27) and in order to investigate the pattern of polymorphisms that exist in OCT1 and OCT2 genes, various studies have screened for genetic variants of these genes in different populations, including Caucasian (27), Japanese (28) and Korean (29). A number of polymorphisms were found in the studied populations, and subsequent functional analysis of the reported variations showed reduced activities of those variants towards their substrates (24-25, 30-31). Nearly all of the studies were conducted on healthy individuals (24-26, 29, 32), and to our knowledge only one employed diabetic subjects utilizing a small sample size (21). Furthermore, no study has occurred in the Arab population. Thus, in order to investigate genetic polymorphisms and their role in clinical pharmacokinetics, therapeutic effects and safety a large scale prospective study in patients with diabetes is required which should include different ethnic groups from different genetic backgrounds.

1.1 Prevalence of diabetes

The worldwide prevalence of diagnosed diabetes has increased dramatically over the past 40 years. In 1985, there were approximately 30 million people with diabetes worldwide (8), by 1995, this number had escalated to 135 million (8), and by 2030, it is projected that the incidence of diabetes will increase by 40%, affecting 336 million people (4). The published age and sex specific estimations of current and future diabetes prevalence have shown differences in numbers;

however, the dramatic escalating prevalence of diabetes was not debated amongst all of the studies.

Epidemiological studies have revealed that the age structure of the diabetic populations of developed and developing countries were noticeably different. The majority of the cases diagnosed in the developed countries were in the oldest age group ≥60 years (4-5, 9). In contrast, the majority of people with diabetes in developing countries were in the 45 to 64 year old age group (4-5, 9). Thus the economic productivity, fertility, and reproduction of these communities especially in developing countries would be significantly affected. Figure 1 shows diabetes cases in 2000 and projections for 2030 (33).

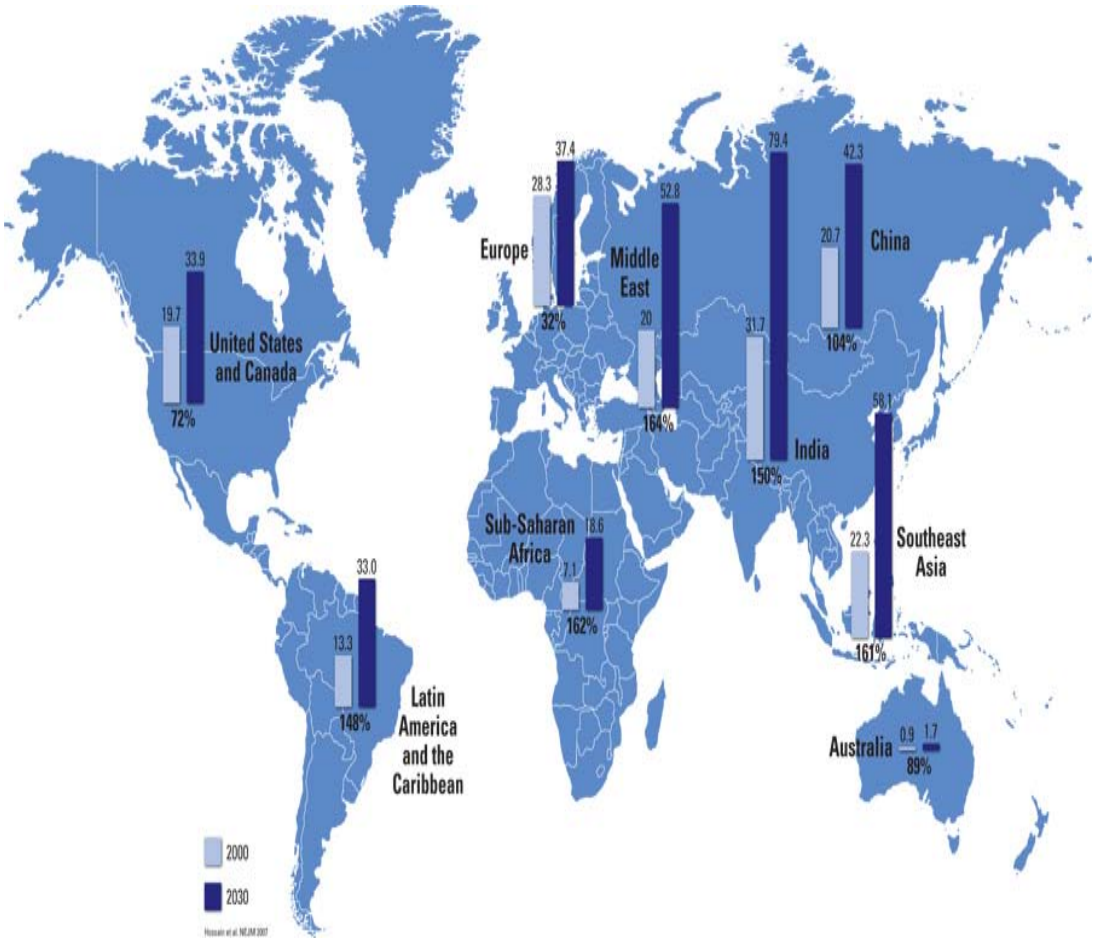


Figure 1: Millions of cases of diabetes in 2000 and projections for 2030, with projected percent changes

1.2 Economic burden of diabetes mellitus

People with diabetes are more likely to use health services than people without diabetes (9, 34), and to use them more often and for longer periods of time [35-37]. Most of the global epidemiological data utilized the prevalence of DM as the basis for projections of economic impact of the disease (9, 34, 38). To assess the economic impact of its prevalence, most of the economic studies have used direct, indirect, and intangible cost approaches (9, 34, 38). The direct costs of the disease are those associated with hospitalization, ambulatory care, and medication. The indirect costs represent lost productivity due to morbidity and, possibly, premature mortality. Finally, the intangible costs are those to which it is difficult to attach a monetary value, such as reduced life expectancy and quality of life (39-41).

According to American Diabetes Association (ADA) (36) diabetes cost the United States (US) an estimated $132 billion in 2002 in medical expenditure and lost productivity. This estimation was 74% higher than 1997 (6). This estimated cost underestimates the true burden of diabetes, because it omits intangibles, such as pain and suffering, care provided by non-paid caregivers, and several areas of health care spending where people with diabetes probably use services at higher rates than people without diabetes; such as dental care, optometry care, podiatric visits, and the use of licensed dieticians. Moreover, this cost estimation excluded undiagnosed cases of diabetes. Worldwide, the direct cost of diabetes increased from 1.7 billion US dollars in 1969 to 4.4 billion US dollars in 1997 (38). In

Australia, direct health care expenditure on diabetes was estimated to be 784 million Australian dollars in 2000-01 (42).

1.3 Classifications and pathophysiology of DM

DM is a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (43-44).

The new classification system identifies four types of DM: type 1, type 2, "other specific types" and gestational diabetes (45).

The alterations of metabolism in patients with type 2 diabetes partially overlap with those alterations of metabolism seen in patients with type 1 diabetes. Absolute or relative deficiency of insulin is common to both disorders, as is a severe disturbance of the patterns of glucose and lipid levels in plasma (46). However, the fluctuations of plasma glucose are less extreme in type 2 diabetes than in type 1 diabetes, and the exaggerated catabolic state of severe insulin deficiency often seen in type 1 is uncommon in type 2 (46-47). The pharmacological therapy is limited in type 1 DM to insulin dependency. This study will focus entirely in type 2 DM. Therefore any further discussions in the following sections relate only to type 2 DM.

The pathophysiology of type 2 DM is characterized by three abnormalities: impaired insulin secretion, peripheral insulin resistance, and excessive hepatic glucose production (43-44, 48). Obesity, particularly visceral or central (as

evidenced by the hip-waist ratio), is very common in type 2 DM (49). In the early stages of the disorder glucose tolerance remains normal despite insulin resistance, because the pancreatic beta cells compensate by increasing insulin output (50-51). As insulin resistance and compensatory hyperinsulinemia progress the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. Impaired glucose tolerance (IGT), characterized by elevation in postprandial glucose, then develops (43-44). A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Ultimately, beta cell failure may ensue.

1.4 Prevention of diabetes

The escalating prevalence of DM with its high morbidity and excess mortality is imposing a worldwide burden on healthcare systems (9, 34, 38). Effort and attention needs to be given to prevention programs that will help greatly in reducing the costs of health expenditure on diabetes and its complications and burden to governments, public and private companies, and individuals.

The Finnish Diabetes Prevention Study (DPS)(52) was one of the first large controlled, randomized study to show that in a high risk population, lifestyle intervention was associated with a 58% decrease in the risk of progression of type 2 DM ($p< 0.001$). Similar observations were reported in the American Diabetes Prevention Program (DPP)(53). However DPP randomized subjects to intensive lifestyle intervention and standard lifestyle intervention plus metformin 850 mg twice daily. The DPP study revealed that treatment with metformin and

modification of lifestyle were two highly effective means of delaying or preventing type 2 DM with a 31% and 58% reduction of diabetes risk, respectively. This study showed for the first time that metformin could be used in the prevention of type 2 diabetes especially with individuals who fail to comply with intensive lifestyle intervention.

1.5 Prediction of diabetes

1.5.1 Metabolic syndrome

The clinical importance of metabolic syndrome (MetS) is related to its putative impact on cardiovascular morbidity and mortality and it's possibility of predicting type 2 DM. Diabetes by itself is considered to be a risk factor for cardiovascular disease (CVD), thus increased CVD risk occurs for individuals suffering from both DM and MetS (54) (55) (56) .

MetS has received increased attention in the past few years. It is a constellation and clustering of interrelated risk factors of metabolic abnormalities including glucose intolerance (type 2 DM, IGT, or impaired fasting glycaemia [IFG]), insulin resistance, central obesity, dyslipidaemia, and hypertension, which appear to directly promote the development of atherosclerotic cardiovascular disease (54-60). In an effort to gain recognition of its existence, several organizations have attempted to formulate and develop an internationally recognized definition of the MetS (Table 1).

All of the above definitions are sex-specific, with different thresholds for both waist circumference and high density lipoprotein (HDL) cholesterol between males and females.

Many studies compared the prevalence of MetS using different criteria. Most of the published studies reported the prevalence of MetS based on WHO and NCEP ATPIII definitions. Despite of the differences in the study designs, sample selection, year that they were undertaken, precise definition of the MetS used, and age and sex structure of the population of the studies, most of them came out with a consistent finding that the prevalence of the syndrome is highly age-dependent. This pattern was clear in the study conducted in the USA where the prevalence increased from 7% in participants aged 20-29 years to 44% and 42% for those aged 60-69 years and at least 70 years, respectively (61). Similar finding was observed in Greece studies (62-63), the Botania study in Finland and Sweden (54), and in Cameron *et al.* study (64) who published a detailed review about the worldwide prevalence of the syndrome with different criteria.

Table 1: Comparison of definitions of the metabolic syndrome

WhO (1998) (65)		NCEP ATPIII (2001) (57)	NHLBI/AHA (2005) (66)	IDF (2005) (67)
Diabetes or IFG or IGT or insulin resistance (hyperinsulaenemic, euglycaemic clam-glucose uptake in lowest 25%) plus two or more of the following		Three or more of the following	Three or more of the following	Central obesity- waist circumference- ethnicity specific
Obesity/central obesity				
Body mass index (BMI)	>30			
Waist-to-hip ratio	>0.9 (men) or >0.85 (female)			
Waist circumference Men		>102 cm	>102 cm	≥94 cm
Women		>88 cm	>88 cm	≥80 cm

Hypertension	BP>140/90 mm Hg	BP≥135/85 mm Hg	BP≥135/85 mm Hg or specific medication	BP≥135/85 mm Hg or specific medication
Triglycerides	≥1.7 mmol/L (≥150 mg/dl)	≥1.7 mmol/L (≥150 mg/dl)	≥1.7 mmol/L (≥150 mg/dL) or specific medication	≥1.7 mmol/L (≥150 mg/dL) or specific medication
HDL-cholesterol men	<0.9 mmol/L	<1.03 mmol/L (40 mg/dl)	<1.03 mmol/L (40 mg/dL) or specific medication	<1.03 mmol/L (40 mg/dL) or specific medication
Women	<1.0 mmol/L	<1.29 mmol/L (<50 mg/dL)	<1.29 mmol/L (<50 mg/dL) or specific medication	<1.29 mmol/L (<50 mg/dL) or specific medication
Fasting plasma glucose (FPG)		≥6.1 mmol/L (≥110 mg/dL)	≥5.6 mmol/L (≥100 mg/dL)	≥5.6 mmol/L (≥100 mg/dL)
Microalbuminuria	Albumin excretion >20 µg/min			

1.6 Complications of diabetes

Diabetes is an important and independent risk factor for the development of atherothrombosis and related cardiovascular events (68-70). It is characterized by specific changes in microvessels, thus causing diabetic microangiopathy, namely nephropathy, retinopathy, and neuropathy. (71-74)

Diabetes noticeably reduces life span; many observational studies have shown that CVD is a major factor in reducing longevity in persons with diabetes (7, 70-71, 75). About 80% of all diabetic patients die from CVD (7, 70, 74-76), 75% of such deaths are due to coronary heart disease (CHD), and the remaining 25% to cerebrovascular, peripheral or other macrovascular diseases (69-70).

The beneficial effects of improved glycemic control on microvascular complications, including retinopathy, nephropathy, and neuropathy, have been documented in several randomized studies published during the last 20 years. In order to document the effect of improved glycemic control on reduction of macrovascular complications a meta-analysis study was performed by pooling data from several randomized trials (77). Ten studies that included 14 randomized comparisons of intensified and conventional treatment were included. The result showed that improved glycemic control (measured by HbA1c %) translated into substantial reductions in macrovascular risk in type 2 DM (combined incidence rate ratios was 0.81). Moreover, they found that in type 2 DM, substantial effects were observed for peripheral vascular disease (PVD) and stroke, whereas cardiac events

were not found to be reduced significantly. This finding could be explained by the fact that the metabolic abnormalities typical for type 2 DM not only lead to insulin resistance and hyperglycemia but also to dyslipidemia, arterial hypertension, endothelial dysfunction, and increased platelet hyperactivity and coagulability (68-69, 78-80). Improving blood glucose control without also addressing the other abnormalities, most importantly hypertension, dyslipidemia, and platelet hyperactivity, may therefore produce only limited benefit (71, 77).

Recent findings from the US (61, 81) and the United Kingdom (UK) (82) have shown an unfavourable trend in CVD mortality in younger people (35-44 years). In this age group (in woman from the US and men from the UK), CVD mortality increased significantly for the first time over two decades ($p< 0.0001$) (61, 81-82). These adverse outcomes were considered to be related to the recent epidemic of MetS and DM (61, 81).

As the nature of the syndrome being a cluster of multiple diseases, the management also requires multiple interventions targeting individual risk factors. Education about MetS is an essential element in the management plan and it should involve both physicians and patients. The overall assessment should weigh the risk and benefit of non-pharmacological and pharmacological interventions that significantly improve CVD risk factors among patients.

1.7 Management of type 2 DM

Weight control and long term management of obesity are central to the prevention of diabetes and the optimal management of people with diabetes. Accordingly, lifestyle interventions, irrespective of the concomitant use of pharmacologic anti-diabetic therapy, remains the cornerstone of the management of type 2 DM (83-84).

Treatment goals for patients with type 2 DM specify targets for glycemic and other cardiovascular risk factors (78, 83-85). For most patients, lifestyle intervention will be tried first, although commencement of pharmacological treatment may be necessary in patients with poor glycemic control and low body weight (10, 85). The long term efficacy of lifestyle intervention is often poor. For example, diet based therapy in the United Kingdome Prospective Diabetes Study (UKPDS) controlled HbA1c to <7.0% in only 25% of patients after 3 years, 11% after 6 years and 9% after 9 years of treatment (15).

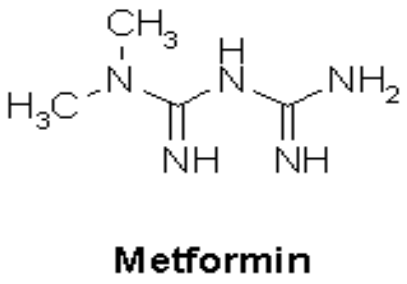
Pharmacological management of type 2 DM includes insulin therapy and oral hypoglycaemic therapy. To date, seven types of medications are commonly used to treat type 2 DM; insulin, metformin, sulphonylureas, thiazolidinediones, meglitinides, dipeptidyl peptidase-4 inhibitors, and alpha glucosidase inhibitors.

According to the UKPDS (15) intensive treatment with metformin reduced stroke, diabetes related end points, and all cause mortality when used as mono-therapy in overweight, newly diagnosed type 2 diabetic individuals, compared with intensive treatment with insulin or sulphonylureas, despite similar glycemic control (15, 71).

Consequently, metformin is regarded as the drug of choice for the treatment of overweight newly diagnosed type 2 diabetic subjects.

1.8 Metformin

Metformin is a biguanide (1, 1-dimethylbiguanide)(Figure 2) used as an oral hypoglycemic agent in the treatment of type 2 DM (86). It is the only biguanide available in the market, and is marketed as Glucophage®, Diaformin®, Glucomet®, and Diabex® (87-88). It is used as mono-therapy or in combination with other types of oral antidiabetic agents or insulin (86, 89-90).



Metformin

Figure 2: Metformin chemical structure

1.9 Combining metformin with other hypoglycaemic agents

Metformin is considered the first choice of mono-therapy in patients with type 2 DM (8, 15-16). However, some patients fail to achieve target HbA1c level with metformin mono-therapy (15, 17). Treatment options for metformin mono-therapy failure include the addition of other available hypoglycemic agents. The efficacy of

different hypoglycemic drugs in combination with metformin was investigated in several randomized controlled trials. Overall, sulphonylureas (91-94), alpha glucosidase inhibitors (95-97), thiazolidinedione (98-99), and glitinide (11, 100-101) were all effective and tolerable as add on therapy to metformin. However, sulphonylureas, alpha glucosidase inhibitors, and glinides, seems to have similar efficacy when used as add on therapy in patients with type 2 diabetes inadequately controlled with metformin mono-therapy. Thiazolidinediones often have lower effects on HbA1c in the first six months, although their efficacy could be equal to or higher than other agents in the long term (98-99). The limited number of randomized trials available does not allow any definitive conclusion regarding the efficacy of different agents in patients failing metformin mono-therapy (11). Some factors, including initial HbA1c or duration of diabetes, dietary, and physical activity could affect the extent of the reduction of HbA1c in individual studies.

Thus in clinical practice, the choice of drugs to be added to metformin mono-therapy is mainly individualized and sometimes subjected to the clinician's preference and the guidelines they follow. For example, the Australian guidelines (102) recommend sulphonylureas as the first add on medication to metformin therapy where patients fail to achieve the target HbA1c level with metformin mono-therapy. However, it does not clearly specify the next add on medication in case of failure with both metformin and sulphonylureas; it is left to physician's preference and judgment.

1.10 Metformin pharmacokinetic properties

The pharmacokinetic properties of metformin have been investigated in patients with diabetes (103-104), in healthy volunteers (104-106), and in diabetic patients with impaired renal function (107), using the oral and intravenous routes of administration. The pharmacokinetic properties of metformin have been well characterized in different populations (104-106, 108-111) and no significant difference was observed in the metformin pharmacokinetic parameters among those populations.

Compartmental analysis has indicated that metformin pharmacokinetics is best described by a two compartment open model (103, 112). Elimination from the central compartment is relatively rapid ($t_{1/2}$ $\alpha \approx$ 2 hours), with slower elimination from the deep compartment ($t_{1/2}$ $\beta \approx$ 12 to 14 hours), involving only small fraction (<5%) of the administered dose (113). Erythrocyte was suggested as a marker for a deep compartment to measure metformin distribution and potential accumulation in chronic metformin therapy (114-115).

1.10.1 Absorption

Gastrointestinal absorption of metformin is incomplete with 20 to 30% of an oral dose being recovered in the faeces (103, 105-106). Metformin is slowly absorbed from the proximal small intestine and mainly excreted from urine without undergoing liver metabolism (105-106, 113). Because the rate of metformin absorption is slower than its rate of elimination , intestinal absorption is the rate

limiting step of metformin disposition (106, 113), thus making the absorption of metformin incomplete and dose-dependent (104, 106, 113).

Metformin doses of 500 mg to 1500 mg have an absolute bioavailability of 50% to 60%, and decreases with increased dose (105-106). It was observed that metformin bioavailability decreased from 86% with 250 mg of metformin HCl to 42% with 2000 mg, and the 1500 mg dose had lower bioavailability than the 500 mg metformin dose (104-106, 108, 116). These observations show that the absorption of metformin is dose dependent and is subject to a saturable active transport process (104, 116). Food slightly reduced the rate and extent of metformin absorption with a 20% lower area under the plasma concentration-time curve (AUC) value and 35% lower maximum concentration (Cmax) value (103, 112).

1.10.2 Distribution

Metformin is rapidly distributed however, a slow transfer to a deep compartment also seemed to occur (103, 105-106). Metformin accumulated in the esophagus, stomach, duodenum, salivary glands and kidneys (105, 112). Protein binding is negligible. Following a single oral doses, the mean apparent volume of distribution (Vd) of metformin ranged from 63 to 276 L (105-107, 112, 117).

1.10.3 Metabolism

Metformin does not undergo metabolism, but is excreted largely in the unchanged form (103, 105-106). It is a strong base (pKa 11.5) hence the drug is protonated at physiologic pH (118-119).

1.10.4Excretion

Metformin undergoes rapid renal excretion through active secretion. It has a plasma elimination half life (t1/2 β) ranging from 1.5 to 4.5 hours after intravenous injection, and from 2.0 to 6.0 hours after oral administration in healthy volunteers (105-107).

1.11 Plasma concentrations and therapeutic efficacy

Metformin plasma levels are not usually monitored in order to determine the effectiveness of the treatment, and it has little clinical value except when lactic acidosis is present (103). The normal therapeutic level of metformin is not defined; however some studies have investigated the relationship between metformin levels and clinical outcome in patients with type 2 DM. A therapeutic range of 0.6 ±0.5 mg/L in the fasting state had been suggested by Lalau *et al*. (120) . In this study patients with type 2 DM (n= 58) were assumed to have achieved the therapeutic range for metformin because they had been receiving well-tolerated chronic metformin therapy at the recommended dosage of 1,700 to 2550 mg/day and were well controlled at the time the study was conducted. Their arterial lactate levels were significantly low (1.09 ±0.06 mmol/L). However, the study did not give details of the glucose and HbA1c levels or the mean duration in which their glycemic control was stable. Other studies have also suggested the same concentration range of metformin at steady state level (93, 113, 121-122).

1.12 Pharmacokinetics in special conditions

1.12.1 Renal failure

The kidney plays an essential role in the elimination of metformin, therefore the relationship between renal function impairment and metformin pharmacokinetics has been studied.

Sirtori *et al*. (105) analysed the kinetic parameters of metformin in volunteers with normal and altered renal excretory function (estimated by creatinine clearance, Clcr). Following an IV injection to patients with impaired renal function the mean plasma $t_{½}$ was 4.94 ±1.11 hr longer than for normal volunteers ($p< 0.005$). They also observed a significant inverse correlation between the plasma $t_{1/2}$ of unchanged metformin and Clcr in individuals with normal and impaired renal function ($r= 0.88$, $p< 0.001$). This significant correlation indicated the possibility of accumulation of metformin in severe renal impairment. If metformin was taken at eight hour intervals, the accumulation of unchanged drug after chronic treatment would be approximately 20% higher in a patient with $t_{1/2}$ of 3 hours (i.e., with a Clcr between 30 and 60 mL/min) than in one with a normal $t_{1/2}$ of 1.5 hour. Doubling of accumulation would occur only with $t_{1/2}$ values above 7 hours, i.e., in patients with Clcr of 30ml/min or less. Only patients with extremely impaired renal function are likely to significantly accumulate the drug.

Another open labelled study (107) evaluated the independent contribution of kidney function and ageing on the pharmacokinetics of metformin. As shown in Figure 3,

the pharmacokinetics of metformin was influenced by kidney function. When the results for subjects with moderate and severe renal impairment were compared with those in the healthy young/middle age group, it was found that the total clearance was 77% lower in the latter group. As for metformin renal clearance, the result was estimated to be 78% lower in the moderate and severe renal impairment groups than in the healthy young/middle-age groups, again the difference was significant. Cmax was estimated to be 173% higher, and AUC was estimated to be 390% greater in subjects with moderate and severe renal impairment than in the healthy young/middle-age group, this was also noted to be a significant difference.

Moreover, Tucker *et al.* (106) described the pharmacokinetics of metformin administered via oral and IV routes in normal subjects and in patients with type 2DM. Three groups of subjects were studied, consisting of healthy male volunteers, patients who were newly diagnosed with type 2 DM and eight type 2 DM patients (with impaired renal function, estimated by Clcr, which ranged from 47 mL/min to 107 mL/min). A highly significant linear correlation was observed between the renal clearance of metformin and creatinine clearance ($r=0.85$, $p< 0.001$).

Data summarising these findings are shown in Tables 2-4.

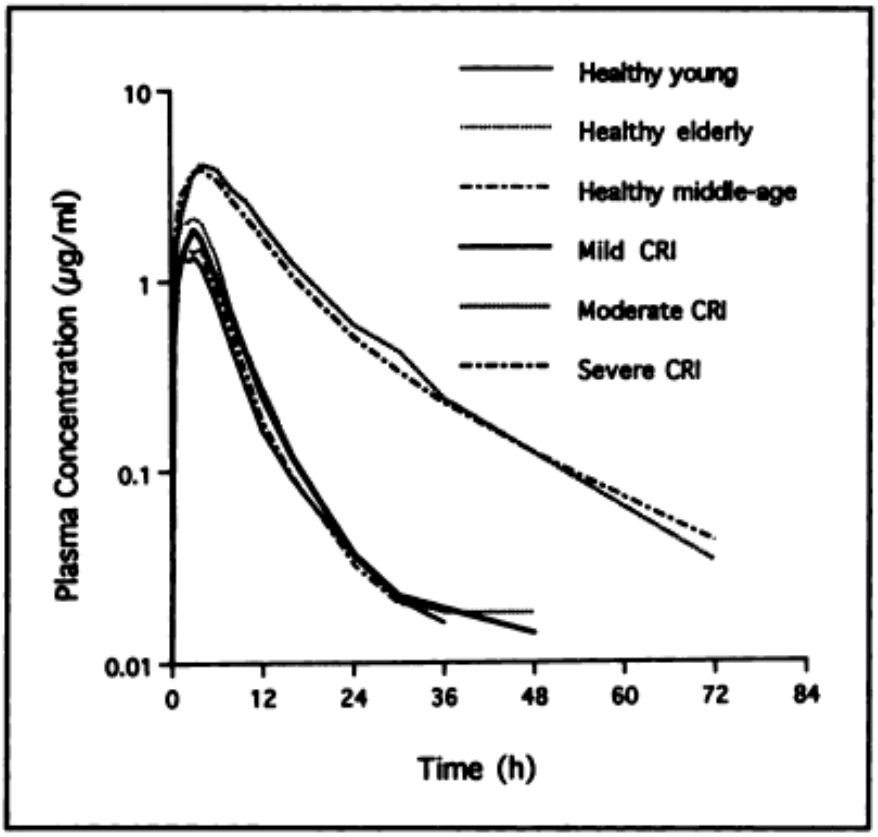


Figure 3: Metformin plasma concentration versus (Vs) time curves of mean data after a single, 850 mg oral dose of metformin HCL (adapted from [(107)])

Table 2: Parameters describing the kinetics of metformin after a single oral 850 mg metformin dose in healthy, moderate renal impairment, and severe renal impairment subjects [(107)]

	Healthy volunteers	Renal (moderate impairment) subjects	Renal (severe impairment) subjects
Number of subjects (n)	21	4	6
Clearance total (mL/min)	1155 ±273	238.3 ±109.9	257.3 ±129
Renal clearance (mL/min)	636 ±84	108.3 ±57.2	129.5 ±90.3
$t_{1/2}$(hrs)	6.9 ±4.2	16.2 ±7.6	17.2 ±9.1
C max (µg/mL)	1.39 ±0.33	4.12 ±1.83	3.93 ±0.92

Table 3: Comparison between kinetic parameters of metformin between healthy volunteers and renal impaired subjects following IV administration of metformin [**(105)**]

	Healthy volunteers	Renal impairment subjects
Number of subjects (n)	5	5
Clearance total (mL/min)	441 ± 40	88.4 ± 11.8
Renal clearance (mL/min)	335 ± 46	60.4 ± 78
t ½ (hrs)	1.52 ± 0.13	4.94 ± 1.11

Table 4: Comparison between kinetic parameters of metformin between healthy individuals and renal impairment subjects after oral administration [**(106)**]

	Healthy volunteers	Renal impairment subjects
# of subjects	4	8
Clearance total (mL/min)	1435.5 ± 370	718 ± 203
Renal clearance (mL/min)	522 ± 201.5	224 ± 58
t ½ (hrs)	5.7 ± 2.9	NA*
C max (μcg/mL)	2.06 ± 0.64	3.24 ± 1.46
Dose (mg)	1850 ± 1	1000
AUC $_{-24}$ (μcg/mL.hr^{-1})	12.5 ± 4.17	24.84 ± 6.68

*NA: not available

1.12.2 Elderly patients

Nearly 50% of individuals with type 2 DM are over the age of 65 years (123), and there is a number of changes often seen with advancing age that may complicate the management of DM, e.g. hypodipsia, anorexia, visual disturbance, altered renal and hepatic function, depression, impaired basoreceptor response and multiple medications (44). However, certain specific changes associated with physiological ageing have potentially greater effects on the metabolism of oral hypoglycaemic agents (124). The most important change interfering with the pharmacokinetic profile of metformin is the progressive decrease of renal function with ageing (107, 125-126). Serum creatinine (Srcr) levels decrease with age because of the decline in lean body mass, despite an age-related decline in glomerular numbers and function (125, 127), therefore glomerular filtration rate (GFR) declines with age. As previously discussed in the study by Sambol *et al*. (107) the combined effect of

kidney function and age on the pharmacokinetics of metformin used multivariate regression analysis on the combined data of all groups. It showed that both renal function (as measured by Clcr) and age were predictors of metformin clearance. Whereas Clcr as a single covariate was significant, age was only significant when Clcr was considered. These results indicated that, given the same Clcr an elderly individual was expected to have a lower metformin clearance than a younger individual. The authors commented that it was unlikely that age had an effect on the clearance of metformin entirely independent of renal clearance; it was more likely that overall renal function and the function of various subcomponents (i.e., GFR and renal tubular secretion) may not be able to be predicted entirely from Clcr. They concluded that the prediction of metformin clearance was enhanced by adding age to Clcr as an independent variable, because in elderly individuals, relative to young, renal function was overestimated by Clcr alone.

1.13 Metformin side effects

The most common adverse effects due to metformin are gastrointestinal in nature and often occur during initial therapy (103, 128). These include diarrhoea, abdominal discomfort, anorexia, nausea and rarely, a metallic taste in the mouth (129-131). However, in order to minimize these adverse effects, metformin should be taken with meals, and as the symptoms are dose related, metformin should be started at a lower dose. More than 50% of patients can tolerate the maximum dose (2250 mg/day), but about 5% cannot tolerate any dose of metformin (128).

Metformin can also interfere with the absorption of vitamin B12 (86, 132) and folate (132).

1.13.1 Lactic acidosis

Lactic acidosis is a rare and potentially fatal metabolic condition that can occur whenever substantial tissue hypoperfusion and hypoxia exist (133-134). The mortality rate in reported cases is approximately 42-50% (129, 135).

Lactate is produced from anaerobic glycolysis principally by the skeletal muscle, brain, erythrocytes and the renal medulla and eliminated by the liver, kidney and heart (136). The normal fasting serum lactate has a range of : 0.4−1.2 mmol/L (137). Pathological hyperlactataemia with acidosis may occur through over-production of lactate (and hydrogen ions), reduced lactate clearance or a combination of both (133). It is a life threatening disorder of intermediary metabolism and defined as a metabolic acidosis in which the arterial blood lactate is greater than or equal to 5mmol/L and the arterial pH is less than or equal to 7.35 (136, 138). Associated symptoms are nonspecific, but include malaise, myalgia, somnolence, abdominal discomfort, and respiratory distress.

1.13.2 Types of lactic acidosis

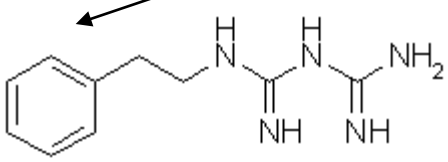
Classically lactic acidosis is subdivided into two groups: type A or anaerobic, and is due to hypoxia from the hypoperfusion of tissues, whereas type B or aerobic, is due to metabolic conditions such as liver disease, sepsis or DM. Both lead to lactic acid overproduction and /or defective excretion in the aerobic state (139).

1.14 Metformin –associated lactic acidosis (MALA)

The true incidence of MALA is not known (140-141). The Food and Drug Administration (FDA) has estimated the rate of fatal and nonfatal lactic acidosis to be five cases per 100,000 persons treated over the course of one year (135). Population based studies have estimated a rate of two to nine cases of lactic acidosis in metformin users per 100,000 person years (142-143), while data from France, Sweden, and Switzerland suggested rates of 1 to 15 cases per 100,000 person-years (144). However, most of the reported cases have occurred in patients with severe acute conditions such as renal failure, septicaemia or cardiovascular collapse (shock), that could in themselves have caused lactic acidosis (128, 135, 145-147). Phenformin, a biguanide agent, was withdrawn from the market because it was associated with a reported rate of lactic acidosis in 40-64 cases per 100,000 patient-years (128-129, 139, 148). However, metformin differs from phenformin in molecular structure and pharmacokinetics (129, 147, 149). Phenformin is a mono-substituted compound and has a rather long side chain. Metformin (1,1-dimethylbiguanide) is bi-substituted and the methyl side chain is short (103). (Figure 4) The mono-substituted, long chain, lipophilic phenformin is metabolized by aromatic hydroxylation in the liver (103, 128, 150). In contrast, the bi-substituted, short chain, hydrophilic metformin is not metabolized, and is eliminated by renal excretion (103, 151-152). These pharmacokinetic differences result in a poor binding of metformin to mitochondrial membranes , therefore metformin does not influence lactate turnover or oxidation in either basal or insulin-stimulated states (149). Thus, metformin does not increase lactate production in

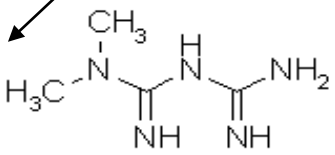
skeletal muscle (153) and any metformin- related increase in blood lactate levels does not arise from peripheral tissues (103, 154). Data indicates instead that metformin increases lactate production via the extrahepatic splanchnic bed (130) with animal studies favouring the small intestine as the site of origin (154). The magnitude of this effect is known to be small when the drug is prescribed appropriately (129). Therefore, the incidence of metformin associated lactic acidosis was estimated to be 10-20 times lower than phenformin (144).

Long lipophilic side chain causes it bind to mitochondrial membranes and to be metabolized by aromatic hydroxylation in the liver



Phenformin

Bisubstituted with two small groups with less lipophilicity on the molecule



Metformin

Figure 4: Chemical structural differences between metformin and phenformin

1.15 Studies of MALA

Numerous clinical studies, including randomized, double-blind, placebo-controlled trials, have demonstrated that metformin is safe to be used if contraindications and warnings are respected (155) (156) (157) (158) (86) (159). In all previous studies there was no case of fatal or nonfatal lactic acidosis reported in the metformin group, this was supported by a meta-analysis study performed by Salpeter *et al.* (140-141). They reviewed published reports of controlled trials and cohort studies to assess the risk of fatal and nonfatal lactic acidosis associated with metformin use in persons with type 2 DM. It was observed that when the data from cohort studies were combined with the prospective comparative trials there was no case of fatal or nonfatal lactic acidosis reported in either the metformin (totalling 35,619 patient-years) or the non-metformin groups (representing 30,002 patient-years). When statistically analysed with 95% confidence, the upper limit for true incidence of metformin associated lactic acidosis was 8.4 cases per 100,000 patient-years, and the upper limit for the incidence of lactic acidosis in the non-metformin group was 9 cases per 100,000 patient-years. When the combined data from metformin and non-metformin groups were considered together the upper limit for the true incidence of lactic acidosis in all patients with type 2 DM was 4.6 cases per 100,000 patient-years. Tables 5, 6 and 7 describe the characteristics and limitations of MALA studies.

Table 5: Characteristics of MALA studies, with sample size, duration of the studies, inclusion and exclusion criteria's, intervention and measured outcomes

Study	Design	Duration	# of subject			Age (years)
			Treatment	Comparison	combination	
Horton *et al* .(155)	Randomized placebo controlled double-blind	6 months	178	351	172	56-59
Herman *et al* .(156)	Randomized controlled double-blind	6 month	38	34	72	60
COSMIC (157)	Randomized open label	12 month	7,227	1,505	NA	≥ 18
Yki-Jarviene *et al.* (158)	Randomized control	12 month	NA	19	23	NA
DeFranzo *et al* .(86)	Randomized control multicenter	29 weeks	P1:143 P2: 210	P1: 146 P2: 209	P 2: 213	40-70

Table 6: Characteristics of MALA studies, with sample size, duration of the studies, inclusion and exclusion criteria's, intervention and measured outcomes

Study	Interventions		Outcomes	Inclusion	Exclusion
	Treatment	Comparison			
Horton *et al.* (155)	Metformin 500 mg three times/day or metformin + nateglanide	Nateglanid or placebo	HbA1c	Type 2 DM	Renal impairment, significant diabetic complications
Herman *et al* .(156)	Metformin dosage adjusted clinically	glibenclamide	HbA1c FBG	Type 2 DM	Contraindication to medication
COSMIC (157)	Metformin 500-2500 mg	Usual care	HbA1c FBG	Type 2 DM	Normal renal function, normal hepatic function, no history of metabolic acidosis
Yki-Jarviene *et al.* (158)	Bed time insulin + metformin 2000 mg/day Bed time insulin + glyburide	Bedtime insulin+ metfromin + glyburide Bed time insulin + morning insulin	FBG, insulin sensitivity	Type 2 DM BMI<30 kg/m^2 FBG> 8 mmol/L	Congestive heart failure (CHF) MI Stroke Epilepsy Liver disease Srcr < 120μ mol/L

					Proliferative retinopathy Severe maculopathy Excessive alcohol consumption >20 g/day
DeFranzo *et al.* (86)	Metformin 2550 and 2500 mg	Glyburide and Placebo	FPG BMI	Type 2 DM Obese FPG>7.8 mmol/L Srcr ≤ 124 µmol/L men, ≤115µmol/L women Normal liver function	Symptomatic cardiovascular disease Diastolic blood pressure (BP) > 100 mmHg Insulin therapy ≥ 3 ounce alcohol/day

Table 7: limitations of MALA studies and the National Health and Medical Research Council (NHMRC) level of evidence for each study

Study	Limitations	NHMRC level of evidence (160)
Horton *et al*. (155)	Maximum metformin dose used in the study was lower than what is normally used in clinical practice (1500 compared to 2500-3000 mg/day). Excluded patients who are contraindicated to metformin use.	II (randomised controlled trial)
Herman *et al* .(156)	Small sample size in each assigned group. The conditions of contraindications to the drug were not clearly defined. Dietary habits and physical activity were not mentioned which might contribute to weight loss, thus increase lactate levels and affect the results and outcomes. Excluded patients who are having contraindication to metformin use such as renal impairment.	II (randomised controlled trial)
COSMIC (157)	Exclusion of patients with abnormal hepatic or renal function from the study which themselves might cause lactic acidosis.	III 2 (comparative study with concurrent controls)
Yki-Jarviene *et al* (158)	Small sample size in each group.	II (randomised controlled trial)
DeFranzo *et al*. (86)	Exclusion of any conditions which might be regarded as a contraindication to metformin use.	II (randomised controlled trial)

1.16 Is it safe to use metformin with the presence of contraindications?

Classically metformin was classified to cause type B lactic acidosis , however, data from one study (120) indicated that pure type B lactic acidosis occurred only in exceptional cases and that most metformin treated patients present with a mixed (A + B) lactic acidosis (i.e. metformin accumulation with concurrent disease) (148). As indicated earlier lactic acidosis with metformin therapy has been found to occur exclusively in patients with contraindications to the drug's use, which leads to the supposition that this adverse effect might be avoided through strict adherence to prescribing guidelines (153). However, in clinical practice it has been shown that prescribing doctors tend not to always comply with the recommended contraindications to metformin use listed in the guidelines (161), believing that these have been developed from experience with phenformin. Therefore it has been argued that metformin contraindications are overzealous (141).

In order to weigh the risk and benefit of metformin use with stated contraindications in the available guidelines, several clinical studies have evaluated the safety of continued use of metformin in patients with contraindications to metformin. Rachmani *et al.* (162) recruited 393 patients who had been treated with metformin alone or in combination with other hypoglycaemic agents, and who were found to have one or more contraindications to metformin according to the guideline recommendations (147, 163). Patients were then randomized to continue or to stop metformin. The patients were followed annually or more frequently if

clinically indicated. The authors concluded that metformin was safe in patients with recognized contraindications to its use. Moreover, they observed that discontinuation of metformin resulted in weight gain, worsening of glucose control as evidenced by a rise in HbA1c level, and a modest rise in low density lipoprotein (LDL) compared to those who continued the drug. These disadvantages of discontinuance were balanced by no advantage. They further recommended that patients who have been on metformin and who tolerated the drug well, should probably be allowed to continue the drug despite the development of one or more of the contraindications stated in the available guidelines.

Another population based study of adherence to prescribed guidelines (164), retrospectively studied the incidence of contraindications to metformin in 4600 patients with type 2 DM using metformin. They observed that 24.5% of patients receiving metformin had contraindications to its use which was in accordance with advice in the manufacturers data sheet and the British National Formulary (BNF) (165). One episode of lactic acidosis was identified in the study group, this was a 72-year-old patient on metformin with previously normal renal function (creatinine 78 μmol/L, 20 days previously), who was admitted to hospital with an extensive myocardial infarction (MI) demonstrated on electrocardiography who then developed acute renal failure (urea 31.2 mmol/L, creatinine 152 μmol/L), lactic acidosis (serum lactate 17.9 mmol/L) and died the same day. Based on this patient's clinical scenario, they suggested that it was likely that the lactic acidosis was secondary to the MI rather than a direct consequence of inappropriate metformin use. They concluded that patients commonly remained on metformin despite the

development of conditions considered in the data sheet to be contraindications. They observed that in their region at least, metformin was rarely stopped when contraindications developed, but despite this, lactic acidosis remains a rare complication.

Moreover, from 164 prospective studies reviewed recently in a meta-analysis study (141), they observed that 156 (95%) studies allowed for the inclusion of patients with at least one contraindication to metformin, 16% of all participants were estimated to be older than 65 years with no adverse effects observed among those groups. However, they concluded that it was not clear how many participants with contraindications were included in the trials; hence the safety of metformin in the presence of standard contraindications cannot be assessed.

A recent study from the UAE (166) assessed the risks and benefits of metformin use in diabetics with the presence of contraindications; 106 diabetic patients were recruited in the study. Metformin plasma concentration, lactate concentration, C-peptide, HbA1c, and insulin were measured. Current medical condition, demographic data, and current medication were also reported. They observed that almost 67% of the patients had medical conditions that hindered metformin usage, yet no case of lactic acidosis was observed or reported in their patients. They concluded that the prescribing patterns did not conform to the guidelines, and that the available guidelines appeared to be misleading. They recommended that the contraindications to metformin as stated in the guidelines should be reviewed, and unambiguous guidelines were required to prescribe metformin rationally.

The above mentioned studies failed to confirm an absolute safety of metformin in the presence of contraindications to its use. However, in order to confirm metformin safety and to maximize its clinical efficacy and minimize the unwanted side effects, a large prospective population study to investigate factors might positively or negatively impact on the occurrence of fatal adverse effects such as lactic acidosis in the presence of other contraindications is required.

1.17 Mechanism of action of metformin

Despite being in the market for decades, the molecular site of action of metformin has been a research area of interest. Unlike sulphonylurea agents, the mechanism of action of metformin does not involve the stimulation of insulin secretion by the endocrine pancreas, although it's clinical efficacy requires the presence of insulin, hence, hypoglycaemia is not observed (86, 131, 167-168). Whereas, most studies have shown that the glucose lowering effects of metformin are secondary to a decrease in hepatic glucose production (86, 130, 149, 167-169) a significant body of data has also suggested that metformin increases glucose disposal in skeletal muscle (128, 130-131, 149, 170).

Adenosine monophosphate activated protein kinase (AMPK) provides a candidate target capable of mediating the beneficial metabolic effects of metformin. AMPK belongs to the family of energy-sensing enzymes that are activated by cellular stresses resulting in Adenosine Tri-Phosphate (ATP) depletion, thus acting like a "fuel gauge" (171-173). On activation, AMPK functions to restore cellular ATP by inhibiting ATP consumption processes, but accelerating ATP generation processes.

The cascade is activated by physiological stress, such as contraction or prolonged exercise (174-176) or electrical stimulation in skeletal muscle (174, 176-177), ischemia in heart muscle (178-179), heat shock (180) as well as through inhibition of the tricarboxylic acid cycle or oxidative phosphorylation (181).

AMPK is a heterotrimeric protein comprising catalytic subunit (α) and two regulatory subunits (β and γ)(182-183). The regulatory subunit plays an important role in maintaining stability of the heterotrimer complex (171, 183). Both α and β subunits have two isoforms each, AMPKα1 and AMPKα2, and AMPKβ1 and AMPKβ2, while γ subunit exists in three isoformic forms, AMPKγ1, AMPKγ2, and AMPKγ3 (184-186). AMPKα1 is widely distributed, while AMPKα2 is found mainly in skeletal muscle, heart, and liver (185) .

AMPKα consists of 548 amino acids and it contains a catalytic domain (1-312 amino acid), an autoinhibitory domain (312-392 amino acid), and a subunit binding domain (392-548 amino acid)(185, 187). The catalytic domain has the site of phosphorylation at Thr172 which is required to be phosphorylated for its activation by AMPK kinase. Removal of both the autoinhibitory domain and subunit binding domains leads to constitutive active AMPK retaining one third of activity compared to full AMPK complex whereas, removal of subunit binding domain leads to a complete loss of activity (187-188). AMPK can also be activated through other mechanisms, allosteric activation, and a decrease in the inhibitory action of phosphatases(171, 182, 189). The increase in AMPK activity results in the stimulation of glucose uptake in muscle, fatty acid oxidation in muscle and liver,

and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis (190-192).

The activation of AMPK initiates a cascade of metabolic effects that resembles metformin gluco-regulatory effects; therefore, several *in vitro* and *in vivo* studies have tested the hypothesis that activation of AMPK mediates the beneficial effects of metformin.

Zhou *et al.* (193) postulated that AMPK activation was the mechanism of metformin action. They measured the activity of AMPK in cultured rat hepatocytes and isolated muscles that were incubated in control, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), and metformin media. They found that after one hour treatment, 500 µm metformin was required to significantly activate AMPK, whereas after a 7 hour treatment, 50 µm was sufficient to significantly activate the enzyme; a result that was consistent with the time dependent effects of metformin. They also found that metformin as well as AICAR induced AMPK Thr172 phosphorylation demonstrated by using an anti-phospho-AMPK antibody. Moreover, both metformin and AICAR were able to inhibit cumulative glucose production in primary cultured rat hepatocytes stimulated with glucagon, and incubation of isolated muscles with metformin resulted in an increase in the activity of both catalytic subunits of AMPK. Importantly, co-incubation of hepatocytes with compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl])-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine), a potent reversible AMPK inhibitor, was able to reduce the effects of metformin to decrease glucose production in these cells. In conclusion, the study

showed that metformin significantly increased AMPK activity in cultured rat hepatocytes and isolated muscles, and the activation of the enzyme was associated with diminished glucose production by the hepatocytes and higher muscle glucose uptake.

In order to investigate whether the activation of AMPK by metformin was a likely mechanism for its metabolic effects in subjects with type 2 diabetes, Musi *et al.* (194) examined if treatment with metformin modified the activity of the AMPK α1 and α2 isoforms in skeletal muscle. Eight subjects with type 2 diabetes (seven males and one female) were studied before, during, and after 10 weeks of treatment with metformin. The subjects discontinued their antidiabetic therapy three weeks before the study. Three days before the study, they were given a diet composed of 55% carbohydrates, 30% fat, and 15% protein. Blood samples for FPG, HbA1c%, lipids, lactate, and free fatty acids, were taken at zero week, and week 10 of the study. Muscle biopsies were taken at week zero, week four, week 10 three hours after a 1000 mg dose of metformin to insure metformin concentrations were at their highest (t_{max} 2.5-3.3 hr), and to determine whether potential changes in AMPK activity would persist when blood metformin concentrations were low. Six subjects continued treatment at 1000 mg twice a day and underwent a fourth muscle biopsy within seven days of the 10 week biopsy. The subject's commenced metformin on 500 mg oral dose once daily, the dose was then increased weekly to a maximum of 1000 mg twice daily. All subjects tolerated metformin without any adverse effects. They found that AMPK α2 activity increased by 52% after four weeks of treatment and further increased to 80% after 10 weeks of treatment. Interestingly, the

activation of AMPK α2 by metformin continued after an overnight withdrawal. In contrast, AMPK α1 activity did not change during treatment. Together, these findings in animal tissue (193) and human skeletal muscle (194) strongly supported the role of AMPK in mediating the metabolic actions of metformin.

The mechanism by which metformin activates AMPK remains controversial, although some studies (195-196) have demonstrated that metformin activates AMPK by decreasing cellular energy by acting as an inhibitor of complex 1 of the respiratory chain, which might also explain metformin associated lactic acidosis. However, recent studies (197-201) do not support this model especially since metformin in these studies activated AMPK without affecting the Adenosine Mono-Phosphate (AMP)/ATP ratio. Alternatively, genetic and biochemical studies reported that serine threonine kinase (STK11/LKB1) can phosphorylate and activate AMPK, hence emphasized the role of LKB1 in metformin action.

Summary of the chain for metformin action:

- AMPK is activated by ATP depletion.
- Upon activation AMPK decreases ATP consumption and increases ATP generation. Acting like a “fuel gauge”, as shown in Figure 5.
- AMPK activation results in (Figure 6) -:
    - Stimulation of glucose uptake in muscle.
    - Stimulation of fatty acid oxidation in muscle and liver.

- Inhibition of hepatic glucose production.
- Inhibition of cholesterol and triglyceride synthesis.
- Inhibition of lipogenesis.

- Metformin gluco-regulatory effects resemble activated AMPK metabolic effects.

- AMPK activation was postulated as a mechanism of metformin action.

- AMPK activated by phosphorylation which is mediated by LKB1.

AMPK

ATP consumption process

ATP generation process

Figure 5: Diagram illustrating how AMPK acts as a “fuel gauge”.

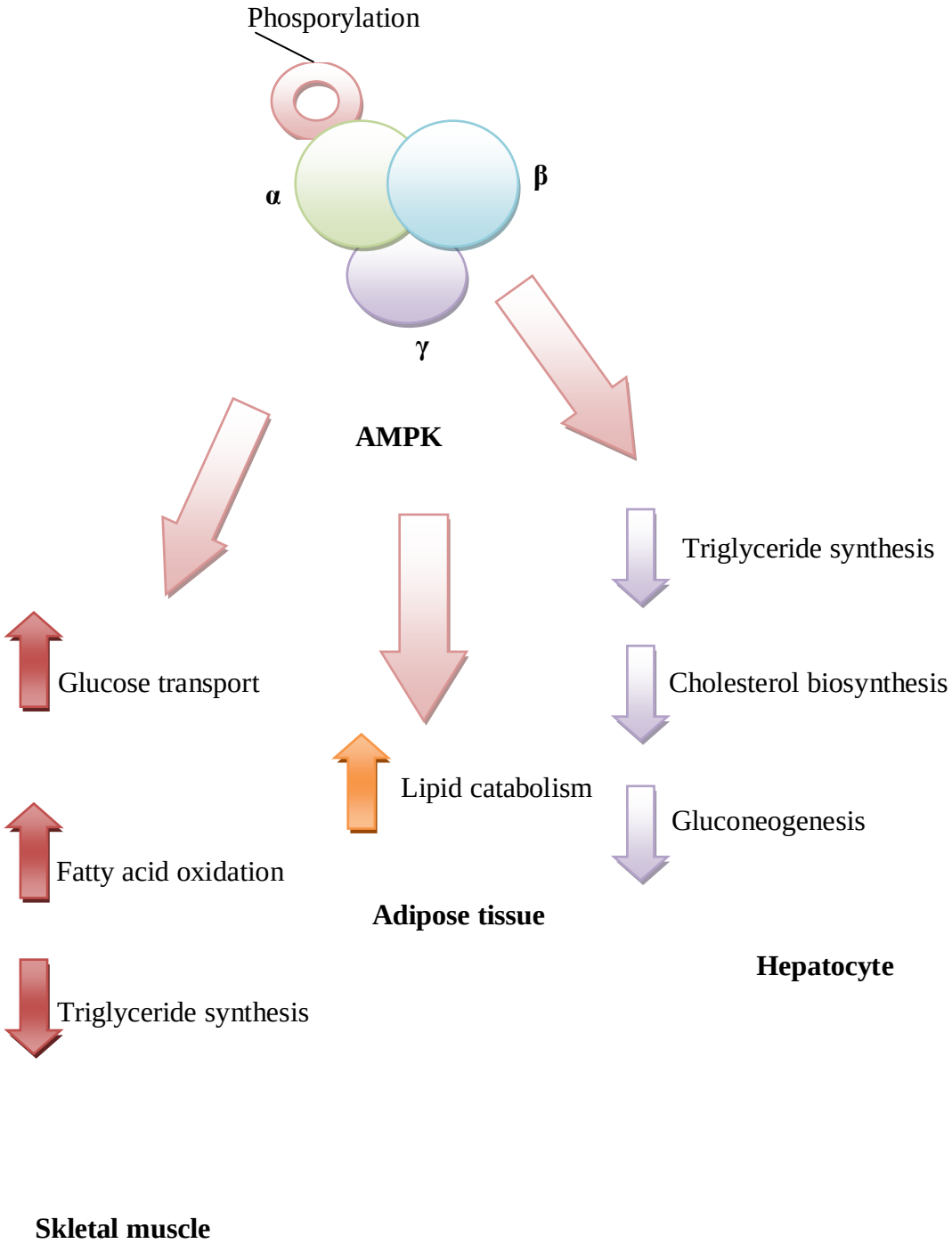


Figure 6: Metabolic cascade upon AMPK activation

1.17.1STK11/LKB1

LKB1, a 50-KDa serine/ threonine kinase, was originally identified as the product of the gene mutated in the autosomal dominantly inherited Peutz-Jeghers cancer syndrome (PJS) (202-203). A number of groups have demonstrated that knocking out one of the LKB1 alleles in mice is sufficient to induce a cancer syndrome similar to PJS in humans (204). In resting cells, LKB1 is reported to be predominantly located in the nucleus (204-206). LKB1 is a heterotrimeric complex with regulatory proteins termed STRAD and M025, which are required for its activation and cystolic localization (204, 206). LKB1 has been shown to mediate Thr172 phosphorylation of AMPK both *in vitro* and in intact cells (197, 200). *In vivo* studies have shown that mice lacking LKB1 expression have significantly inhibited AMPK activity (198). Importantly metformin has shown to activate AMPK in wild type mice but failed to activate AMPK in livers deficient in LKB1(201, 207). Moreover, metformin treatment lowered blood glucose in obese mice by 40%, however, there was no reduction in blood glucose in the mice in which LKB1 had been deleted in the liver (201, 207). Xie *et al.* (207) demonstrated that; in order to activate AMPK, LKB1 must be exported from the nucleus into the cytoplasm. They used specific antibodies against LKB1, and found that LKB1 was located mainly in the nucleus of non-stimulated human umbilical vein endothelial cells. However, in the cell treated with metformin, LKB1 was mainly detected in the cytosol; an observation that was confirmed by Western blot analysis. Accordingly, they concluded that, metformin increased LKB1 phosphorylation at ser 428 that induced the translocation of LKB1 from the nucleus to cytoplasm

where it activated AMPK. Moreover, they observed that mutation of ser 428 into alanine was able to prevent this translocation and hence abolished metformin enhanced association of LKB1 with AMPK. Thus, ser 428 phosphorylation might play a crucial role in regulating AMPK activation. Therefore knowing the allele frequencies of this mutation among different ethnic groups, as well as identifying the significance of this mutation in the clinical setting will help the clinicians to predict or modify a patient's response to metformin therapy.

1.18 Inter-individual response to metformin

Inter-individual differences in response to metformin can be due to a variety of factors, including age (107), renal function (105-107) and metformin dose (208-209). Pharmacogenomic studies indicate that genetic differences should be taken into consideration when large inter-individual variability in the intensity and duration of drug effects and side effects are observed. Several genetic studies have revealed that genetic differences contribute to the variability of metformin effects (210-211). As discussed previously, metformin is a strong base that protonated at physiological pH. Metformin has been well characterized *in vitro* as a substrate of OCTs (20, 212-214), including OCT1 (215-217), which is primarily expressed in the liver (218-220) and its paralog OCT2 (18-19, 221), which is mainly expressed in the renal basolateral membrane (18, 221-222). In contrast, *in vivo* studies have shown that the uptake of metformin was higher in rat OCT1 transfected cells than in vector transfected cells. Moreover, it was observed that the plasma and liver concentrations of metformin were three times higher in OCT1-/- mice than

OCT1+/+ mice (215). These results were compatible with other *in vivo* and *in vitro* studies (216-217, 223-224).

The OCT1 and OCT2 transporters are subtypes of the solute carrier transporter family (SLC), subfamily 22 (SLC22A) (225-226). The SLC22A family includes three organic cation transporters, OCT1, OCT2, and OCT3 (225, 227). In human the genes coding for OCT1, OCT2, and OCT3 are localized within a cluster on chromosome 6.q26-7 (228-230). Each of the three genes comprises 11 exons and 10 introns (229, 231-232)

Several genetic and molecular studies have demonstrated that OCT genes exhibit polymorphic variation in the coding region (24-27). In order to investigate the pattern of polymorphisms exhibited with OCT1 and OCT2 genes, various studies have screened for genetic variants in different populations. Recently, genetic testing was performed with 57 Caucasian subjects. A number of 25 single nucleotide polymorphisms (SNPs) have been identified in the OCT1 gene, which were dispersed through exons, introns, and the promoter region (24-25). Eight of these polymorphisms were non-synonymous, resulting in an amino acid exchange of the OCT1 carrier protein. Subsequent functional analysis of 5 coding variants revealed diminished *in vitro* transport activity of one variant and nearly completely lost uptake function of two other mutants. Another study investigated for novel SNPs by sequencing all the exons and the surrounding introns of OCT1 from 116 Japanese individuals (26). Twenty variants were found including 7 novel ones. Eight detected variants were similar to the previous Caucasian study with different

frequencies between these two populations. One novel non-synonymous SNP that resulted in amino acid substitution was identified in one heterozygous subject, however, the functional effect of this SNP on transport activity was not investigated.

Moreover, Shu *et al*. (25) screened all exonic, introns, and the promoter region of the OCT1 gene. They detected 14 non-synonymous polymorphisms and one deletion variant from five different ethnic groups, African-Americans (100 subjects), European- Americans (100 subjects), Asian- Americans (30 subjects), Mexican – Americans (10 subjects), and Pacific- Islanders (7 subjects). The allele frequencies of these SNPs were different among all five different ethnic groups (Table 8). Of 14 variants, five exhibited decreased transport activities (OCT1-R61C, OCT1-G220V, OCT1-P341L, OCT1-G401S, and OCT1-G465R) while one had increased transport activities (OCT1- S14F) which was exclusively found in the African-American population sample.

Table 8: The allele frequencies of OCT1 SNPs reported among five different ethnic groups in the Shu *et al.* (25) study.

	Allele frequency‡						
Amino acid change	Total (n= 494)	AA (n=200)	EA (n=200)	AS (n=60)	ME (n=20)	PA (n= 14)	Function*
S14F	0.013	0.013	0	0	0	0	+/+
R61C	0.031	0	0.072	0	0.056	0	+/-
G220V	0.002	0.005	0	0	0	0	-
P341L	0.047	0.082	0	0.117	0	0	+/-
G401S	0.008	0.007	0.011	0	0	0	-
G465R	0.016	0	0.04	0	0	0	-
‡ AA: African American; EA: European American; AS: Asian American; ME: Mexican American; PA: Pacific Islander, n is the number of chromosomes.							
* ++: increased function relative to the reference OCT1; +: function similar to that of reference OCT1; +/-: reduced function; -: no function.							

Recently Kang *et al.* (29) evaluated genetic polymorphisms of OCT1, and OCT2 genes in healthy Korean subjects and investigated the functional properties of the non-synonymous SNPs. They screened all 11 exons and flanking intronic sequences of 150 Korean subjects. Four non-synonymous SNPs were identified of the OCT1 gene (F160L, P283L, P341L, and M408V). Two of them showed decreased transport activities (P283L and P341L). Whereas, they reported three non-synonymous SNPs of the OCT2 gene (T199I, T201M, and A270S) of which all showed decreased transport activities. Furthermore, they compared the allele frequencies of the major functional SNPs of the OCT1 and OCT2 genes in the

study population with three other ethnic groups, namely; 100 Vietnamese, 100 Chinese, and 100 white German. They observed that OCT1-P283L, OCT2-T199I, and OCT2-T201M SNPs were detected very rarely and showed no differences between Asians and white Germans. The allele frequencies of OCT1-P341L, and OCT2-A270S in the Korean population were similar to those in other Asian populations and significantly higher than those in the white German population. Despite being genetically classified as Caucasian, one genetic variant found in the white German population (OCT1-P341L) was not detected in a white European-American population (24-25). In addition, the OCT2-A270S mutation revealed different allele frequencies between the white European- American and white German populations (15.7% (233), 2.5%, respectively). Kang *et al.* concluded that OCT1 and OCT2 genes exhibited genetic polymorphisms in Korean populations that were similar to those in other Asian populations but different than white German populations. The functional analysis of the detected non-synonymous SNPs showed significant decreased transport activities. The results emphasize the role of genetic factors in observed inter-individual variability with response to clinically used drugs. Interestingly, the allele frequencies of major SNPs could vary among the same descent. However, all of the above mentioned studies have not examined the functional consequences of synonymous SNPs on the OCT1 substrate transport activity, and haplotype linkage analysis was not performed on the detected SNPs.

In order to investigate the role of genetic variation of the OCT gene on metformin pharmacokinetics, several *in vivo* and *in vitro* studies have been undertaken.

Recently, Shu *et al.* (223) measured the cellular uptake of metformin in primary mouse hepatocytes. They have found that metformin uptake was significantly lower (3.4 fold, $p< 0.0001$) in OCT1-/- cells compared with those in cells with a functional OCT1 allele, and the hepatic accumulation was significantly greater in wild type mice (4.2 fold, $p< 0.001$) than OCT1-/- mice. Moreover, the phosphorylation of AMPK by metformin was substantially reduced in OCT1-/- hepatocytes in comparison to those in wild type hepatocytes. They also observed that metformin significantly suppressed glucagon stimulated glucose production in hepatocytes from wild type mice (30% suppression, $p< 0.001$) but not in hepatocytes from OCT1-/- mice. Based on these results they concluded that OCT1 plays a key role in determining one of the major pharmacologic effects of metformin, inhibition of hepatic gluconeogenesis. In order to study the effect of non-synonymous OCT1 polymorphism on metformin uptake and response in cellular assays, Shu *et al.* (223) measured the uptake of metformin in stable cell lines expressing empty vector, OCT1 reference, and in 12 OCT1 non-synonymous variants. Compared with OCT1 reference, seven OCT1 variants exhibited significantly reduced or lost metformin uptake, despite similar mRNA levels. Finally they compared the clinical effects of metformin in an oral glucose tolerance test (OGTT) in volunteers carrying the reference OCT1 allele and those carrying the OCT1 polymorphisms. They found that after OGTT the glucose plasma levels and plasma glucose AUC in volunteers carrying reference OCT1 alleles and those carrying a reduced function polymorphism of OCT1 were similar. However, volunteers with reference OCT1 alleles had significantly lower plasma glucose

levels than those carrying OCT1 polymorphisms. The results of this study showed that OCT1 is required to facilitate cellular uptake of metformin, and genetic polymorphisms of the OCT1 gene may contribute to a reduced therapeutic response to metformin.

As noticed, the above study was unable to determine the effect of genetic variation in the OCT1 gene on the pharmacokinetics of metformin, therefore Shu *et al.* (32) recently conducted another study in 20 healthy European American volunteers. Subjects were classified into OCT1 variants (n= 12) and reference group without OCT1 variant (n= 8). All subjects received two separate doses of oral metformin 850 mg and 1000 mg. The plasma concentrations of metformin were measured for 24 hours after the second dose. They found that metformin plasma concentrations, AUC, and Cmax were significantly higher in the individuals in the OCT1 variant group than those in the OCT1 reference group ($p< 0.05$, $p= 0.01$, $p= 0.004$ respectively). Moreover, subjects with variant OCT1 showed significantly lower volume of distribution compared to those in the OCT1 reference group ($p= 0.022$). Similar trends were observed in an animal study as OCT1-/- mice (n= 6) tended to have higher metformin plasma concentrations, AUC, and Cmax compared to those in OCT1+/+ mice (n= 5). Unlike the previous study, this study showed that the pharmacokinetics of metformin was significantly affected by OCT1 activity or genotype in healthy subjects. However, this study was conducted in healthy volunteers with a small sample size, and in order to verify the role of OCT1 polymorphism in metformin action a large study on diabetic patients is required. Moreover, this study has not examined the functional consequences of synonymous

SNPs in the studied population that might reveal an association with metformin action on recruited subjects.

In another study, the role of OCT1 polymorphisms on the clinical response to metformin therapy was studied in diabetic patients (21). Patients were classified as responders (n= 24) or non-responder (n= 9) to metformin therapy. The classification was based on their HbA1c results after more than three months of metformin treatment. Unpredictably, they observed no significant contribution of OCT1 polymorphisms to metformin efficacy. The discrepancy between Shikata *et al.* (21) and Shu *et al.* (32) results can be due to many reasons. Firstly, they followed different approaches to identifying patients, because Shu *et al.* (223) used OCT1 genotype while Shikata *et al.* (21) used metformin response (phenotype-genotype study), and therefore the relative contribution of OCT1 is unknown. Secondly, the studies were conducted in different ethnic groups having different genetic backgrounds (European American, Japanese subjects) hence, as discussed earlier, the allele frequencies of major SNPs could vary among the same descent and the role of these SNPs might be clinically different among different subgroups. Finally, both studies had small numbers of subjects (20 and 33 patients respectively) and in order to confirm the role of genetic polymorphism in drug therapy larger sample sizes are usually required.

A recent study investigated the effect of genetic polymorphisms in the OCT1, OCT2, and OCT3 on the renal clearance of metformin (22). They analysed all known non-synonymous coding variants with frequencies >0.5% among 103

unrelated healthy, male Caucasian volunteers. They observed that there were no significant associations between the renal clearance of metformin and the variants analysed in the OCT2 and OCT3 genes. On the contrary, they found that two SNPs in the OCT1 gene were significantly associated with the renal clearance of metformin, namely; synonymous Ser (rs1867351) and non-synonymous 465Gly>Arg SNPs (p= 0.03 and p= 0.003 respectively). However, after adjustment for age and Clcr, the significance of the association between rs1867351 SNP and metformin renal clearance was no longer evident. Subsequently, they analysed the combined effect of the four amino acid variants known to abolish OCT1 activity partially (Met420del) or fully (Arg61Cys, Ser401Gly, and Gly465Arg). They found that the renal clearance of metformin increased significantly with the number of inactive OCT1 alleles (p= 0.038), and the association remained significant after age and Clcr adjustments (p= 0.032). They also found that the net clearance by tubular secretion of metformin increased significantly with the number of inactive alleles. In order to explain the association between OCT1 polymorphism and the renal clearance of metformin, they further analysed OCT1 mRNA expression in the kidney in relation to liver and small intestine. It was found that the highest OCT1 mRNA expression was observed in the liver, and the second highest OCT1 expression was found in the kidney, which was fivefold higher than in the small intestine. They suggested that OCT1 localization in the luminal membrane in distal tubules may contribute to metformin reabsorption in the tubule, thus OCT1 genetic variations were associated with increased renal elimination of metformin. In contrast, they were unable to confirm the effects of genetic variations in OCT2 on

renal clearance. This study was conducted on young healthy volunteers, and it only studied the effect of genetic variations of the OCT gene on renal elimination of metformin that might be affected by other variables such as GFR which was not investigated.

As discussed previously, GFR declines with age (107, 125-127) and diabetes by itself may reduce renal function and cause nephropathy (90, 234-235), and as it was shown the authors in the above mentioned study failed to associate OCT2 genetic variations and renal elimination of metformin. This indicates that GFR may play an important role that overcomes OCT2 contributions to renal elimination of metformin especially in diabetic patients.

In contrast, those with OCT1 gene, human genetic variants of OCT2 were investigated comprehensively by screening all 11 exons of the gene as well as flanking intronic sequence in a collection of 247 ethnically identified genomic DNA samples (233). Twenty eight variable sites were identified, of which 16 were coding region variants and 12 were non-coding variants. Eight of the variants caused amino acid changes, while one variant was a single nucleotide insertion (134-135insA) that leads to a premature stop codon at amino acid position 48. Four of the non-synonymous variants, Met165Ile, Ala270Ser, Arg400Cys, and Lys432Gln, were polymorphic, with ethnic-specific allele frequencies $\geq$1%. The remaining four non-synonymous variants, Pro54Ser, Phe161Leu, Met165Val, and Ala297Gly, as well as the insertion variant were found on only one of 494 chromosomes screened. Met165Ile and Arg400Cys were present only in the

African-American population sample, and Lys432Gln was present in both the African-American and Mexican-American population samples. Ala270Ser was present in all of the populations screened and had a particularly high allele frequency over all populations (12.7%). Leabman *et al.* determined the activity of the four polymorphic non-synonymous variants of OCT2 and observed that all of these variants retained function as measured by uptake of the prototypical organic cation substrate, [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+), in Xenopus laevis oocytes expressing the variant transporters (233). However, differences in MPP^+ uptake activity and kinetic differences in interactions with organic cations was observed for the common variants. The 134-135insA variant identified appeared to be a loss of function variant as assayed by MPP^+ uptake in oocytes; individuals with this variant may exhibit reduced renal clearance of organic cations.

The contribution of a genetic component to variation in the renal clearance of metformin was estimated to be particularly high (>90%) (210-211). Another study of six monozygotic twins showed that genetic factors contributed substantially to the renal clearance of metformin (236).

Moreover, the OCT2 gene polymorphisms were detected in a group of 48 unrelated Japanese individuals (27) and 116 arrhythmic Japanese patients (28, 237). In the former study, 27 SNPs and 5 deletion polymorphisms in the OCT2 gene were identified (27). Two synonymous variants, also reported by Leabman *et al.*(233) were identified at amino acid positions 130 and 150. The remaining SNPs and deletion polymorphisms appeared in introns, 3` untranslated regions and 3` flanking

regions of the OCT2 gene. In the later study, 33 genetic variants including 14 novel ones were found (28, 237). Two non-synonymous were identified (Thr199Ile and Thr20Met) and other SNPs, insertion, and deletion polymorphisms were located in exons, 3`-untranslated region of exon 11, introns, and 3`-flanking region. Functional effects of these polymorphisms have not been investigated.

Unlike the OCT1 gene, fewer studies have evaluated the effect of OCT2 variants on the *in vivo* pharmacokinetics of metformin, and most of these studies were on an Asian population.

As discussed, Shikata *et al.* (21) assessed the functional significance of genetic polymorphisms of the OCT1 gene with regards to the efficacy of metformin in Japanese diabetic patients, at the same time they also assessed that with OCT2 polymorphisms. They observed two non-synonymous variants with the OCT2 gene (T201M, and A270S) and the prevalence of these variants were not different among responders and non-responders. However, the discriminant functional analysis found one of the OCT1 variants as a positive predictor of metformin efficacy (M408V) but none of the OCT2 variants were shown to be predictors for metformin efficacy. However, this study recruited only a small number of subjects, and utilized a phenotype-genotype approach to investigate the role of OCT1 and OCT2 genes on metformin effects; therefore the relative contribution of OCT2 was unknown. Moreover, OCT2 is mainly expressed in the renal basolateral membrane making it more reliable to investigate the functional analysis of OCT2 variants with regards to metformin pharmacokinetics, namely excretion and its role with

metformin accumulation but not with metformin action which is primarily facilitated by the OCT1 gene. Furthermore, they haven't measured metformin plasma levels to estimate the relationship between OCT2 variants and inter-individual variation to metformin therapy.

Some of these drawbacks were considered in a recent study (238) that evaluated the effect of genetic variants of the OCT2 gene identified in a Korean population on the *in vitro* transport activity of metformin and on the *in vivo* pharmacokinetics of metformin in healthy human subjects. They determined metformin uptake in cell lines that expressed the OCT2 reference and OCT2 variants. It was shown that metformin uptake mediated by the OCT2 reference gene was saturable and higher than that mediated by OCT2 variants, and the intrinsic clearance of metformin was lower in OCT2 variants expressing cells in comparison with that in OCT2 reference expressing cells. In order to evaluate the *in vivo* pharmacokinetics of metformin in healthy human subjects, 26 subjects were recruited in the study; 9 in the OCT2 reference group, and 17 in the OCT2 variants group. A single 500mg dose of metformin was given to the subjects, blood and urine samples were collected after 12 hours. The pharmacokinetic parameters showed significant differences among reference and variant groups. Subjects with OCT2 variant had significantly higher Cmax and AUC values, but lower clearance, volume of distribution, renal clearance, and secretion clearance values as compared to the reference group. In conclusion, the results of the study indicated that the genetic variations of OCT2 gene will result in decreased renal excretion of metformin and subsequently increased metformin plasma concentration. Therefore, genetic variants of OCT2

should be considered with metformin therapy. However, small sample sizes and the use of healthy subjects were drawbacks.

Recently, Chen *et al.* (23) determined the effect of the most common genetic variation in OCT2 (A270S) on the renal elimination of metformin in healthy volunteers of 94 unrelated European and 66 unrelated African-American ancestries. Subjects were given a single oral dose of 850mg of metformin. Blood and urine samples were drawn at different times for metformin pharmacokinetic parameters and DNA genotypes. By using multivariate analysis, they tested the effect of the OCT2 genotype, sex, age, race, and Clcr on metformin renal elimination. They found that only genotype (p= 0.009) and Clcr (p=0.023) were significant predictors of metformin clearance. It was also shown that individuals carrying the A270S variant were associated with a greater metformin clearance compared with the reference allele. However, this study investigated the effect of only one non-synonymous SNP (A270S) on young healthy volunteers; the situation might however differ in an older diabetic population. Moreover, the fact that this study examined one non-synonymous SNP does not exclude the effect of other synonymous and non-synonymous SNPs which could occur in one or more haplotype blocks and subsequently predict metformin clearance.

1.19 Organic cation transporters and diabetes

Nephropathy is a major cause of morbidity and mortality in patients with type 2 diabetes mellitus (234, 239-240). Ethnicity has a role in the prevalence of diabetic nephropathy in certain regions (240). Nephropathy is defined by persistent

microalbuminuria (31-299 mg/l), declining glomerular filtration rate and hypertension (BP>135/85mmHg) (241). Structural changes in the proximal tubule included basement membrane thickening and glycogen accumulation (241). The proximal tubular transport defects may indeed occur in diabetes as suggested by data from investigation of the so called “protection” phenomenon seen in streptozotocin-diabetic rats injected with nephrotoxic agents (242-244). Grover *et al.* (241, 245) studied the association between diabetes, diabetes duration, and insulin treatment in the function and expression of OCT1 and 2 in the streptozotocin (STZ)-induced diabetes rats. They observed a decrease in both protein levels of OCT1 and 2 in samples obtained from rats after 14, 21, and 42 days of diabetes compared with the non-diabetics. The results also showed that the expression of OCT1 was not significantly different in diabetic compared with rats in the non-diabetic group. On the other hand, OCT2 was significantly decreased within 14 days of diabetes and maximally decreased by about 50% after 21 days. Moreover, there was a decrease in the uptake of tetraethylammonium (TEA) in the STZ-induced diabetic rats compared to the control group of non-diabetic. Based on these results, another experiment was conducted to test whether early insulin treatment could prevent the observed changes in transporter expression. The results showed that there was no significant difference in expression of OCT1 mRNA among the three groups (non-diabetic, untreated diabetic, and diabetic + insulin). On the other hand, insulin treatment significantly attenuated the diabetes-related decrease in OCT2 mRNA expression. The authors of the mentioned study

concluded that uncontrolled diabetes caused a progressive decrease in OCT at the basolateral membrane.

To date it is unknown if there is a difference in OCT expression in human kidney's between controlled and uncontrolled diabetics. Thus, if OCT is less expressed in an uncontrolled diabetic's kidney as it is in a rat kidney, metformin renal elimination will decrease and subsequently lead to increased plasma concentrations which might expose patients to unwanted adverse effects.

1.20 Gender differences in the expression of OCT gene

Urakami *et al.* (246) examined whether rat rOCT1 and/or rOCT2 contributed to the gender differences in organic cation transporter activity in the rat kidney. They examined accumulation of TEA by slices of kidneys from male and female rats. The TEA accumulation rate was approximately two fold greater in renal slices of male rats than female rats. In order to ascertain if there were gender differences in TEA transport activity of the renal basolateral membranes, TEA uptake was examined using isolated basolateral membrane vesicles from male and female kidneys. Again it was observed that TEA uptake was greater in the vesicles from the male kidney cortex compared to the female kidney. They also examined mRNA expression of rOCT1, rOCT2, and rOCT3. The amount of rOCT2 mRNA was much lower in females than in males and concluded that rOCT2 was responsible for the gender differences in renal organic cation transporter activity in the basolateral membrane of rat kidney. The same group further studied whether gender differences in rOCT2 expression was due to sex hormones or not (247). They

injected male and female rats with either testosterone or estradiol for 7 days. They then observed that administration of testosterone to male and female rats significantly increased in rOCT2 mRNA expression, while estradiol caused a slight, but not significant decrease in rOCT2 mRNA expression in males, but not in females. These hormone treatments had little effect on rOCT1 mRNA expression. They also examined the impact of hormonal treatment on organic cation transport activity, and reported that TEA accumulation was higher in slices from testosterone treated male and female rat kidneys compared to controls. However, the accumulation of TEA was lower in slices from estradiol treated male rats compared to controls. Treatment with estradiol had no significant effect on the TEA accumulation in slices from female rats. The expression of rOCT2 in the rat kidney was under strong stimulatory regulation by testosterone which had no effect on the expression of rOCT1 mRNA. The results of these two studies were in concordance with another study (248) which determined the tissue distribution of OCT expression in 20 tissues from male and female rats and examined whether OCT mRNA expression was affected by ageing. Only OCT2 mRNA levels in the kidney was significantly different with gender.

To date it is unknown if there is a gender difference in OCT2 expression in the human kidney. If OCT2 is also differentially expressed in human kidney as in the rat kidney, the pharmacokinetics and subsequently, the efficacy and dispositions of OCT2 substrate drugs will vary between males and females.

In summary, metformin is considered to be an important and commonly used hypoglycaemic agent. It was observed that patients respond differently to metfomin treatment, thus inter-individual variations in the efficacy of metformin can be influenced by genetic factors, especially the impact of genetic variations in the drug transporters such as OCT1 and OCT2. Population studies have demonstrated significant associations between non-synonymous SNPs of OCT1 and OCT2 and metformin pharmacokinetics, and efficacy. Nearly all of the studies have been conducted on healthy individuals, very few were on diabetics usually with small sample sizes. However, in order to determine the effect of genetic variations of drug transporters on pharmacokinetics, therapeutic effects, and safety of important drugs such as metformin, a prospective large scale study in patients with diabetes is required which should be conducted in different ethnic groups from different genetic background. To date no genotyping studies of OCT1 and OCT2 genes have been conducted on the Arab population, particularly the UAE population. Thus, the role of OCT1 and OCT2 polymorphisms in metformin pharmacokinetic and efficacy is not known in this population.

This study therefore has investigated the role of OCT1 and OCT2 genetic polymorphisms and studied their impact on metformin plasma concentrations in the UAE diabetic population.

2 Aims

The main aims of this project were to:

- Investigate factors influencing metfomin plasma concentrations.
- Investigate the association between genetic variations in OCT1, OCT2, and LKB1 drug transporters and metformin plasma concentrations among a diabetic group of patients from the UAE population.
- Identify the frequency of polymorphisms in OCT1, OCT2, and LKB1 genes among diabetic (test) and non-diabetic (control) groups from the UAE population.

2.1 Research hypothesis

- There is no significant genetic difference in polymorphisms in OCT1, OCT2, and LKB1 transporters between the UAE population and the Caucasian population.
- There is no significant difference in metformin plasma concentrations in the UAE population exhibiting polymorphic patters in the OCT1, OCT2, and LKB1 genes.

2.2 Significance of the study

In clinical practice it has been shown that patients respond variably to metformin treatment. Inter-individual differences in response to metformin can be due to a variety of factors, including age (107), renal function (105-107) , metformin dose (208) and genetic differences (210-211). Genetic variability in drug transporters involved in renal secretion, hepatic uptake, and disposition of metformin may explain part of the pharmacokinetic variability.

This is the first study to investigate the impact of genetic factors on metformin pharmacokinetics in an Arab population, particularly the UAE population. If genetic polymorphisms of all candidate genes involved in metformin pharmacokinetics were known then it may be possible in the future to genetically profile patients into responders and non-responders to metformin use and that would be clinically relevant information for the treatment which will assist in establishing metformin doses especially in elderly and renal impaired patients.

3 Methodology

This study was conducted at four different hospitals in the UAE, Fujairah Hospital and Al-Madina Medical centre, Khurfakan Hospital, Kalba Hospital, and Shaikh Khalifa Medical city at Abu-Dhabi UAE.

3.1 Ethical considerations

The research was approved by the Curtin University of Technology Human Research Ethics Committee Australia, the Ministry of Health, UAE, and Abu-Dhabi Health Authority, UAE.

Patients were required to give written informed consent in order to participate in the study. Data from patients were coded alphanumerically and stored on a computer hard disc which was password protected. Then the information that linked patients with their alphanumeric code was stored on a separate removable memory flash and kept in a secure place. That information was only available to the principle investigator.

3.2 Study period

The data collection commenced in the UAE in February 2007 and was completed on June 1st 2008.

3.3 Study personnel

The chief investigator and the supervisor managed the study. Other staff included clinical pharmacists, the in-charge nurse and specialists for patient identification, pathology and nurses for collecting and labelling blood samples.

3.4 Settings

Diabetic out-patients who met the selection criteria and were visiting a diabetic clinic at any of the selected hospitals or health centres in the UAE during the data collection period were asked to volunteer for the study.

Control non-diabetic subjects were randomly selected from different clinics at selected hospitals and health centres in the UAE.

Both non-diabetic and diabetic subject were UAE citizens and were part of the local population.

3.5 Subjects/Participants

Non- diabetic (control group)

Inclusion criteria:

- Not diabetic, confirmed by medical history from the medical file.
- FPG ≤ 6 mmol/L (85, 249).

- Out-patient.
- Consent to participate in the study.

Exclusion criteria:

- Less than 18 years old.
- On corticosteroids (increase plasma glucose level).
- Refused to give consent for any reason.
- They were unable to give informed consent (e.g. patients with cognitive dysfunction, and language barrier).

II. Diabetic patients

Inclusion criteria:

- Type 2 DM diagnosis confirmed.
- Prescribed metformin therapy for at least seven days.
- Outpatients.
- Consented to participate in the study.

Exclusion criteria:

- Type 1 DM.

- Women diagnosed with polycystic ovary syndrome.
- Less than 18 years.
- Ceased metformin treatment for any reason.
- Refused to give consent for any reason.
- Unable to give informed consent (e.g. patients with cognitive dysfunction, and language barrier).

Subjects were withdrawn from the study if:

- Consent was withdrawn.
- The medical or surgical team, who was responsible for the subject's care did not wish the subject to continue participation in the study.

3.6 Data collection

For control non-diabetic subjects, basic demographic data including age, gender, BMI, and ethnicity were collected. Diabetes free cases were confirmed by medical history through patient's medical file and by FPG level.

For the diabetic -test- patients, basic demographic data including metformin dose, medical history, BP, current medications, and duration of diabetes, smoking status, the level of physical activities, and demographic data (age, gender, BMI, and ethnicity) were collected. Patients BMI ([1]) were calculated using a standard

equation. Modification of Diet in Renal Disease Study (MDRD) equation (250-253) ([2]) was used to estimate GFR. Patients body surface area was adjusted using DuBois' formula (254) ([3]), and the website (http://www.halls.md/body-surface-area/refs.htm) was used to calculate body surface area. One venous fasting blood sample (10mL) was taken from each diabetic subject in order to determine the following:

- Srcr.
- FPG.
- HbA1c%.
- Plasma metformin concentration.
- Lipid profile (LDL, HDL, total cholesterol, and triglyceride).
- DNA genotyping.

(1) BMI= weight (kg)/ height * height (m^2)

(2) GFR (mL/min/1.73 m^2) = 186 x $(S_{cr})^{-1.154}$ x $(Age)^{-0.203}$ x (0.742 if female) x (1.212 if African-American) (conventional units)

(3) Body surface area (m^2) = weight (kg) $^{0.425}$ x height (cm) $^{0.725}$ x 0.007184

3.7 Study design

This was a prospective cross-sectional study, diabetic patients and non-diabetic subjects were asked to participate in the study. The identification of non-diabetic subject's for possible inclusion in the study was coordinated by the central laboratory consultant in all selected hospitals. While the identification for the diabetic patient was coordinated by specialists, and in-charge nurses in the selected diabetic clinic at different hospitals and health centre.

Type 2 DM patients on long term treatment with oral metformin orally for at least seven days were asked to participate in the study.

After the patient had been identified, the chief investigator met the patient and gave the required information of the study verbally and in written format (Appendix A). Before signing the consent form (Appendix B) by the patient, the chief investigator answered any questions related to the study. A request form was then filled in by the chief investigator to order a fasting blood sample, and then the co-ordinator nurse escorted the patients to the pathology department for a fasting blood sample. The chief investigator had designed a stamp labelled with "metformin study" which helped staff from the pathology department identify the patients who required their blood to be taken for the purpose of the study. The stamp was approved by the ethic committee in each participated hospital and a circular was distributed accordingly to the pathology department f. In order to observe patient confidentiality as required by ethics, the blood collection form did not contain the name of the patient and codes were used to de-identify the patients.

Tests for FPG, $HbA1_C$, Scr, and lipid profile including LDL, HDL, total cholesterol, and triglycerides were carried out in the pathology department at each hospital and was performed using the equivalent machines, equipment, reagents and protocols approved by the UAE Ministry of Health. Samples for measuring plasma metformin levels were frozen at -20°C and were transported to Australia by the DHL express courier. In Australia, the blood samples were analysed at the PathCentre Laboratory at Sir Charles Gairdner Hospital. The imported samples complied with all international and domestic requirements concerning the safe handling, transport and labelling of biological material. Safety precautions were also maintained during shipment and handling to prevent the dissemination of pathogens.

For the study purpose, renal function was classified into five groups depending on the degree of renal impairment (255):

- Group one: individuals with estimated GFR > 90 mL/min/1.73m^2
- Group two: individuals with estimated GFR between 90-60 mL/min/1.73m^2
- Group three: individuals with estimated GFR < 60 and >30 mL/min/1.73m^2
- Group four: individuals with estimated GFR between 30-15 mL/min/1.73m^2
- Group five: individuals with estimated GFR < 15 mL/min/1.73m^2

Metformin plasma level was also classified into two categories (120-122, 256):

- Category one: patients with metformin plasma levels between 0.01-1.0 mg/L

- Category two: patients with metformin plasma levels more than 1.0 mg/L

Moreover, BMI was classified into four categories (257-260):

- Category one: individuals with BMI between19.00-24.99 kg/m^2
- Category two: individuals with BMI between 25.00-30.00 kg/m^2
- Category three: individuals with BMI between 30.10-39.99 kg/m^2
- Category four: individuals with BMI $\geq$ 40.00 kg/m^2

Based on the above mentioned references (257-260), individuals in the category two were considered overweight, and individuals in the category three were considered obese, while individuals in the category four were considered morbid obese.

Patient's physical activity was classified into three groups according to the level of physical activity (261-262):

- Sedentary: individuals who were not involved in any kind of physical activity.
- Irregular: individuals who were walking for less than 150 minutes per week
- Regular: individuals who were walking for $\geq$ 150 minutes per week.

Furthermore, individuals were classified based on the duration of diabetes into three groups:

- Group one: individuals who had been diagnosed with type 2 DM between one week and less than 4 years

- Group two: individuals who had been diagnosed with type 2 DM between 4 and 10 years

- Group three: individuals who had been diagnosed with type 2 DM for > 10 years

Ethnicity was sub-divided based on subjects surnames (263-264), country of birth (264), language spoken at home (264), and self identification information into:

- Arabs : Individual of pure Arab origins

- Asians: Individuals whose origins included any of the original peoples of the Far East, Southeast Asia, of the Indian Subcontinent, including, for example, India, Malaysia, Pakistan, and the Philippines.

- Persians: Individuals whose origins were Iranian

- Africans: Individuals whose origins were in any of the black racial groups of Africa

Immediate metformin dosage form was used by all recruited patients. Metformin prescribed daily dose was further divided by defined daily doses (DDD) (265) into three groups:

- Group one: patients who were taking daily metformin dose between 500- < 1900 mg/day.

- Group two: patients who were taking daily metformin dose of 1900 and < 2500 mg/day.

- Group three: patients who were on daily metformin dose between 2500-3000 mg/day.

The DDD is a standardized dosing measure representing the recommended daily dose for the main indication in an adult.

3.8 Analytical Technique for Metformin

The blood sample analysis for determination of metformin concentrations was done at the Western Australian Centre for Pathology and Medical Research. (PathCenter). Blood samples (5ml of heparin blood in tubes not containing gel separator) were collected centrifuged and the plasma transferred to a second tube (5ml plastic). These were then stored at -200C until analysed.

The sample preparation for analysis involved precipitation of the plasma with acetonitrile containing an internal standard (dihydrocodeine). The organic/aqueous layer was transferred to a second tube and washed with n-hexane. The hexane was aspirated to waste and the acetonitrile/aqueous layer taken to dryness in a water bath at 450C using a nitrogen stream. The residue was reconstituted in the eluting solvent and an aliquot injected onto the HPLC for analysis. Column used was a

Merk LiChrospher RP select B (5um) 250 x 4 mm Solvent was 72% acetonitrile in 0.01M potassium dihydrogen phosphate that had previously been adjusted to pH 8.0 with diethylamine. Flow rate = 1.5 ml/min. Detector wavelength was 235nm.

The venous lactate levels were determined by an enzymatic method with standard automated techniques.

3.9 Genotyping

Initially the LKB1 gene was not included in the study methodology; however, after understanding the upstream regulators of OCT genes, and the postulated mechanism of action of metformin, the decision was made to include the LKB1 in the genetic screening and analysis of the studied population. In this study, all exonic and flanking intronic regions of OCT1, OCT2, and LKB1 were screened and compared with the observed SNPs in this population with the reported SNPs in other populations.

As mentioned previously, in the human the genes coding for OCT1 and OCT2 are localized within a cluster on chromosome 6q26 (228-230), and each of the two genes comprises 11 exons and 10 introns (229, 231, 266). The Human LKB1 is located on chromosome 13.3p19, and comprises 10 exons (266). The forward and reverse primers were designed based on the flanking intronic region to amplify the full exon sequences. The sequence data were fitted by the DNAMAN sequence alignment software and compared against the NCBI reference sequences to investigate for genetic variations. The genotypes were confirmed by two

independent observers. In order to compare the genotypes of the UAE populations with the reported SNPs in other populations, SNP data of OCT1 and OCT2 genes from the Genome Variation Server database version 5.09 (267) were downloaded.

3.9.1 Genomic DNA extraction

Blood samples from control non-diabetic individuals and diabetic patients were collected in K3EDTA BD Vacutainer® Blood Collection Tubes (1 Becton Drive, Franklin Lakes, NJ USA). The samples were frozen at -20^{0}C until they were ready for processing.

Protocol: The stored blood samples were used for DNA isolation.

1. Approximately 500 µl of blood samples were added to 500 µl of digestion buffer containing proteinase K and incubated overnight at 65^{0}C for protein digestion for 1 hour in a water bath.
2. After incubation the mixture was heated to 85^{0}C for 10 minutes to inactivate proteinase K.
3. The suspension was centrifuged at 10,000 revolutions per minute (rpm) for 3 minutes to collect the supernatant.
4. The supernatant was then transferred to a new tube and an equal volume of phenol was added directly & mixed gently.
5. The above mixture was centrifuged at 12,000 gravity (g) for 7 minutes at 4^{0}C.

6. The aqueous layer was taken out carefully in an Eppendorf® tube and an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added.
7. The above aqueous mixture was centrifuged at 12,000 g for 10 minutes at 4^0C.
8. The aqueous layer was separated and chloroform: isoamyl alcohol (24: 1) was added and centrifuged at 12,000 g for 10 minutes at 4^0C.
9. The aqueous layer was separated and DNA was precipitated by adding twice the volume of chilled 100% ethanol and $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2), by incubating it in a -20^0C freezer overnight.
10. The precipitate was pelletted by centrifugation at 10,000 rpm for 10 minutes, and washed twice with 70% ethanol and air-dried.
11. 50 µl of 1 X TE buffer (pH 8) was used for dissolving the stored DNA for later use.
12. DNA concentration and purity of samples was measured with a Nanodrop 1000 (ThermoFisher Scientific, USA) and run on 0.8% agarose gel for quality assessment.

3.9.2 Polymerase Chain Reaction

In this study, PCR was used to amplify the specific exons. Specific primers were designed using Primer3 software (268) to amplify the entire exonic region and the specificity of the primers were confirmed by UCSC *in silico* PCR Genome Browser

website (269). The primers were ordered from Metabion International AG and the sequences of all primers and the annealing temperatures used are given in Table 13.

1. 1 µl of 10-50 ng of genomic DNA were aliquoted into 96 well plates.

2. PCR master mixes were prepared for the specific primers in excess to correct for pipetting errors.

3. The master mix comprised of 0.25 µM of each primer; 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, 1 unit of GoTaq Hot Start Polymerase (Promega, USA), 200 µM of each dNTP.

4. PCR was performed on a final volume of 25 µL using a Thermal Cycler 9700 (Applied Biosystems, Foster City, CA, USA).

5. The thermocycling protocol consisted of an initial incubation at 95°C for 10 minutes followed by 35 cycles at 94°C for 30 seconds; 30 seconds at annealing temperature for each primer (Table 13) and elongation at 72°C, and a final extension step of 72°C for 10 minutes.

6. The amplified DNA fragments were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide, and then visualized under UV light.

3.9.3 Sequencing

Protocol: The PCR products were transferred to optical plates (Applied Biosystems, USA) and sequenced using both forward and reverse primers. Prior to

sequencing, to dephosphorylate any residual amplification nucleotides and the unused primers, the PCR products were purified with Shrimp Alkaline Phosphatase in combination with Exonuclease I.

1. 2 μL of the PCR products were purified with 0.5 U Shrimp Alkaline Phosphatase (SAP) (USB Corporation, USA) in combination with 5 U of Exonuclease I (ExoI) (USB Corporation, USA).

2. The above mixture was incubated at 37°C for 30 minutes, followed by 15 minutes at 85 °C to inactivate the enzyme (performed in a Thermal Cycler).
3. 5 μL of the purified PCR products were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in both directions with the same primers as used for PCR.
4. The cycling conditions were as follows;

    - 96^0C for one minute, followed by 25 cycles of 96^0C for 10 seconds, then 50^0C for 5 seconds, and finally 60^0C for 4 minutes.

5. Sequencing reactions were cleaned using the ethanol/EDTA/sodium Acetate precipitation method following the instructions provided by the BigDye® Terminator v3.1 Kit.
6. The precipitated cleansed sequencing reaction products were kept in the dark and then air dried.
7. Samples were re-suspended in 15 μL Hi-Di™ formamide (Applied Biosystems, USA) and run in an ABI3730 DNA Analyser (Applied Biosystems, USA).

3.10 SNPs discovery and Statistical analysis

Sequencing data were collected through an ABI3730 DNA Analyser and analysed by Sequencing Analysis Software Version 5.1 (Applied Biosystems, USA) using mixed base identification. Multiple sequence alignment was performed using DNAMAN sequence analysis software (Lynnon Biosoft, Canada). The suspected SNPs detected in DNAMAN multiple nucleotide sequence alignments were confirmed by observation of the electropherogram in SeqScape (Applied Biosystems, USA). The intron/exon and protein coding region of genes were compared with gene sequences in the NCBI database. GENEPOP software Statistical analysis was carried out using GENEPOP (270). Minor allele frequency (MAF) and Hardy Weinberg Equilibrium (HWE) and FIS was analysed using the standard options available in the program. The Genome Variation Server version 5.09 (GVS) was used to compare the distribution of minor allele frequencies and heterozygosity in the European (CEU), Japanese (JPT), Chinese (CHB) and the African (YRI) populations (267).

3.11 Statistical analysis

3.11.1 Sample size estimation

Based on the analysis of two articles (120, 256), the mean values for metformin plasma levels and their standard deviations for different levels of renal dysfunction are shown in Table 9.

Table 9: The mean plasma metformin concentrations and their corresponding creatinine clearance

Clcr (mL/min)	Metformin level (mg/L) (mean ± standard deviation (SD)
Group 1 (<10)	42.1 ± 26.3
Group 2 (11-20)	7.6 ±7.3
Group 3 (21-30)	3.3 ± 2.9
Group 4 (> 30)	1.6 ±1.6

Based on the above data, a sample size of 239 patients will initially be selected as it is sufficient to detect a difference of 20% in metformin level with over 80% power if the standard deviation was 50% of the mean or less.

Data were analysed using SPSS® software version 17 for Windows. Metformin plasma concentration was considered as the dependent variable. Age, gender, height, ideal body weight, BMI, metformin dose and GFR were considered as independent variables. The Chi-square test was used to detect any significant differences between metformin plasma concentration and other variables. Logistic regression was used to test the independent association between metformin plasma concentration and associated factors of interest including age, gender, BMI, metformin dose, HbA1c, and OCT1 and OCT2 and LKB1 polymorphisms. Correlation studies were done using Pearson's correlation. A p value of < 0.05 was considered to be statistically significant. Data were presented as mean ± standard error (SE) unless otherwise stated. All metformin levels measured were trough concentration at steady state. For the purpose of the current study, concentrations of

0.01 to 1.0 mg/L were considered as a therapeutic reference range for metformin plasma levels (120-122). Any concentration above that was considered to favour the accumulation of metformin.

4 Results

A total of 461 eligible diabetic subjects and 185 control non-diabetic subjects were identified in all the study hospitals in the UAE.

Of the 461 diabetic subjects, 374 were approached by the investigator (81.1%). Of the 374 subjects, 323 volunteered to participate in the study (86.4%) of which 31 were excluded for different reasons, the remaining 292 patients had a blood sample taken and analyzed in the study as demonstrated in Figure 7. While out of 196 identified non-diabetic individuals, only 170 approved to participate in the study and had a blood sample taken for FPG, HbA1c, and DNA analysis.

4.1 Demographics of diabetic subjects

From 292 patients 64% were females. The mean age for this group of patients were 54 ±0.7 years (Table 10).

4.2 Demographics of control non-diabetic subjects

From 170 control non-diabetic subjects 60% were females, the mean age at recruitment was 49 ±0.84 years. Table 10 illustrates the demographic data of the recruited diabetic and control subjects.

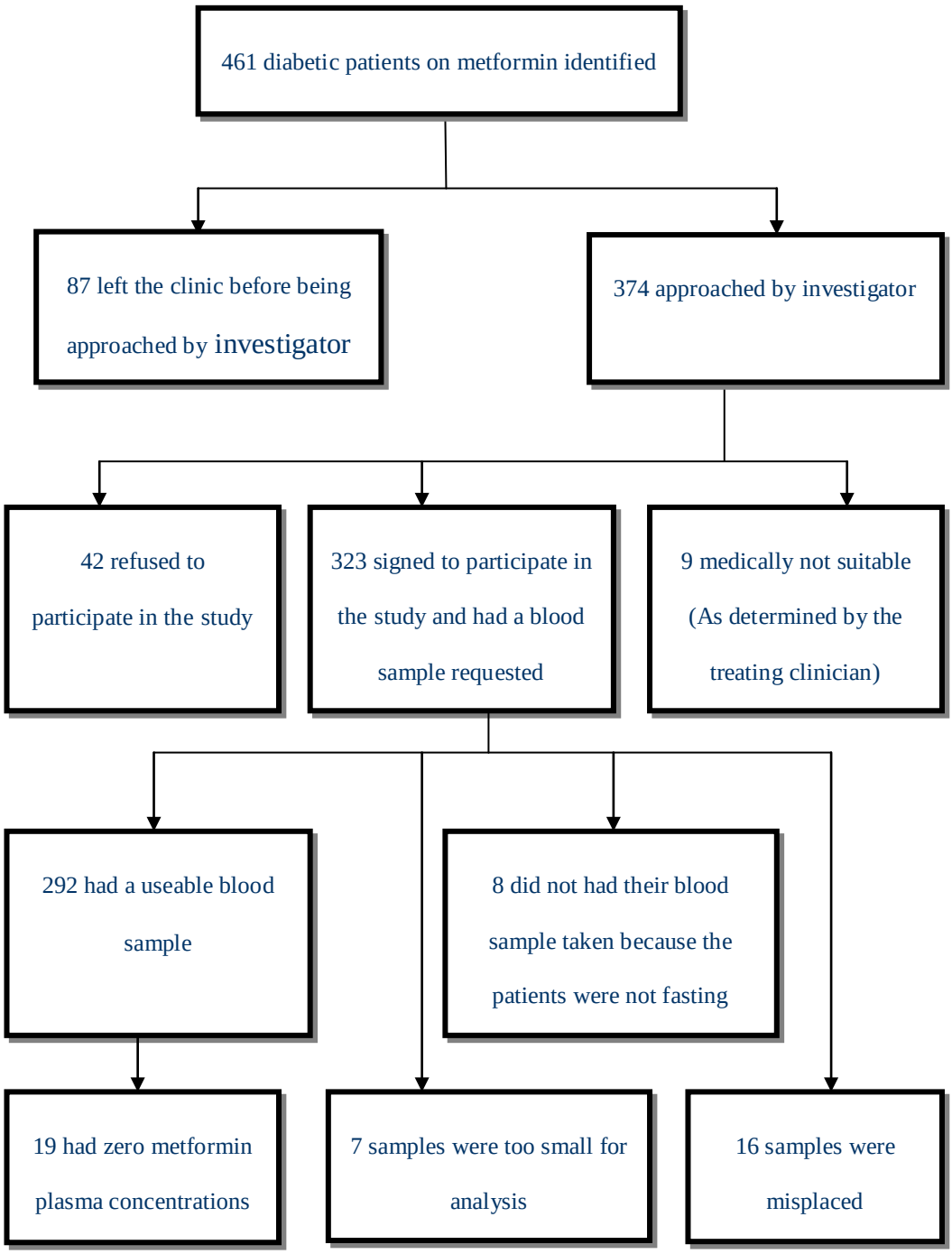


Figure 7: Illustrations of the result of patient's recruitment

Table 10: Clinical parameters and demographic data of diabetic UAE patients (n= 292) and control subjects (n= 170) recruited in the study. Results are mean ±SE

Demographic/clinical parameters			*P* value
	Diabetic (test)	**Non-diabetic (control)**	
Sample number (n)	292	170	
Female (n, %)	187 (64%)	102 (60%)	0.076
Male (n, %)	105 (36%)	68 (40%)	0.076
Age (Years)	54.00 ± 0.7	49.00 ± 0.8	0.002
BMI (Kg/m^2)	30.36 ± 0.35	30.46 ± 0.31	0.314
FPG (mmol/L)	9.26 ± 0.21	4.63 ± 0.04	< 0.0001
HbA1c%	8.15 ± 0.12	4.50 ± 0.04	< 0.0001
Cholesterol (mmol/L)	4.70 ± 0.19	NA*	NA
Triglyceride (mmol/L)	1.82 ± 0.06	NA	NA
LDH (mmol/L)	2.65 ± 0 .06	NA	NA
HDL (mmol/L)	1.40 ± 0.02	NA	NA
GFR (mL/min/1.73m^2)	88 ± 1.6	NA	NA
Metformin concentration (mg/L) ‡	0.62 ± 0.03	NA	NA
Metformin dose (mg/day)	1622 ± 28	NA	NA

*NA: not applicable

‡ The mean value for this parameter was based on 273 patients; subjects with zero metformin plasma concentrations were excluded

4.3 Metformin plasma concentrations

The mean metformin plasma concentration for the 273 diabetic groups of patients was 0.62 ±0.03 mg/L with a minimum of 0.01 and a maximum of 4.28 mg/L, of which 83% had metformin plasma concentrations between 0.01-1.0 mg/L. Nineteen patients recorded zero metformin level, and they were excluded from further regression analysis because they were considered non compliant to therapy. Thus, any analysis between the investigated variables and metformin plasma concentrations included 273 patients, excluding those clearly non-complaint. However, demographic, biochemical and genetic baseline data were estimated on the total of 292 diabetic patients.

4.4 Association between metformin plasma concentration with demographic variables and clinical parameters in the diabetic group

As stated in the methodology section, each variable was categorized into different groups, and then a cross tab chi-square test was used to investigate the possible association between those variables and metformin plasma concentrations in the 273 diabetic patients. If an association was established by a significant *p* value (< 0.05) then further multiple regression was performed on independent significant variables.

4.4.1 Patient's age

The mean age of the 292 diabetic patients who were prescribed metformin therapy was 54.00 ±0.7 years with a minimum of 19 years and a maximum of 100 years old. There were 53 patients aged ≥65 years (18%) with mean GFR of 21.2 mL/min/1.73m^2. Of the recruited patients 82% were in the age group between 46-64 years.

The result of the study showed that there was no significant association between patients age and metformin plasma concentration (p= 0.15), thus patient age was not included into further analysis.

4.4.2 Patient's gender

It was shown that 64% of the recruited diabetic population was female. Of the 273 diabetic patients, 11% of the females had metformin plasma concentration more than 1.0 mg/L, while 5% of the males had metformin plasma concentration more than 1.0 mg/L.

It was found that there was no significant association between patient's gender and metformin plasma level (p= 0.33), thus gender was not included in further regression analysis in this study.

4.4.3 Patient's BMI

The mean BMI for the recruited 292 diabetic patients was 30.36 ±0.35 kg/m^2, with a minimum of 19.00 and maximum of 56.00 kg/m^2. It was found that 76% of the

patients were overweight or obese. There was a significant difference between males and females in BMI measures (p= 0.001), the female population was more obese than the male population.

The results showed no significant association between BMI and metformin plasma concentration (p= 0.13). Although there was a significant difference between males and females in BMI, however, the results showed that there was no significant association between the average male BMI and average female BMI and metformin plasma concentration (p= 0.14 and p= 0.47 respectively). Therefore, BMI was not included in any further analysis.

4.4.4 Patient's ethnicity

Of the 292 recruited populations, a majority were of Arab origin (74.7%), (Figure 8); with 15% of the Arab population having a metformin plasma concentration more than 1.0 mg/L, (Table 11).

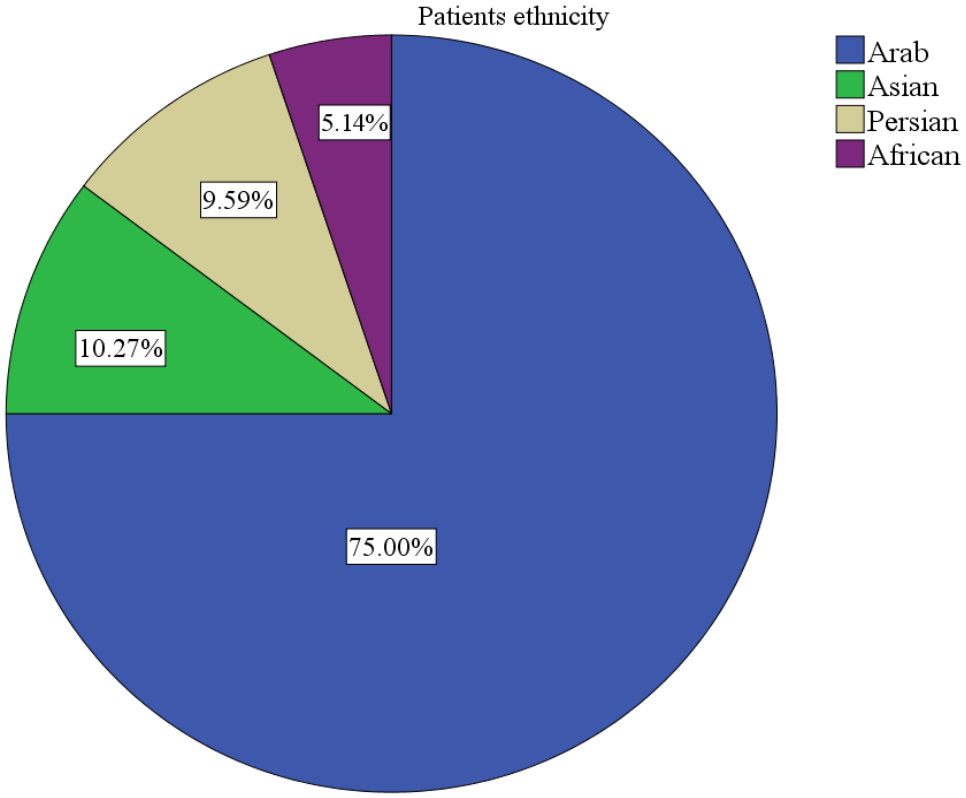


Figure 8: Representation of different ethnic groups in the diabetic population of the UAE, n= 292

Table 11: Ethnicity Vs metformin plasma concentration (mg/L) $p = 0.71$, n= 273

			Metformin category		Total
			0.01-1.0 (mg/L)	> 1.0 mg/L	
Ethnicity	Arab	Number (%)	173 (85%)	31 (15%)	204
	Asian	Number (%)	23 (77%)	7 (23%)	30
	Persian	Number (%)	20 (83%)	4 (17%)	24
	African	Number (%)	13 (87%)	2(13%)	15
Total			229	44	273

The results demonstrated that all ethnic groups had poor glycemic control (HbA1c=8.15 ±0.12%); however 93% of African origin recorded higher HbA1c level compared to 69% Arabs and 83% in both Asian and Iranian ethnic groups.

Patients ethnicity was not significantly associated with metformin plasma concentration (p= 0.71). With the exception of Arab ethnicity that would be included in sub-analysis with any observed significant variables with metformin plasma concentrations; patient ethnicity was not included in further analysis.

4.4.5 Patient's physical activity

Of the recruited 292 patients 52% led a sedentary lifestyle, with the remaining 37% and 11% involved in irregular and regular physical activity groups respectively. There were no significant differences between males and females in the level of physical activity.

The results showed that there was no significant association between patients physical activity and metformin plasma concentration (p= 0.29), therefore, patients physical activity was not included in further analysis.

4.4.6 Duration of diabetes

According to the methodology used in this study, patients were classified into different categories based on the duration of diagnosed diabetes. They ranged from being diagnosed from one week to more than 30 years. Patients were distributed almost evenly in each group, and most of them had metformin plasma levels between 0.01 to 1.0 mg/L, thus, the results showed that of the 273 diabetic patients there was no significant association between the duration of type 2 DM and metformin plasma level (p= 0.11).

4.4.7 HbA1c

The mean HbA1c for the recruited 292 patients was 8.15 ±0.12%, with a maximum of 15.3% and a minimum of 3.3%. The results revealed that the HbA1c variation between males and females didn't reach statistical significance (p= 0.055).

The results also showed that in the 273 patients there was no significant association between glycemic control measured by HbA1c level and metformin plasma concentration (p= 0.65), thus HbA1c was not included in further regression analyses.

4.4.8 Lipid profile

Triglycerides: Mean triglyceride was 1.82 ±0.06 mmol/L. No significant association was observed between triglyceride levels and metformin plasma concentration (p= 0.09).

LDL: Mean LDL level was 2.65 ±0.06 mmol/L. There was no significant association between LDL plasma level and metformin plasma level among patients (p= 0.13).

Cholesterol: Mean cholesterol level was 4.70 ±0.19 mmol/L. There was no significant association between cholesterol level and metformin plasma concentration (p= 0.97).

HDL: Mean HDL level was 1.40 ±0.02 mmo/L. There was no significant association between HDL level and metformin plasma concentration within the 273 diabetic patients (p= 0.20). Therefore, lipid profiles were not included in further regression analyses with metformin plasma concentration.

4.4.9 Metformin dose

The mean metformin dose in the 273 diabetic patients with measured metformin plasma concentrations, was 1622 ±28 mg/day, with a minimum of 500 and a maximum of 2550 mg/day. According to the methodology followed in this study, metformin daily dose was classified into three groups. The result of this study showed that metformin dose was significantly associated with metformin plasma level (p= 0.012). Patients with higher metformin doses had recorded higher metformin plasma levels. Thus, metformin dose was included in the regression analyses.

4.4.10 GFR

The mean GFR using MDRD formula (250-253) was 88 ±1.6 mL/min/1.73m^2 with a minimum of 17 and maximum of 229 mL/min/1.73m^2.

The results showed a highly significant association between patients GFR and patients age (p< 0.0001) (Table 12), it also revealed a significant association between GFR and metformin plasma concentration (p= 0.001), thus, GFR was considered for further regression analysis with metformin plasma concentration.

Table 12: The association between patient's age category and GFR group, n= 292

GFR mL/min/1.73m^2	Age (years)			
	18-30	31-46	47-64	≥65
> 90	5%	31%	57%	7%
90-60	1%	16%	61%	22%
< 60-15	0%	1%	51%	48%

4.5 Complications of diabetes, CVD

Almost 33% of the recruited patients were diagnosed with CVD (MI, angina, and CHF) of this group 10% had metformin plasma concentrations above 1.0 mg/L (p= 0.002), and 78% were diagnosed with hypertension and were on different groups of antihypertensive medications. Moreover, 74% of the diabetic patients were on statin antihyperlipidemia treatment. Of the diabetic and hypertensive patients 39% were diagnosed with CVD and were on medications prescribed to control this complication, hence, there was a significant association between hypertension and CVD (p< 0.0001). Of all the diabetic patients 7% were smokers and 26% of them quit smoking either recently, 6 weeks or as long as 20 years earlier. Of the 7% smokers, 38% were diagnosed with CVD, thus, there was a significant association between smoking status and the presence of CVD in the diabetic patients (p< 0.0001). Furtheremore, 26.5% of the diabetic patients who were diagnosed with CVD had HbA1c> 7% (p= 0.002). Figure 9 summarizes the effect of different variables on the presence of CVD in the diabetic patients.

The result of this study showed that out of 292 diabetic subjects, 45 patients were on metformin therapy mono-therapy, the remaining 247 patients were prescribed combinations of metformin with other hypoglycaemic agents, Table 13.

Table 13: Combination of metformin therapy with other oral hypoglycaemic agents represented with number of patients in each combined regimen, n= 247

Combined therapy	Number of patients (n)
Metformin plus sulphonylureas	166
Metformin plus rosiglitazone	17
Metformin plus insulin and sulphonylureas	28
Metformin plus insulin	27
Metformin plus sulphonylureas and rosiglitazone	9

There was no significant association between treatment regimen as mono and multi-hypoglycaemic therapy (p= 0.52), however, there was a significant association between the duration of the disease and treatment regimen as mono-therapy with metformin and multi-therapy with other hypoglycaemic agents (p< 0.0001); as an increase in the duration of the disease led to a poor glycemic control and subsequently addition of other hypoglycaemic agents into the treatment regimen.

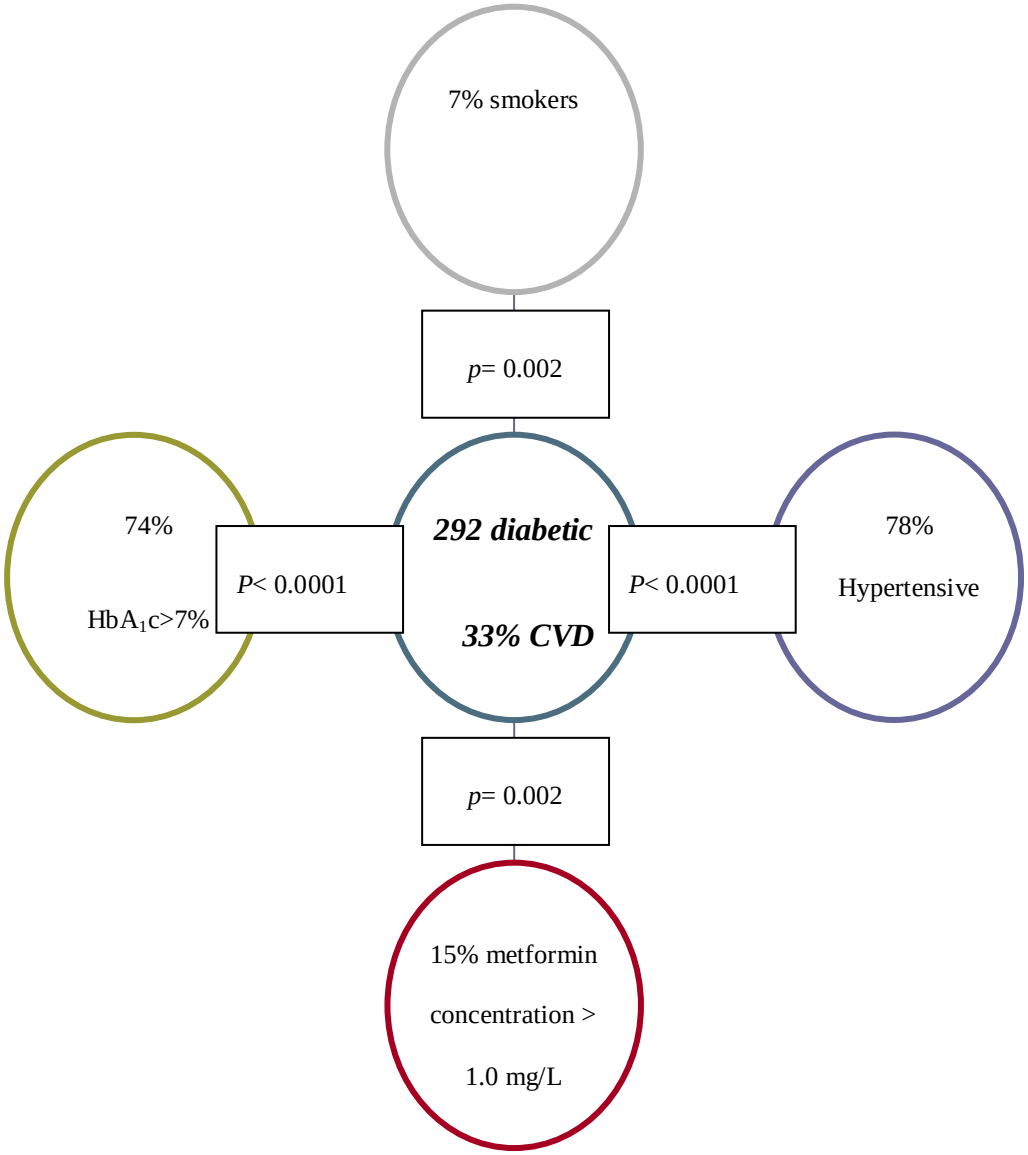


Figure 9: The percentage of CVD in the diabetic population and the percentage of other variables and the association between these variables with the presence of CVD represented by *p* value.

In summary, among all demographic and clinical data that were collected and analysed; within the diabetic patients, metformin dose and GFR showed a significant association with metformin plasma concentrations, hence were included in the multiple regression analysis.

4.6 Genotypes

The genotyped alleles for the candidate genes were categorized into homozygotes (both alleles are the same) and heterozygotes allele (both alleles are different). The reported allelic variations (polymorphism) were classified into synonymous and non-synonymous polymorphism based on the NCBI SNP reference (http://www.ncbi.nlm.nih.gov/). Genotype was carried on all of the UAE population, including diabetic and non-diabetic groups. However, in order to investigate the association between metformin plasma concentrations and genetic polymorphisms of the candidate genes, only the diabetic group was considered in the analysis. Genetic variations were categorized into two group homozygotes and heterozygotes. A cross tab chi-square test was used to investigate the possible association between genetic variation and metformin plasma concentrations in the 273 diabetic patients. If an association was established by a significant p value ($p< 0.05$) then further multiple regressions was carried on independent significant SNPs.

Table 14 shows the list of primers used in the study along with the sequences, annealing temperatures and expected product size.

4.6.1 OCT1 (SLC22A1)

The results of the study showed that of the reported 28 SNPs in the NCBI data base 12 were polymorphic in the UAE population. Table 15 illustrates the details of the SNPs, exonic location, functional consequence of amino acid change, and the percentage of MAF reported in the study population. Among the reported polymorphic SNPs in the OCT1; 9 were with MAF> 0.01%. No novel SNPs were found in any of the OCT1 exons. MAF of the observed SNPs in the UAE population were not always matching with those reported in Caucasians, although it was sometimes comparable with other ethnic populations, such as African or Asian. Table 16 demonstrates a summary of the polymorphism observed in the OCT1 gene among the UAE population in comparison with the other major population groups in the GVS database.

4.6.2 OCT2 (SLC22A2)

It was observed that of the 18 reported SNPs in the database, five were found to be polymorphic in this population, of which three were with MAF> 0.1%. Table 17 shows genotyped polymorphisms in the OCT2 gene along with their exonic location, NCBI SNP reference ID, functional consequence of amino acid change and MAF observed in the UAE population. A novel synonymous Ser 279 (A/G) variation was found in exon one not reported in any other population, with an MAF 1.03%. The electropherogram for this SNP is shown in Figure 10. Moreover, the majority of the reported SNPs in this population were comparable with Caucasians. Furthermore, some of the observed SNPs in the study population were also reported

in the Caucasian population but with different MAF. Table 18 demonstrates the summary of polymorphisms with MAF> 0.1% observed in the OCT2 gene among the UAE population in comparison with the other major populations in the GVS database.

4.6.3 LKB1

The genotype results of the LKB1 gene showed of four reported SNPs in the database, two polymorphisms were found in the UAE population; the non-synonymous 125Ile>Thr (rs11552325) and synonymous Tyr (rs9282859) SNPs with MAF= 0.01%. Table 19 discloses genotyped polymorphisms in the LKB1 gene along with their exonic respective location, NCBI SNP reference ID, functional consequence of amino acid change and MAF observed in the UAE population.

Table 14: List of primers used in the study along with the sequences, annealing temperatures and expected product size.

Gene	Exon	Primer sequence Forward (5´-3´)	Reverse (5´-3´)	Annealing Temperature (°C)	Product size (bP)
OCT1	1	cttggttgccttccagatgt	tcccaggaactcccatgtta	57	590
	2	cctcttgccgtggtatgact	tccagcagcttctgcagtta	57	250
	3	atttcaacctctcccacctg	ccatcccattctaccctgag	57	248
	4	gggaaggagaaatgggagac	cgttatgcatgtggacacca	60	495
	5&6	ccgaggaaaatgccagatag	ctggagagacgcacaggaac	57	499
	7	gggcccttctaggacactct	cctcatctttgttctcattcca	55	492
	8	caccatggcctctcacagta	gggttcacagccatttcact	55	231
	9	tcctcatggttcctcctgac	cgagctgcaaaagaaggaat	55	323
	10	ttggctggctgtgattatttc	tgatattaggccccttttgg	55	249
	11	ttgcaacagttccatcatcaa	agcaccaacagctttcccta	55	385
OCT2	1	tttgggaagtgcaggaaggac	ccthcttgcttccttgagaa	55	698

	2	agggcaagccttttggttat	gaaaggatgggattcaagca	55	578
	3	cagtctgtgcctcctggatt	gaagctgggtccctttctt	55	391
	4	ctgctaagttaccaagccattt	cccaaagagaaaggacatttg	55	850
	5	atccagtccttgacccctct	gaagctcctcacgtgacctt	55	697
	6	ctagtgctgggcatgagatg	atttgcccaagaaaaacacg	60	392
	7	cacagccagccactgaagta	gctggccatatgaatttgct	55	554
	8	catgagcctatagaagaagtcatt	tcacgttattttcaatgcacaa	55	693
	9	caattgctcctcccatcatt	acatccaggaagaacgcaag	55	660
	10	tggtggcaatgtcctctacc	gaccttctccacgtgaaagttta	55	574
	11	ttctttctcccctctccattt	ttttaaaatccacaaatgttaagaca	60	836
LKB1	1	ggccgtgttcatacttgtcc	cgctaccagggcattttaac	58	966
	2	gttgggctctccaggtgtg	gaaccatcagcaccgtgac	60	394
	3	ccaagagtcagccctgtcc	aacttgggccttcatgtcaa	58	431
	4&5	gtgtgcctggacttctgtga	accaccatctgccgtatgag	58	650

6	ctaccccgtagcctccacta	cagtcctctcaatgcctgct	60	425
7	ccttaggagcgtccaggtatc	cctcactcagaccccagttc	60	400
8	tcctgagtgtgtggcaggta	gaagctgtccttgttgcaga	62	387
9	tgcctcctactcgtgaggtt	cgactcagcctcagccatac	60	996
10	acctcttccgtcttccttcc	gttgagacgcgaacaaaacc	55	900

Table 15: Summary of genetic variations detected in the OCT1 (SLC22A1) gene with minor allele frequencies (MAF) in the UAE population, n= 462

No	Location	NCBI SNP ID	Functional Consequence	Allele change	mRNA position	MAF (%)
1	Exon 1	rs35888596	Non-synonymous	A/G	218	3.3
2	Exon 1	rs1867351	synonymous	C/T	261	28
3	Exon 1	rs12208357	Non-synonymous	C/T	286	4.3
4	Exon 1	rs55918055	Non-synonymous	C/T	367	0.4
5	Exon 2	rs683369	Non-synonymous	C/G	585	11.2
6	Exon 3	rs34134157	synonymous	C /T	663	0.2
7	Exon 6	rs2282143	Non-synonymous	C/T	1127	3.4
8	Exon 7	rs628031	Non-synonymous	A/G	1327	6.3
9	Exon 7	rs34888879	synonymous	A/G	1344	0.3
10	Exon 9	rs35270274	Non-synonymous	G/T	1568	4.1
11	Exon 10	rs41267797	synonymous	A/G	1608	3.3
12	Exon 11	rs16891138	synonymous	A/C	1752	1.3

* Highlighted data indicates the SNPs of MAF≤ 0.01

Table 16: Summary of genetic variation observed in OCT1 (SLC22A1) gene among the UAE population in comparison with the other major populations reported in the genome variation server (GVS) database

Location	NCBI SNP ID	CEU*	JPT*	HCB*	YRB*	UAE
Exon 1	rs1867351					
	Alleles	C/T	C/T	C/T	C/T	C/T
	Minor allele, MAF (%)	C (21)	C (45)	C (44)	C (29)	C(28)
	Heterozygosity	0.33	0.50	0.49	0.41	0.3
	Hardy-Weinberg chi-Square	0.15	0.06	0.05	0.42	0.73
Exon 2	rs683369					
	Alleles	G/C	G/C	G/C	G/C	G/C
	Minor allele, MAF (%)	G (20)	G (10)	G (13)	1	G (11.2)
	Heterozygosity	0.37	0.18	0.22	0.03	0.198
	Hardy-Weinberg chi-Square	2.16	1.87	2.68	0.02	0.84
Exon 6	rs2282143					
	Alleles	C	T/C	T/C	T/C	T/C
	Minor allele, MAF (%)	mono	T (12)	T(11)	8	T (3.4)
	Heterozygosity	mono	0.21	0.19	0.14	0.06
	Hardy-Weinberg chi-Square	NA	0.00	6.77	0.71	0.079
Exon 7	rs628031					
	Alleles	A/G	A/G	A/G	A/G	A/G
	Minor allele, MAF (%)	A (44)	A (17)	A (22)	A (24)	A (6.4)
	Heterozygosity	0.49	0.29	0.34	0.37	0.119

	Hardy-Weinberg chi-Square	0.97	1.25	11.72	0.06	1.45
Exon 11	rs16891138					
	Alleles	A	A	A	C/A	C/A
	Minor allele, MAF (%)	mono	mono	mono	C (6)	C (1.3)
	Heterozygosity	mono	mono	mono	0.11	0.025
	Hardy-Weinberg chi-Square	N/A	N/A	N/A	0.42	0.67

MAF is the fraction of total alleles of the given marker that are minor alleles; mono = monomorphic SNP, NA not applicable. GENEPOP software was used to estimate MAF and Hardy-Weinberg chi-Square.

* CEU: Europeans, JPT: Japanese, HCB: Chinese, and YRB: Africans.

Table 17: Summary of genetic variations detected in the OCT2 (SLC22A2) gene with MAF in the UAE population, n= 462

No	Location	NCBI SNP ID	Functional Consequence	Allele change	mRNA position	MAF (%)
1	Exon 1	rs624249	synonymous	G/T	560	33.4
2	Exon 4	rs316019	Non-synonymous	G/T	978	10.4
3	Exon 6	rs45599131	Non-synonymous	T/G	1222	1.0
4	Exon 8	rs3907239	Non-synonymous	A/G	1558	1.0
5	Exon 10	rs316003	synonymous	C/T	1676	12.2

*Highlighted data indicates the SNPs of MAF≤ 0.01

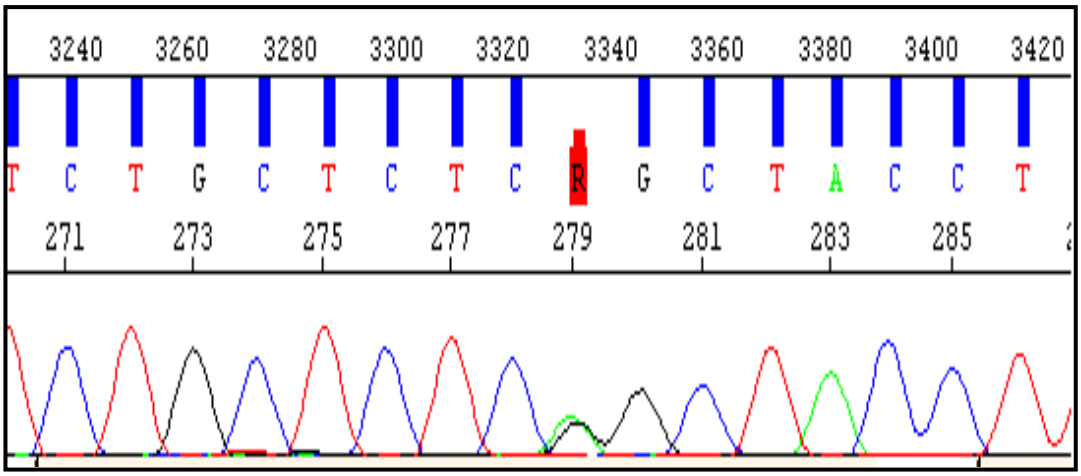


Figure 10 : Electropherogram of novel synonymous Ser 279 (TCTCAGCTA) SNP in exon1 of OCT2 (SLC22A2) gene that was found in the UAE population.

Table 18: Summary of genetic variation observed in OCT2 (SLC22A2) gene among the UAE population in comparison with other major populations reported in the genome variation server (GVS) database

Location	NCBI SNP ID	CEU	JPT	HCB	YRB	UAE
Exon 1	rs624249					
	Alleles	A/C	A/C	A/C	A/C	A/C
	Minor allele, MAF (%)	A (40)	A(20)	A (17)	A(24)	A (33.4)
	Heterozygosity	0.48	0.32	0.28	0.36	0.44
	Hardy-Weinberg chi-Square	0.03	0.14	0.23	0.03	0.46
Exon 4	rs316019					
	Alleles	A/C	A/C	A/C	A/C	A/C
	Minor allele, MAF (%)	A(10)	A(12)	A(11)	A(16)	A(10.3)
	Heterozygosity	0.18	0.20	0.20	0.27	0.184
	Hardy-Weinberg chi-Square	0.02	0.00	0.00	0.00	0.00
Exon 7	rs8177516					
	Alleles	G	G	G	A/G	G
	Minor allele, MAF (%)	mono	mono	mono	T (1)	mono
	Heterozygosity	mono	mono	mono	0.02	mono
	Hardy-Weinberg chi-Square	NA	NA	NA	0.00	NA
Exon 8	rs8177517					
	Alleles	T	T	T	G/T	T
	Minor allele, MAF (%)	mono	mono	mono	G (6)	mono
	Heterozygosity	mono	mono	mono	0.11	mono

	Hardy-Weinberg chi-Square	NA	NA	NA	0.23	NA
Exon 10	rs316003					
	Alleles	C/T	C/T	C/T	T/C	C/T
	Minor allele, MAF (%)	C (19)	C (14)	C (19)	T(46)	C (12.2)
	Heterozygosity	0.31	0.24	0.31	0.50	0.214
	Hardy-Weinberg chi-Square	0.44	0.05	0.15	0.53	1.42

Table 19: Genotyped polymorphisms in LKB1 gene along with their exonic location, NCBI SNP reference id, functional consequence of amino acid change and Minor Allele Frequencies (MAF) in the diabetic patients, n= 462

Gene	Location	NCBI SNP ID	Functional Consequence	Allele change	mRNA position	MAF (%)
LKB1						
	Exon 1	rs56354945	synonymous	A/C	1379	mono
	Exon 2	rs11552325	Non-synonymous	C/T	1489	1.0
	Exon 6	rs9282859	synonymous	C/T	1931	1.0
	Exon 8	rs59912467	Non-synonymous	G/C	2177	mono

MAF = Minor Allele Frequency (%), mono = monomorphic, *p* value < 0.05 was considered significant.

4.7 Association between metformin plasma level and OCT1 variations

Among all of the 9 SNPs reported in the study population only two were found to be significantly associated with metformin plasma concentration, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) with p= 0.001 for both variables. It was also observed that 33% of the diabetic patients were carrying OCT1-E1 synonymous Ser SNP (rs1867351) variants of which 27% had metformin plasma concentration above 1.0 mg/L. While 5% of the patients had polymorphism in OCT1-E9 non-synonymous 488Arg>Met (rs35270274) of them 53% recorded metformin plasma concentration above 1.0 mg/L. Table 20 illustrates OCT1 variations along with the percentage of heterozygocity in association with the percentage of metformin plasma concentration that was above 1.0 mg/L and the p value of the association of those reported SNPs with metformin plasma concentration.

Table 20: demonstrates OCT1 genetic variations and the percentage of the OCT1 heterozygocity reported in the diabetic patients. It also shows the percentage of the patients with the polymorphic alleles in the higher metformin plasma concentration category (above 1.0 mg/L).

OCT1 variation	% Heterozygocity	% of polymorphic patients with metformin level >1.0 mg/L	*p* value
E1 23Leu>Val rs34570655	<1	0	1
E1 38Gly>Asp rs35888596	4	9	0.81
E1 Ser rs1867351	33	27	0.001
E1 61Arg>Cys rs 12208357	33	15	0.87
E1 88Cys>Arg rs55918055	<1	0	1
E2 160Leu>Phe rs 683369	17	13	0.49
E3 Asn rs34134157	<1	0	1
E6 341Pro>Leu rs2282143	8	7	0.57
E7 408Met>Val rs628031	6	29	0.30
E7 Ala rs34888879	<1	0	1
E9 488Arg>Met rs35270274	5	53	0.001
E10 Val rs41267797	12	9	0.27
E11 Ser rs16891138	3	2	0.85

4.8 Association between metformin plasma level and OCT2 variations

The OCT2 gene was less variant than OCT1 gene, the results of the study revealed that OCT2 genetic variations were not significantly associated with the metformin plasma concentration as indicated by *p* value. Table 21 demonstrates OCT2 exonic variations with the percentage of heterozygocity, percentage of patients with polymorphism that had metformin plasma concentration above 1.0 mg/L, and the *p* value for each of the OCT2 reported SNP along with metformin plasma concentration.

4.9 Association between metformin plasma level and LKB1 variations

As it was reported in the database, LKB1 gene was less variant than the other two genes; only four SNPS were reported in all studied populations. The result of the current study showed that the genetic variation of the LKB1 gene was not associated with metformin plasma concentrations (p= 0.377) thus LKB1 was not included in further regression analysis.

Table 21: OCT2 variations with the percentage of the heterozygocity reported in the diabetic patients and the percentage of the patients with the polymorphic allele in the higher metformin plasma concentration category (above 1.0 mg/L).

OCT2 variation	% Heterozygocity	% of polymorphic patients with metformin level >1.0 mg/L	*p* value
E1 Thr rs624249	37	15	0.82
E4 270Ala>Ser rs316019	18	20.5	0.58
E6 351Leu>Trp rs45599131	<1	0	0.66
E8 463Arg>Lys rs3907239	2	33	0.24
E10 Ala rs8177521	7	26	0.21

4.10 Regression model between metformin plasma concentration and the associated significant variables

As previously stated, metformin dose, patient's GFR, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) were found to be the only factors that were associated with metformin plasma concentration in the diabetic patient group. Table 22 demonstrates the association between each significant variable and metformin plasma concentration represented by *p* value.

Table 22: Association between metformin dose (mg/day), GFR (mL/min/1.73m^2), OCT1-E1 Ser synonymous SNP (rs1867351), and OCT1-E9 Arg>Met non-synonymous SNP (rs35270274) and metformin plasma concentration (mg/L), n= 273

Variables	*p* value
Metformin daily dose (mg/day)	0.012
GFR (mL/min/1.73m^2)	0.001
OCT1-E1 synonymous Ser SNP (rs1867351)	0.001
OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274)	0.001

Furthermore, in order to test the significance of independent significant variables with metformin plasma concentration a multiple regression test was applied to examine the effect of metformin dose, GFR, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) on metformin plasma concentration. The model was found to be highly significant ($p< 0.0001$), and all of the four variables were found to be significantly associated with metformin plasma concentration. Table 23 summarizes the effects of each variable on metformin plasma concentration represented by a *p* value.

Table 23: Multiple logistic regression between metformin dose (mg/day), GFR (mL/min/1.73m^2), OCT1-E1 Ser synonymous SNP (rs1867351), and OCT1-E9 Arg>Met non-synonymous SNP (rs35270274) and high metformin plasma concentration as dependent variable (mg/L) with odds ratio, 95% CI, and *p* value, n= 273

Variable	N with high metformin level	Odds ratio	95% CI‡	*P*
Metformin dose (mg/day)				
500-< 1900	4	2.19	1.34-3.63	0.002
1900- < 2500	10			
2500-3000	30			
GFR (mL/min/1.73m^2)				
>90	9	2.08	1.23-3.49	0.06
90-60	26			
<60- >30	9			
30-10	1			
OCT1-E1 synonymous Ser SNP (rs1867351)				
Homozygotes	20	2.82	1.30-5.47	0.007
Heterozygotes	24			
OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274)				
Homogygotes	37	6.04	1.66-21.98	0.006
heterozygotes	7			

‡CI: confidence interval.

However, in order to generalize the result to the Arab population, a sub-analysis of the data using multiple regression analysis to include only Arab ethnicity (75% of the study population). The result confirmed the above observation that metformin dose, GFR, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) were predictive factors for variability in metformin plasma concentration with p= 0.002, p= 0.035, p= 0.022 and p= 0.016 respectively (Table 24)

Table 24: Multiple logistic regression between metformin dose (mg/day), GFR (mL/min/1.73m^2), OCT1-E1 Ser synonymous SNP (rs1867351), and OCT1-E9 Arg>Met non-synonymous SNP (rs35270274) and metformin plasma concentration (mg/L) odds ratio, 95% CI and *p* value in the Arab ethnic group, n= 219

Variable	N with high metformin level	Odds ratio	95% CI‡	*P*
Metformin dose (mg/day)				
500-< 1900	3	2.59	1.42-4.72	0.002
1900- < 2500	7			
2500-3000	21			
GFR (mL/min/1.73m^2)				
>90	7	1.94	1.05-3.59	0.035
90-60	17			
<60- >30	6			
30-10	1			
OCT1-E1 synonymous Ser SNP (rs1867351)				
Homozygotes	14	2.73	1.16-6.45	0.022
Heterozygotes	17			
OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274)				
Homogygotes	26	6.74	1.43-31.6	0.016
heterozygotes	5			

*Research basic data are available upon request.

5 Discussion

According to a recent IDF report (271), diagnosed type 2 DM is highly prevalent among both sexes in member states of the Eastern Mediterranean region, and highest among member countries of the Gulf Corporation Council including the UAE. One of the major health care challenges in the UAE is the lack of national data, thus it is difficult to estimate the prevalence of diagnosed diabetes among the UAE population. However, based on a national survey conducted jointly by WHO and the UAE Ministry of Health between 1998 and 2000 on 2360 UAE nationals (272) the overall percentage of type 2 diabetes among UAE citizens was 24%, therefore UAE was classified as the second highest country with the highest diabetes prevalence within the adult population (20.1%) (271). The prevalence of diabetes among the UAE population could be higher than 24% especially with increasing levels of obesity (272), changes of lifestyle towards a sedentary pattern (272), and with increased prevalence of undiagnosed diabetes (271-272).

Most type 2 diabetic patients were overweight or obese (273-275). According to available guidelines, Metformin is recommended as first line pharmacological therapy in newly diagnosed overweight type 2 diabetic subjects (15-16, 83, 249). It is of note that with recent global diabetes management guidelines metformin is recommended as the initial pharmacological management option for all patients (83, 276). In this study, it was observed that 41% of the diabetic patients had BMI values between 30-40 kg/m^2 and 78% of the recruited diabetic subjects were prescribed antihypertensive medications. These figures reflect community

ignorance about diabetes risk factors, endemic of diabetes, and subsequently the risk of diabetes complications, and further identifies the need for a prompt government decision to initiate national diabetes prevention programs and enhance community awareness about this chronic disease. As the antihyperglycemic efficacy of metformin does not vary with adiposity to a clinically significant extent (277) it is not recommended to adjust metformin dose according to patients BMI. This recommendation is supported by the results of this study which observed no significant association between metformin plasma concentrations and patients BMI.

Metformin is considered the drug of choice for the treatment of overweight newly diagnosed type 2 diabetic patients (15), and it is the most commonly used anti-diabetic drug. In the USA, metformin accounted for 33% of oral antidiabetic prescriptions (278). There has been no national survey regarding the use of metformin among diabetic patients in the UAE, however, because of the high prevalence of DM among UAE citizens, and low cost of metformin; it could be postulated that metformin could be the most common antihyperglycaemic agent used in the UAE. Moreover, the result of this study showed that 461 diabetic patients were prescribed metformin therapy in four different hospitals and health centres in the UAE. It was also observed that out of 292 diabetic subjects, 45 patients were on metformin mono-therapy while 247 patients were prescribed combinations of metformin with other hypoglycaemic agents, which strongly indicates that metformin could be the most commonly used oral hypoglycaemic agent in the UAE.

5.1 Association between metformin plasma concentration and demographic and clinical data

The result of the current study showed that, among all evaluated demographic and clinical parameters, metformin dose and GFR were found to be significantly associated with metformin plasma concentration (p= 0.002, and p= 0.006 respectively).

5.2 Association between metformin plasma concentrations and GFR

Renal disease affects 20-40% of individuals with diabetes (279-281), and diabetic nephropathy is the leading cause of end-stage renal disease in developed countries (282). The evaluation of renal function is therefore of critical importance in diabetic subjects. GFR is the best measure of overall kidney function in health and disease (251-252, 283-284). GFR can be directly measured by infusion of external substances such as inulin or ^{51}creatinine labeled EDTA (285). However, these methods are expensive, not practical and time consuming. The ADA recommends estimation of GFR by either Cockroft Gault or MDRD equations in all patients with diabetes (286). The MDRD model is recommended for use in Australia for the routine estimation of GFR with every request for serum creatinine in adults (287). A body of evidence has suggested that the MDRD formula is more accurate and better performing than Cockroft Gault equation in estimating GFR in diabetic patients (250-252). It was also reported that MDRD is less influenced by body

weight unlike Cockroft Gault equation (288-290). In the current study we have used GFR to measure patients kidney function and because the majority of subjects were obese (mean BMI 30.36 ±0.35) we estimated GFR using the MDRD equation (284) which is less biased in obese patients.

Pharmacokinetic studies have shown that metformin is rapidly eliminated by the kidney by combined glomerular filtration and tubular secretion (104-106). The results of this study are in agreement with metformin pharmacokinetic properties, as the kidney is the major site of metformin excretion. Therefore, it was not surprising to observe that patients with decreased GFR had higher metformin plasma concentrations than those with normal kidney function. As a result, one can postulate that patients with severe renal impairment might be at higher risk for metformin accumulation in the plasma, therefore, subjecting those group of patients to unwanted side effects such as gastrointestinal adverse effect and possibly lactic acidosis (105-107).

5.3 Association between patients age, GFR and metformin plasma concentration

Although patient age was not significantly associated with metformin plasma concentration, it was significantly associated with patients GFR ($p< 0.0001$). It is known that the most important changes interfering with the pharmacokinetic profile of metformin is the progressive decrease of renal function with ageing (107, 125-126), thus GFR declines with age. In a study by Sambol *et al.* (107) they

found that both renal function (as measured by Clcr) and age were predictors of metformin clearance. In the current study, GFR was a significant predictor of metformin plasma level as a single covariate. As mentioned, in this study the MDRD equation was used to estimate GFR (253) . Age was included as a function in the equation for estimating GFR (253), although patient age was not significantly associated with metformin plasma level, thus age should not be ignored when prescribing metformin therapy, particularly for elderly patients with decreased GFR.

5.4 Association between patients gender and metformin plasma concentration

This study indicated that there was no significant difference between males and females in measured metformin plasma concentrations. This result was in agreement with other studies that have observed no gender differences in metformin pharmacokinetics (23, 104, 108).

5.5 Relationship between poor glycemic control and diabetic complications

Poor glycemic control is linked to increased risk of diabetic complications (15, 235). In the current study, it was shown that 33% of the patients were diagnosed with CVD (MI, angina, and CHF), of which 85% were on combined hypoglycaemic therapy. Since this study was not designed to investigate the

prevalence of diabetes complications among the recruited subjects, therefore all of the medical information was retrieved from patient's medical record in the diabetic clinics only. These medical records barely include other medical conditions such as patient cardiovascular or microvascular complications, and the diagnosis of diabetic complications were confirmed either by the treating doctors in the diabetic clinic or by the patient. Thus, the prevalence of diabetes complications could be higher than that observed in this study.

5.6 Association between metformin plasma concentration and metformin dose

It was shown that metformin dose was significantly associated with metformin plasma concentration (p= 0.002). Patients with lower metformin doses had lower metformin plasma concentrations and vice versa. This result was consistent with other studies, where highly significant association have been reported (105-106, 113). This observation indicated that prescribing doctors tended to increase metformin dose when patients response to metformin was not as desired, and that might lead to an addition of another hypoglycaemic agent to metformin therapy. The result of this study showed that of 292 diabetic patients only 45 patients were on metformin mono-therapy. It was also shown that despite maximizing metformin dose and adding other hypoglycaemic agents to metformin therapy, patients had poor glycemic control (mean HbA1c=8.15 ±0.12%). This might be due to several reasons, including failure to maintain physical activity; because it was observed that 51% of the recruited patients led a sedentary lifestyle. Another reason could

be non-compliance to drug therapy, 6% of the patients had zero metformin plasma concentration despite self reporting of good compliance to the prescribed medications. It could also be genetic factors that might affect drug transporters and metabolizing enzymes.

5.7 Association between metformin plasma concentration and OCT1, OCT2, and LKB1 genes

Metformin is well characterized as a substrate of OCT1 and OCT2 drug transporters (18-20), and genetic studies have demonstrated that OCT genes exhibit polymorphic variation in the coding region (24-27). *In vivo* and *in vitro* studies have demonstrated the important role played by OCT1 and OCT2 genes in the pharmacokinetics, disposition, and efficacy of metformin (21, 32, 223, 233, 291). In clinical practice it has been shown that patients respond differently to metformin treatment (17, 292). Metformin plasma levels are not usually monitored in order to determine the effectiveness of treatment, and they have little clinical value except when lactic acidosis is present (103). The variation in metformin plasma levels could be used as an indicator of metformin accumulation in the plasma, and as a variable that might be associated with other factors which could contribute to the inter-individual variation to metformin response.

Metformin pharmacokinetics have been well characterized in Caucasians (104-106, 113, 293) and in other populations, such as Chinese (108), Arabs (109), Indians (110), African-Americans (293), and Germans (111), no significant

difference was observed in metformin pharmacokinetic parameters among those populations.

One of our study aims was to investigate the correlation between the genetic variations in OCT1, OCT2, and LKB1 genes and metformin plasma concentration in the UAE diabetic population, and to identify the frequency of polymorphisms in the drug transporters among diabetic and non-diabetic populations.

The result of the current study revealed that OCT1, OCT2, and LKB1 genes exhibited a polymorphic pattern in the UAE population. It was also observed that there was a significant genetic difference in polymorphism in OCT1, and OCT2 transporters between the UAE population and the Caucasian population.

A number of studies have compared OCT1 and OCT2 SNPs among various ethnic groups, but most of the reports were from Caucasian and Asian populations (26-29). However, genetic studies are somehow limited in regard to the LKB1 gene.

5.8 OCT1 genotypes

In the OCT1 gene, all non-synonymous SNPs have been identified in various racial populations with different frequencies (Table16). It was observed that although the UAE population as Arab is classified as Caucasian, their observed genotype patterns were different than other Caucasians, and surprisingly the allele frequencies of some of the reported SNPs were similar to other ethnic groups such as Asian and African. For example, the allele frequencies of the non-synonymous SNP 160Phen>Leu, and 341Pro>Leu were closer to the reported Japanese and

Chinese allele frequencies (267). While the allele frequency of the synonymous Ser SNP located in exon 1 (rs1867351) was similar to that reported with Africans. This observation was concurrent with Kang *et al*. (29). Their study evaluated genetic polymorphisms of OCT1, and OCT2 genes in Korean subjects while investigating the functional properties of the non-synonymous SNPs. They also compared the allele frequencies of the major functional SNPs of the OCT1 and OCT2 genes in the study population with three other ethnic groups, Vietnamese, Chinese, and white Germans (29). An interesting observation was the reported OCT1 341Pro>Leu SNP (rs2282143) in the white German population that was not detected previously in white European-American population. In addition, they found that an OCT2 270Ala>Ser mutation revealed different allele frequencies between the white European-American population and white German population (15.7%, 2.5%, respectively) (233). They concluded that despite being genetically classified as Caucasian, the white German population had a different genetic background to the white European- American population, the same result was observed in this study when compared between the UAE population and Caucasians, the MAF for some of the OCT1 SNPs were different between these two populations.

Interestingly, some of the observed SNPs in the UAE population were found to resemble the African population, such as the synonymous Ser SNP identified in exon 1 (rs1867351) and in exon 11 (rs16891138). Although the MAF for (rs186735) SNP was comparable in both populations, however it was observed that the MAF for the (rs16891138) SNP was lower in the UAE population

compared to the African population (1.3% *Vs* 6% respectively) (267). In this study it was found that recruited patients were a blend of different ethnic groups and the higher percentage were Arab (Figure 8), with a lower representation of Africans (5%). If the multiethnic culture in the UAE population is observed, this may not be an unexpected observation. Before oil discovery, the economic life of the UAE depended heavily on pearl diving and sea trade in the Gulf and the Indian Ocean (294-296). This led to the settlement of different ethnic groups from countries along the trade route, such as Iran and India (297). Trade activities with east Africa led to the importation of Africans as labourers in the pearling industry in the late nineteenth century (298). The African and Iranian ethnic populations have been fully integrated as citizens (294-296). It was also observed that among different ethnic groups, Africans recorded very high percentage of patients with high HbA1c level (93%), moreover, 93% of them were on multi-hypoglycaemic therapy, which might indicate poor responds towards the given treatment. One factor that might have influenced this result is an increased BMI (>25 kg/m^2) among African patients. It is known that obesity can lead to poor glycemic control (15-16, 299), and it is also known that Africans respond differently to some of the drugs such as oral antihypertensive agents compared to white Caucasians (300-301). This could draw attention to a similar finding with antihyperglycaemic agents, especially when some hypoglycaemic agents are metabolized in the liver by different enzymes which are subjected to genetic variations among different ethnic groups (302-305). The sample size for the African ethnic group was too small to confirm this observation in this study.

5.9 OCT2 genotypes

In comparison with the OCT1 gene, one novel synonymous Ser 279 (A/G) SNP was found in exon 1 of the OCT2 gene in three Bedouin (original UAE ethnic) subjects belonging to various regions in the northern emirates of the UAE (Figure 10). Although it is a synonymous SNP, it identifies the unique genetic background of this population that was different from other ethnic groups, especially with Caucasians. Other than previously mentioned synonymous SNP, all other polymorphisms observed in the OCT2 gene have been reported in other populations (267). Unlike OCT1, the MAF of some of the SNPs observed in OCT2 in the UAE population were comparable to the Caucasians, such as synonymous SNP in exon 1 (rs624249) and non-synonymous SNP in exon 4 (rs316019) with MAF of 44% and 8% in Caucasians compared to 33.4% and 10.3% in the UAE population (Table 18).

5.10 The effect of the cultural and religious factors on the genetic make up of the UAE population

There are recent reports of haplotype-based association studies to increase the chance of identifying common disease genes, but the selected haplotype-tag SNPs from databases may vary among different ethnic groups (306). Hence, this shows that understanding of ethnicity-specific SNP allele frequencies in the UAE population has a significant role in choosing which SNPs and genes to investigate and to calculate an appropriate sample size for a pharmacogenomics study. The

allele frequencies of SNPs particularly non-synonymous SNPs are shown in Tables 15 and 17. The high similarity in allele frequencies between the UAE and European populations and the presence of certain comparable SNPs with Asians and Africans indicated the early migration and settlement of certain ethnic groups. This result highlights the importance of critical assessment of allele frequencies based on ethnicity. Many of the SNPs reported in the literature and database were found to be monomorphic in the UAE population.

Population substructure will lead to inbreeding-like effects, which can be observed by a reduction in the number of observed heterozygotes (*Het Obs*) when compared to the expected number of heterozygotes (*Het Exp*). The polymorphic SNPs observed in the UAE population as a whole and especially the non-synonymous SNPs, show a reduction in the *Het Obs* compared to the *Het Exp* pooled allele frequencies (Table 25). *Fis* is the amount of inbreeding like effects as measured by F- Statistics and data in Table 26 shows the inbreeding-like effect within the UAE population (307). *Fis* indicates the deficiency of average heterozygotes in OCT1 and OCT2 genes within the entire UAE population and it could be observed that almost all significant heterozygote deficits ($p < 0.05$) may be due to non-random mating within the population. Even though Arabs are usually identified as Caucasians, modern Arab populations especially in Egypt, Palestine, Jordan and Lebanon, are the result of a long history of blending of different human races (308). Although multiple rare mutations may segregate in crossbred population, it is noted that founding events followed by population bottle necks may reduce allelic diversity, in a way that a single mutation dominates the allelic spectrum in

an isolated population (309). Due to cultural and religious practices, most of the Arab nations including the UAE still practice consanguineous marriages within first cousins and the consanguinity rate maybe as high as 50.5% (310) in the UAE population. The major impact of consanguinity is that it increases the inbreeding coefficient (311) and the frequency of genetic disorders. Even though this study did not take into account the consanguinity factor in the UAE population, the presence of largely monomorphic SNPs in the OCT1 and OCT2 genes and the low frequency of polymorphic SNPs in comparison with the other populations in the database indicates that even though there was a mixture of populations in the semi-urban areas, still UAE as a whole is an inbred population due to cultural influences. The results of the study also indicate that the UAE population is a distinct population with some similarities with Caucasians.

Table 25: Summary of polymorphisms in OCT1 and OCT2 genes in the UAE population with Het Obs, Het Exp, and FIS, n= 462

Gene	Location	NCBI SNP ID	Het Exp	Het Obs	Fis	*p* value
OCT1						
	Exon 1	rs1867351	117	106	0.0937	0.1031
	Exon 2	rs683369	84.45	57	0.3254	< 0.0001
	Exon 6	rs2282143	29.01	30	-0.0342	1
	Exon 7	rs628031	35.63	6	0.832	< 0.0001
	Exon 11	rs16891138	9.88	10	-0.0119	1
OCT2						
	Exon 1	rs624249	140.77	107	0.2402	< 0.0001
	Exon 4	rs316019	51.21	27	0.4732	< 0.0001
	Exon 8	rs8177517	6.96	3	0.5684	< 0.0001
	Exon 10	rs316003	72.07	41	0.431	< 0.0001

Het Exp = expected number of heterozygotes, *Het Obs* = observed number of heterozygotes. F-statistics for the UAE population which shows the inbreeding like effect within the UAE population (*Fis*). GENEPOP software was used to estimate the various parameters. $p < 0.05$ was considered significant.

5.11 Association between the genetic variations of OCT gene and metformin plasma concentration

The results of the current study have revealed that among all reported SNPs in the UAE population two SNPs were found to be predictive factors for the metformin plasma concentrations, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-

E9 non-synonymous 488Arg>Met SNP (rs35270274). This observation was at variation with other studies, especially with Shikata *et al.* (21) who concluded that the effect of OCT1 and OCT2 polymorphisms on metformin efficacy may not be as significant as expected. The discrepancy in the results could be due to various reasons. The most apparent is that each study was conducted in different ethnic groups, and as it is known that MAF differ between different populations, common alleles in one population are not necessarily common in another population; subsequently, the influence of genetic variation on some drug responses will significantly differ between different populations (312-313). Arabs are genetically classified as Caucasians, however in the current study, it was observed that the UAE population exhibited different genotype patterns in OCT1 and OCT2 than that reported in Caucasians; accordingly, the UAE population might also respond differently to drug therapy than their antecedents.

Another reason for this discrepancy may be due to different approaches used in each study. Shikata *et al.* (21) used a phenotype-genotype approach to investigate the effect of OCT1 and OCT2 variation on metformin efficacy, while our study analysed metformin plasma concentrations and investigated the possible association between OCT1 and OCT2 genetic variations and the variability in metformin plasma levels. The methods used in classifying patients might have affected the results and the outcomes in both studies. Shikata *et al.* classified patients into responders and non-responders based on HbA1c level. This classification might be vague and confusing, thus patients were not only on metformin mono-therapy, some were on sulphonylurea, or insulin combinations.

Hence, it is not clear which therapy contributed more to decreasing HbA1c levels. Whereas in the current study patients were classified according to metformin plasma concentration into normal (below 1.0 mg/L) and high metformin level (≥ 1.0 mg/L), then the association between metformin plasma level and the genetic variation of the candidate genes was examined. As it can be noticed, this was a direct relationship between metformin and other factors affecting its plasma concentration including genetic variation of OCT1 and OCT2 genes without interference of other agents or factors.

The result of the current study can be generalized to other Arab populations, as it was shown that sub-analysing the data to include only Arab ethnicity revealed the same significant association between metformin dose, GFR, promoter linked SNP rs1867351 and 488Arg>Met SNP and metformin plasma concentrations (p= 0.002, p= 0.035, p= 0.022, and p= 0.016 respectively).

An interesting finding was the association between metformin plasma concentration and a synonymous SNP, OCT1-E1 Ser (rs1867351). There is some controversy that all synonymous SNPs are non-functional because they do not change the encoded amino acid. In fact, evidence increasingly suggests that gene functions and onset of disease could be affected by synonymous polymorphisms (314-315). However, there was a suggestion that investigating single synonymous SNP might not be the best way to reveal its impact on gene function and drug response; instead it has been recommended studying the clinical effects of haplotypes rather than single synonymous SNP. Recently, it was found that in one

multi-drug resistance gene (MDR1) haplotype, there were two synonymous SNP sites that occurred frequently and had a unique contribution pattern in human populations (316-317). *In vitro* and *in vivo* studies have observed that this MDR1 haplotype led to several changes, from mRNA level, protein expression, to substrate specificity, and these changes were dependent on the presence of the synonymous SNPs (318). The allele frequency of this synonymous SNP varies between different ethnic groups thus the haplotype distribution of MDR1 which carries this SNP demonstrated a similar pattern (319-323). The presence of synonymous polymorphisms in drug transporters that have an impact on gene functions is not limited to MDR1 (302), hence, the results of the current study strongly suggests the impact of synonymous SNP on transporter function. As it was shown in this study OCT1-E1 synonymous Ser SNP (rs1867351) was significantly associated with variation in metformin plasma concentration, and based on the previous discussion, it was postulated that if this synonymous SNP was carried together with other synonymous and/or non-synonymous SNPs in one haplotype block, subsequently the affinity of OCT substrate; such as metformin, towards drug transporter will decrease. This will lead to decreased hepatic uptake and therefore decreased metformin efficacy and increased plasma levels. The significance of OCT1-E1 synonymous Ser SNP (rs1867351) on metformin therapy was also observed in a recent study by Tzvetkov *et al. (22)*. They observed that OCT1 rs1867351 SNP was significantly associated with the renal clearance of metformin (p= 0.03). Furthermore, when combined, the effect of promoter-linked rs1867351 along with other low-function OCT1 amino acid substitutions

Arg61Cys, Ser401Gly, and Met420del, resulted in an increase in the renal clearance of metformin concentration by approximately 25%. This observation highlights the importance of haplotype linkage analysis for the reported functional and non-functional OCT1 and OCT2 SNPs.

Since this is the first study that has reported polymorphisms in OCT1 and OCT2 genes in the UAE population, it can be considered as initial data for further linkage analysis studies in other Arab populations.

As it was observed, 19 patients were excluded from further analysis, because they recorded zero metformin plasma concentrations. Thus they were considered non-compliant with metformin therapy. The percentage of non-compliance could be higher than what was observed in this study, especially if we consider that there could be some patients who were not taking metformin dose as prescribed. Therefore, in order to investigate the impact of this masked non-compliance to metformin therapy on the observed results, particularly OCT1 genotypes, a sensitivity analysis of the data was performed. It was found that even after increasing all metformin plasma concentrations by 20%, OCT1-E1 synonymous Ser SNP (rs1867351) and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) retained their significance with *p* value of 0.009 and 0.004 respectively. This indicates the significant and profound impact of the genetic polymorphisms of the OCT1 gene on the metformin plasma concentrations.

The OCT2 gene was less polymorphic than OCT1, and was not associated with metformin plasma concentration. Moreover, one novel synonymous Ser SNP was found in the OCT2 gene that has not been previously reported in other populations.

To our knowledge, no clinical study on diabetic patients has been conducted to examine the effect of OCT2 genetic polymorphisms on metformin plasma concentration and efficacy. However, a recent study examined the effect of non-synonymous SNP 279Ser>Ala on the renal elimination of metformin in healthy volunteers with European and African-American ancestries (324). In the mentioned study, they observed that individuals carrying 270Ser>Ala variant had a higher metformin renal clearance compared to those with the reference allele. Subsequent linkage analysis for this SNP showed that the 270Ser>Ala SNP in the CHB population was linked with four intronic SNPs (rs3912162, rs9346814, rs9364551, and rs16891232). These SNPs were not found in the CEU and the YRI populations (325). The authors concluded that one of the four intronic SNPs might have resulted in changes in OCT2 expression levels in the CHB population, which may have led to different observations in different populations. In this current study, we observed that 270Ser>Ala was the common non-synonymous SNP reported in the UAE population with a MAF of 10.3%. Nevertheless, this SNP was not correlated with metformin plasma concentration. One of the reasons for the discrepancy between these two observations could be due to different study populations (European and African-American vs UAE populations). Moreover, the methods used in both studies were varied, in the current study the association between the genetic variation of OCT2 and metformin plasma concentration was

examined in diabetic patients, in contrast Chen *et al.* investigated the effect of one genetic variant (270Ser>Al) of OCT2 on the renal elimination of metformin in healthy, young volunteers. Furthermore, other factors that could contribute to the variation in metformin plasma concentration were investigated in this study. GFR was significantly associated with the variability in metformin plasma concentration. Thus, it was postulated that in the clinical sitting GFR -not OCT2- may be the major factor that determine metformin renal elimination in patients who are regularly on the therapy.

Although OCT2 seems to regulate renal secretion of organic cations through the entry step at the basolateral membrane, it does not regulate the exit step at the apical membrane of renal tubule cells. Recently, metformin was shown to be a good substrate of the multidrug and toxin extrusion (MATE1) transporter (326). MATE1 is located in the bile canalicular membrane in the hepatocyte and in the brush border of the renal epithelium (327). Thus, MATE1 may contribute to the final step of metformin excretion through bile and urine. Hence, it is possible that genetic variation in MATE1 also contributes to inter-individual variation in metformin plasma concentration, disposition, and efficacy. Both this study and that of Chen *et al.* (23) have not included MATE1 as a possible contributor to metformin plasma concentration and its renal elimination.

As discussed previously, the rate of metformin absorption is slower than its rate of elimination, thus intestinal absorption is the rate limiting factor in metformin disposition. Recently, metformin was characterized as a substrate of a novel

organic cation transporter, plasma membrane monoamine transporter (PMAT) (328) which is localized in the human small intestine (328). Thus, PMAT may play an important role in metformin absorption; therefore, genetic variations of this transporter might contribute to the inter-individual variation in response to metformin therapy.

In summary, the results of the current study may influence the pattern of prescribing metformin in clinical practice. It confirmed the important role played by genetic factors along with other factors on metformin plasma concentration and therefore, its possible efficacy. This finding should augment the rising voices to individualize metformin dose for particular patient's especially elderly and renal impaired patients. Thus, in order to avoid unnecessary increments of metformin dosage especially when no response is observed, it is recommended to perform a genotyping test for the OCT1 gene for this sub-group of patient's who might be at higher risk of adverse effects. It is of note that this recommendation worth considering in the available guidelines when prescribing metformin therapy especially for elderly and renal impaired patients.

6 Conclusion

This is the first study to genotype OCT1, OCT2, and LKB1 genes in the UAE population. It was shown that there was a significant genetic difference in polymorphism in OCT1, OCT2, and LKB1 transporters between the UAE and Caucasian populations. Furthermore, one novel synonymous Ser 279 (A/G) SNP was found in exon 1 of the OCT2 gene.

It is also the first large study to report a significant association between metformin dose, GFR, OCT1-E1 synonymous Ser SNP (rs1867351), and OCT1-E9 non-synonymous 488Arg>Met SNP (rs35270274) and metformin plasma concentrations in the UAE diabetic populations. In addition, it has highlighted the importance of synonymous SNPs of OCT genes in drug response and therefore its efficacy, which could be generalized in other Arab populations.

Like the case of genotyping cytochrome P_{450}2C19 with proton pump inhibitors therapy (329-330), OCT1 genotyping test could be a useful clinical tool for the optimal metformin treatment especially in elderly and renal impaired patients. The cost effectiveness of this genotyping test needs to be verified in a future study.

Since this is the first study that has reported polymorphisms in OCT1, OCT2, and LKB1 genes in the UAE population, it can be considered an initial study for further pharmacogenomic and linkage analysis studies in other Arab and Middle Eastern populations.

The results of this study showed reduced representation of mutations affecting the function of the OCT family genes in the UAE population; the observation would indicate the need to assess genetic polymorphisms in the upstream regulators of the OCT pathway, such as AMPK. It is also suggested that the impact of genetic polymorphisms in other candidate genes involved in metformin pharmacokinetics such as PMAT and MATE that are involved in metformin intestinal absorption and renal excretion are evaluated in further studies.

7 Limitation

- Patient physical activity was based on a self report, and it is possible that patients were underreporting the actual level of their physical activity it could reveal the real reason behind their obesity, the fact that it might affect their social images.

- Patients were not only on metformin mono-therapy, they were on other hypoglycaemic agents as well, thus, the impact of other hypoglycaemic agents on type 2 diabetes can be a factor. As these would be added after metformin, it would be expected metformin had been used at a high dosage.

- Dependence on the diabetic clinic medical files to report patient's diabetes complications. This medical file did not include patients follow up examination in other specialized clinics, such as cardiac clinic. Thus, complications such as CVD may not have been registered in the diabetic clinic records.

- There are few studies that investigated the relationship between metformin levels and factors that might have an impact on it in patients with type 2 DM. The fact limits the possibility to compare this study results with other studies from different settings.

- Functional analysis of the novel Ser 279 (A/G) SNP in exon 1 of OCT2 gene could be a goal of further studies

- Linkage analysis to describe the haplotype structures of the investigated genes could also be considered in further studies.

- The effect of other genes such as MATE1 and PMAT on metformin plasma level could also be an objective for further study.

8 Recommendation

- In order to investigate the association between the genetic polymorphisms of OCT1 gene and metformin efficacy in the clinical practice, a prospective population study involves diabetic patients on metformin therapy only is recommended. Patients could be classified into responders and non-responders based on HbA1c levels which should be reported three months before initiation of metformin and four months after metformin therapy.

- An association study with large sample size and with the description of the haplotype structures of the candidate genes in the UAE population is further recommended.

- In order to examine the role of the novel synonymous Ser 279 (A/G) SNP that was observed in the OCT2 gene in the study population, functional analysis of this SNP and its haplotype structure in relation with other SNPs in the OCT2 gene should be investigated.

- Pharmacoeconomic studies are required to assess the feasibility of applying the results of population pharmacogenomic studies within the clinical settings.

- To individualize metformin therapy in clinical practice, genotyping of OCT1, MATE1, and PMAT are required on different populations with

different ethnic background. Figure 11 illustrate metformin pharmacokinetic pathway and the candidate genes involved in each pathway.

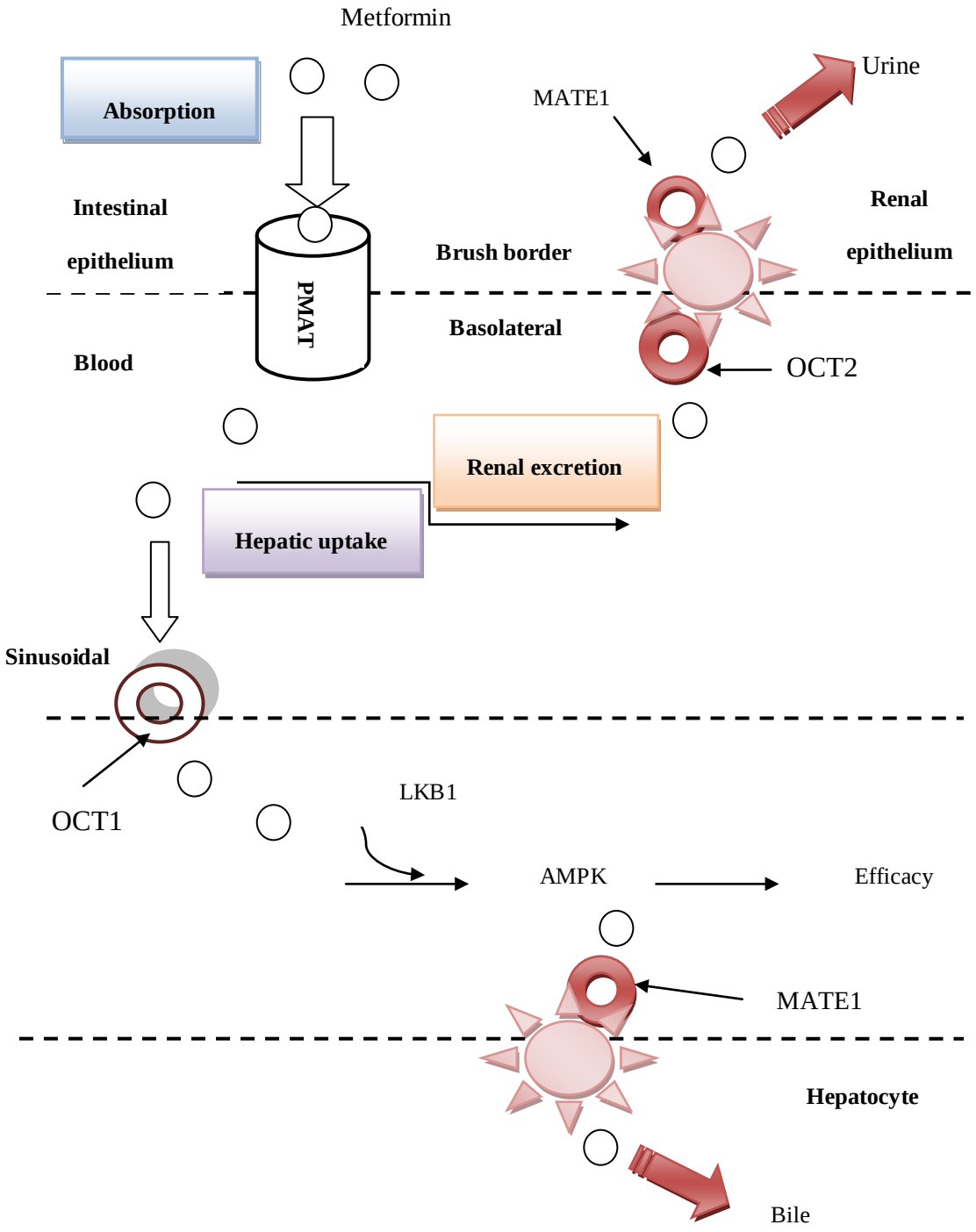


Figure 11: Diagram of candidate genes involved in metformin pharmacokinetics

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10 Appendix A

10.1 Patient information sheet

School of Pharmacy
Curtin University of Technology

INFORMATION SHEET
Factors Affecting Metformin Plasma Concentrations

We invite you to participate in a research study of the medication metformin (also known as Diabex, Glucophage, Diaformin, Glucomet, Glucohexal).This study is being conducted as part of requirement of PHD Degree programme of Sohaila Alawadhi. This study has been approved by the UAE Human Ethics Research Committee. If you decide to take part in this research study, it is important that you understand the purpose of the study and the procedures you will be asked to undergo. Please read this page which will provide you with information about the purpose of the study, potential benefits and precaution of the study.

Nature and the Purpose of the Study

We have asked you to participate in this study as you are currently using metformin for the treatment of diabetes. Metformin is a very effective drug in the treatment of diabetes, and is widely used. The manufacturers, however, usually do not recommend its use in patients with very poor kidney function. This is because if it is used in such cases, a very rare side effect called lactic acidosis may occur. The symptoms of lactic acidosis includes nausea, vomiting, lack of appetite, altered level of consciousness, difficulty breathing, abdominal pain and thirsty.However,many patients with only mild kidney problems can use this drug safely. Our research is intended to investigate factors that affect metformin plasma level and to find out what blood level of metformin is in the circulation of a range of different patients with different levels of kidney function.

Benefits

We hope that identifying factors that affects metformin plasma level will help prescribing doctors to be more confident to adjust metformin doses for particular patients, we also hope that finding out what blood level of Metformin is found in patients with different kidney function, that we can determine a dose of metformin which will be safe for other patients with the same level of kidney problems.

What the study will involve

As part of the study you will have a blood sample taken (10 mL) a procedures that are routinely done in the clinic. Following your consent, a fasting blood sample will be collected before you take any metformin and your breakfast for the day. This sample will be analysed for fasting blood glucose, HbA1c%, lactate level, metformin concentration, kidney function, and DNA analysis. This procedure may be uncomfortable but rarely results in any significant problem. Side effects that have been noted with drawing blood include feeling light-headed or faint, formation of a blood clot, and bruising. You will also be asked to give urine sample and saliva for DNA analysis. We will also collect other information from your doctor, your self and your medical record about your medical history, kidney function and other basic information such as age, height and weight.

Voluntary Participation and Withdrawal from Study

Your participation in this study is entirely voluntary. You may withdraw from the study at anytime without affecting your normal treatment.
If you have any complaints or concerns about the way in which the study is being conducted, you may contact the chairman of the medical centre or the hospital where your blood samples have been taken
For any questions on the study please contact:

Sohaila Al Awadhi.
Pharmacy Department
Fujairah Hospital,
Phone: 092242999
E-mail:s.alwadhi@student.curtin.edu.au

11 Appendix B

11.1 Consent form

SCHOOL OF PHARMACY

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CONSENT FORM
Factors Affecting Metformin Plasma Concentrations.

Patient's Name:..... Date of Birth: .....

1. I agree entirely voluntarily to take part in Factors Affecting Metformin Plasma Concentrations study. I am over 18 years of age.
2. I have been given a full explanation of the purpose of this study, of the procedures involved and of what will be expected of me. The doctor has explained the possible problems that might arise as a result of my participation in this study.
3. I agree to inform the supervising doctor of any unexpected or unusual symptoms I may experience as soon as possible.
4. I understand that I am entirely free to withdraw from the study at any time and that this withdrawal will not in any way affect my future standard or conventional treatment or medical management.
5. I understand that the information in my medical records is essential to evaluate the results of this study. I agree to the release of this information to the research staff and the clinical trial staff on the understanding that it will be treated confidentially
6. I understand that I will not be referred to by name in any report concerning this study. In turn, I cannot restrict in any way the use of the results that arise from this study.
7. I have been given and read a copy of this Consent Form and Information Sheet.

Signature by patient	Signature by Investigator
Signed.....	Signed:.....
Date:.....	Date:.....