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Evaluating the impact of type 2 diabetes mellitus on CYP450 metabolic activities: protocol for a case-control pharmacokinetic study

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ABSTRACT

Introduction: Diabetes affects more than 9% of the adult population worldwide. Patients with type 2 diabetes mellitus (T2DM) show variable responses to some drugs which may be due, in part, to variability in the functional activity of drug metabolizing enzymes including cytochromes P450 (CYP450s). CYP450 is a superfamily of enzymes responsible for xenobiotic metabolism. Knowledge must be gained on the impact of T2D and related inflammatory processes on drug metabolism and its consequences on drug response. The aim of this study is to characterize the activity of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3As in T2DM *vs* non-T2DM subjects following the administration of a cocktail of probe drug substrates.

Methods and analysis: This single-center clinical study proposes the first detailed characterization of T2DM impacts on major CYP450 drug metabolizing enzyme activities. We intend to recruit 42 controlled T2DM patients (A1C<7%), 42 uncontrolled T2DM patients (A1C≥7%) and 42 non-T2DM control subjects. The primary objective is to determine and compare major CYP450 activities in T2DM *vs* non-T2DM patients by dosing in plasma and urine probe drug substrates and metabolites following the oral administration of a drug cocktail: caffeine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3As). Secondary objectives will evaluate the influence of variables such as glycaemia, insulinemia, genetic polymorphisms and inflammation. The value of an endogenous biomarker of CYP3A activities is also evaluated. The first patient was recruited in May 2015 and patients will be enrolled up to completion of study groups.

Ethics and dissemination: Approval was obtained from the ethic review board of the CHUM research center (Montreal, Canada).

Trial registration number: NCT02291666

Strengths and limitations of this study:

- Study population including a control group of non-T2DM subjects and T2DM patients (T2DM patients will be stratified according to their glycemic control) will provide valuable information to better understand T2DM impacts on drug metabolism and to dissect the role of glycaemia *vs* inflammatory factors.
- The use of a validated cocktail of 7 probe-substrates along with highly precise, fast and robust quantification methods will permit the simultaneous determination of phenotypes for 7 major CYP450 isozymes involved in drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5.
- An exhaustive data collection and review of medical and pharmacological records for all participants will permit evaluation of covariables and investigation of mechanisms underlying expected modulation of CYP450 isozyme activities.
- The use of endogenous 4β-hydroxycholesterol plasma concentrations will be assessed as a putative biomarker of CYP3A activity.
- Study population is limited to individuals with a BMI≤35 which, although reducing confounding factors, prevents the extrapolation of our results to morbid obese individuals.

INTRODUCTION

The increasing prevalence of diabetes mellitus, a pathology which is associated with numerous comorbidities, is of major concern due to its important burden on society and health care systems.[1,2] In 2014, diabetes was estimated to affect more than 9% of the worldwide adult population.[3] Type 2 diabetes mellitus (T2DM), the most common form of diabetes, is characterized by insulin resistance, an incapacity of the body to respond adequately to insulin action and by beta cell dysfunction, an inability to respond to a glucose challenge by an appropriate insulin release. The resulting elevated blood glucose levels can lead to the development of costly and serious complications and comorbidities such as neuropathy, nephropathy, retinopathy, heart disease and an elevated incidence of stroke.[4-6] In 2013, diabetes mellitus was the 7th leading cause of death in the United States.[7]

Prevention and treatment of T2DM and its complications and comorbidities often require the use of multiple drugs to address these issues. Polypharmacy, *per se*, increases the risk of multi-drug interactions leading to adverse drug reactions (ADRs) which account for about 6.5% of all hospital admissions.[8] Furthermore, the risk of ADRs and treatment failure appears to be increased in patients with T2DM. Indeed, it has been previously reported that patients with T2DM tend to show highly variable responses to different drugs; while some patients are resistant to some drugs, some are more sensitive to other drugs (*e.g.* warfarin, clopidogrel, cyclosporine, tacrolimus, and antihypertensive agents).[9-14]

Cytochrome P450 (CYP450) is a superfamily of enzymes responsible for drug metabolism that represents a major source of variability in drug pharmacokinetics and response. CYP450 metabolizing activities can be regulated by many intrinsic and extrinsic factors such as genetics and environment. For instance, pro-inflammatory cytokines have been shown to decrease

CYP3A and CYP2Cs activities.[15-17] Thereby, chronic inflammatory diseases such as T2DM can impact CYP450 isoenzymes and modulate the patients' response to drugs.[18] The impact of T2DM on drug metabolizing enzymes could be an important factor to consider clinically to improve treatment of T2DM patients by managing efficiently the use of multiple concomitant drugs to reduce ADRs or to optimize efficacy. To reach an optimized drug utilization, knowledge has to be gained on the influence of T2DM per se on drug metabolism.

Our hypothesis is that T2DM and related inflammatory processes alter activities of CYP450s involved in drug disposition which may explain variability in drug response. The specific aims are 1) to determine CYP450 phenotypes in patients with T2DM vs non-diabetic subjects following a single oral administration of a cocktail of CYP450 probe drugs, and 2) to compare CYP450 phenotypes according to the glycemic control.

METHODS AND ANALYSIS

This clinical study is an open-label explorative pharmacokinetic research investigating the effects of T2DM on major CYP450 activities, namely CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1 and 3As. Study protocol (Trial #14.066) was approved by the ethic review board of the CRCHUM (Centre de recherche du centre hospitalier de l'Université de Montréal, Montreal, Canada) and registered at the US National Institutes of Health website (http://www.clinicaltrial.gov; NCT02291666).

Study population

A total of 126 participants, gender-matched between groups, are to be recruited from the Diabetes Registry at the CRCHUM outpatient clinic to constitute three study groups:

A) 42 T2DM patients with good glycemic control defined here as an A1C $\leq$ 7%;

B) 42 T2DM patients with inadequate glycemic control defined here as an A1C > 7%; and,

C) 42 non-T2DM participants serving as control.

Eligibility criteria include participants ≥18 years old and with a body mass index (BMI) ≤35 kg/m^2. They have to be non-smoker for at least two months prior to study and to abstain from grapefruit juice within two weeks before drug administration. Exclusion criteria also include a review of all subjects' pharmacologic record to control for the use of CYP450 inhibitor or inducer drugs. Detailed eligibility criteria are presented in *Table 1*. Written informed consent is to be obtained from all participants prior to any initiation of study procedure.

Table 1. *Eligibility criteria*

INCLUSION CRITERIA	• Male and female (non-pregnant) aged 18 years and over • Glomerular filtration rate > 50 mL/min/1,73 m2 • Acceptable hepatic function (ALT and AST levels below three times the normal range) • BMI ≤ 35 kg/m2 • Non-smoker for at least 2 months
EXCLUSION CRITERIA	• Use of: ○ antibiotics, antivirals, antiretrovirals, monoamine oxidase inhibitors, immunosuppressants, interferons and antibodies ○ CYP450 inductors (*e.g.*; carbamazepine, phenobarbital, phenytoin, rifampicin, St-John's worth) ○ CYP450 inhibitors (e.g.; amiodarone, fluvoxamine, fluoxetine, verapamil, terbinafine). • Uncontrolled thyroid function • Presence of an important inflammatory condition • A diagnosed gastrointestinal pathology • Patients with an active cancer • Patients who underwent a transplant or a bariatric surgery

Primary endpoints

Our primary endpoint is to compare phenotypes for 7 major CYP450 drug metabolizing enzymes by calculating metabolic ratios in our study groups, *i.e*. T2DM (A1C≤7% and A1C >7%) and non-T2DM (control), by using well-characterized isoform selective orally administered probe-substrates:

a) a CYP450 probe drugs cocktail (CRCHUM-MT cocktail) comprising:

- Caffeine (Wake Ups® 100 mg, Adem); CYP1A2
- Bupropion (Bupropion SR® 100 mg, Sandoz); CYP2B6
- Tolbutamide (Orinase® 250 mg, AA pharma inc.); CYP2C9
- Omeprazole (APO-Omeprazole® 20 mg, Apotex); CYP2C19
- Dextromethorphan (Bronchophan Forte DM® 30 mg, Atlas Laboratories); CYP2D6
- Midazolam (Midazolam injection®, USP, 2 mg, Fresenius Kabi); CYP3As

b) Chlorzoxazone alone (Acetazone Forte® 250 mg, Rougier); CYP2E1

All participants will be studied at the Phase 1 Unit at the CRCHUM starting at 7h00 AM. Subsequent to cocktail administration, blood and urine samples will be collected at specified times (for 24 and 8 hours, respectively). All drug concentration-time data will be analyzed by standard non-compartmental methods using Kinetica® 5.1 (Thermo Kinetica, Thermo Fisher Scientific, USA). The area under the curve 0-8h (AUC_{0-8}) will be obtained by use of the mixed log-linear trapezoidal rule enabling for interpolation and using the limit of quantification (LOQ) to compute last AUC triangle when the 8h time point is below the limit of quantification (BLQ). Phenotypic indices of major CYP450 activities will be determined as previously validated using metabolic ratios (MR) of urinary or plasmatic concentrations.[19-24] Each of our probe drugs are

selective for a specific isoenzyme of the CYP450s and do not affect other isoenzyme activities. All probe-drugs will be administered at the same time as a cocktail (7h00 AM), except for chlorzoxazone which is administered separately in the evening of the experimental day with a urine sample collected overnight and blood sample collected the next morning.

Secondary endpoints

As a secondary endpoint, we will investigate the influence of different T2DM characteristics and covariates on the enzymatic activity of measured CYP450s activities. Principal variables investigated are:

- Insulin resistance markers: Insulin, blood glucose and glycated haemoglobin levels.
- Genetic polymorphisms associated with CYP450s under investigation which are known to modulate enzymatic activity.
- Pro-inflammatory cytokines: interferon-gamma (IFN-γ), interleukin 1-beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels.
- Covariates such as body mass index (BMI), age, co-medication and time since diagnosis.

Another exploratory end point will be to investigate the potential use of the plasmatic ratio cholesterol/4β-hydroxycholesterol as an endogenous biomarker of CYP3A activity. Indeed, recent data suggest that this plasmatic ratio could be used as an endogenous marker of CYP3A activity *in vivo*.[25,26]

Experimental protocol.

On experimental day, participants will be admitted to the clinical research unit of the CRCHUM at 7h00 AM after an overnight fast and will remain fasted until the 4h time point is collected. Serial blood samples will be drawn in 6 mL K2-EDTA vacutainers via indwelling venous catheters

immediately before (time 0) and at 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8 and 24h post administration of the CYP450 probe drugs cocktail for measurement of probe drugs and their metabolites. Blood samples will be kept on ice and centrifuged for 10 minutes at 4°C and 1,500g less than 15 minutes after collection. Plasma samples will then be stored in aliquots at -80°C for later analysis. Urine will be collected prior to probe-drug administration (baseline) and over an 8 hour period after the cocktail administration. Urine will constantly be kept on ice and after measurement of total urine volume, aliquots will be stored at -20°C until analysis. Patients will be discharged after the 8h urine and plasma sample collection. As an outpatient, on the same night, subjects will be asked to take a chlorzoxazone oral dose prior to bedtime and collect all their urine for a minimal period of 8 h after drug administration. This urine collection will be returned on the next morning to the research center. After measurement of total urine volume, aliquots will be stored at -20°C for later analysis.

Two blood samples collected in SST II vials prior to dosing will be sent to the CHUM's biochemistry lab for insulin and glucose measurements. Furthermore a blood sample will be collected in 6 mL K2-EDTA vacutainers prior to dosing for the measurement of inflammatory markers and plasmatic ratio cholesterol/4β-hydroxycholesterol.

Sample size calculations and statistical analyses

Sample size was calculated for a significant difference of 30% between groups, with a power of 80% and an alpha value of 0.05. The required sample size (n=38 subjects per group) was calculated to meet the primary objective considering the reported variances of CYP3A activities found in the literature (greatest changes have been observed with CYP3A4). Considering an anticipated drop-out rate of 10%, 42 subjects per group will be recruited for a total of 126 participants.

Statistical analyses will be used to answer primary objective which consists of determining if T2DM disease modulates CYP450 activities. A stepwise statistical analysis will be performed: 1) each phenotypic probe will be compared between T2DM (including controlled and uncontrolled glycemia) *vs* non-T2DM individuals, and 2) T2DM group will be sub-categorized based on A1C ≤7% *vs* A1C >7% *vs* non-T2DM group. This aim will be tested for significance by performing an analysis of variance (ANOVA) with a post-hoc.

In a secondary analysis, the influence of covariables will be tested using multiple regression analyses. Different variables will be considered in the models: CYP450 genetic polymorphisms, BMI, insulin resistance markers, pro-inflammatory cytokines, age and time since diagnosis. Potential confounding variables will be defined according to: 1) variables associated with a specific CYP450 that have been reported in literature and 2) variables with a correlation coefficient above 0.2 or below -0.2 in T2D groups *vs* non-diabetic group.

Finally, plasmatic concentration ratios of the endogenous markers (cholesterol/4β-hydroxycholesterol) will be compared between the 3 study groups and correlation will be established with activity observed for the probe-substrate, midazolam, administered in the CRCHUM-MT cocktail.

Data management

In this project, confidentiality of collected data will be preserved by using an attributed identification number to all participants. Subjects' identities and numbers will only be accessible to designated investigators and all data will be treated in a non-nominal manner. All documentation related to study will be kept in locked drawers. DNA samples will solely be used for genetic analyses associated with CYP450s or other enzymes/transporters of drugs relevant for T2D, associated co-morbidities and drug response variability. It will not be used to serve any

other purpose than this present study. DNA will be kept for a maximum period of 10 years before complete destruction (hydrochloric acid).

Ethics and dissemination

This study will be carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. All participants will be informed of the objectives, risks and inconveniences of the trial and be provided with written information on the study, contact details of the principal investigators as well as copies of their signed consent/assent forms. Subjects will be clearly informed of their right to withdraw from the study at any time during the process, and this without consequences for their future care. Immediate intervention or treatment is available in case of an acute adverse event, *e.g*. anaphylactic reaction.

A manuscript presenting and discussing all results of the primary objective as well as the covariables analyses will be submitted and published in a peer-reviewed journal. A second distinct manuscript addressing the use of an endogenous marker of CYP3A activity will be submitted in a peer-reviewed journal as well.

Upon completion of the trial and publication of the results, a request for experimental data can be submitted to principal investigator at the CRCHUM in Montreal, Canada.

DISCUSSION

Many drugs in addition to antidiabetic medications are prescribed to T2D patients to treat their numerous co-morbidities. However, few information is available on the efficacy and dosing of these drugs in this sub-population of patients. Indeed, during drug development processes,

pharmacokinetic and pharmacodynamic studies of new medications for various indications are rarely tested in a population of patients with T2D. Available data for this group of patients are often derived from secondary analyses of clinical trials. This research protocol will generate valuable results enabling a better understanding of factors affecting drug metabolism *in vivo*. New knowledge gained on metabolic capacity in patients with T2D will improve our understanding of the underlying mechanisms responsible for the high interindividual variation in drug response observed in this group of patients. Thereby, it will provide new information to help clinicians improving use of drugs to reduce therapeutic failure and toxicity entailed by sub-optimal or inappropriate use of pharmaceutical treatments.

Authors' contributions VM conceived the study hypothesis. JLC, SD, SG, VM and JT contributed to the feasibility of the study. VM designed and wrote the trial protocol. JT and JLC provided revision of the proposed protocol. SG wrote the first draft of this manuscript. VM, JT and JLC revised and edited this study protocol article. All authors approved the final version of this manuscript**.**

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Competing interests None.

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