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Methods and Interventions
with Precision Medicine
Approaches

A Protocol for the Study of Polymorphisms and Response to Metformin in Patients with Type 2 Diabetes in Trinidad

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Background: Metformin is the drug of first choice in people newly diagnosed with type 2 diabetes. Most patients respond to metformin monotherapy, but many others remain uncontrolled even at maximal doses. Although non-adherence is a major contributor to non-response, genetic polymorphisms of organic cation transporters play an important role in clinical response. We hypothesize that genetic variants are partly responsible for non-response.

Objective: This study aims to determine the allele and genotype frequencies of three single nucleotide polymorphisms (SNPs; ATM rs11212617, SLC22A1 rs594709 and SLC47A1 rs2289669) most commonly associated with failure to respond to metformin.

Setting: Ten primary health care facilities in the North Central Regional Health Authority region of Trinidad.

Patients: The study population will include 216 patients with diabetes adherent to metformin monotherapy for at least three months.

Methods: Following a 12-hour overnight fast, blood samples will be taken to measure fasting insulin and HbA1c. DNA would be isolated and PCR will be used to determine the allele and genotype frequencies of these three SNPs in adherent diabetic patients. DNA samples will be stored for future sequencing of these three genes to determine whether other, possibly novel, mutations are associated with poor metformin response in Trinidad.

Clinical Significance: This study will highlight the prevalence of these polymorphisms in our population. Should an association be found between the polymorphisms tested and glycemic control in adherent patients on metformin monotherapy, this will have implications for further research on medication initiation in newly diagnosed patients with diabetes in Trinidad. *Ethn Dis.* 2020; 30(Suppl 1):211-216; doi:10.18865/ed.30.S1.211

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Introduction

Diabetes mellitus is a major global public health problem, with significant burden on national health care systems. The burden of the disease in the Caribbean is significantly higher than in North America and Europe, as highlighted in a review of studies between 1972 and 2013.[1] Researchers concluded that there is a "… higher prevalence of DM (diabetes mellitus) among Caribbean Blacks compared with West African Blacks and Caucasians but lower when compared with South Asian origin groups." They also concluded that morbidity from diabetes-related complications was highest in persons with low socioeconomic status. With a diabetes prevalence of 12.7%, Trinidad and Tobago is ranked among the top ten (per capita) in the world.[2]

Observational studies over the last decade at public primary health care facilities in Trinidad showed that many patients were uncontrolled, even those of polypharmacy. A small study by Bhagirathee and Maharaj,[3] with 253 patients at two public primary health care facilities, showed that <30% of these patients had HbA1c <7%. Slightly better results were found in another small study by Morren and colleagues, with 255 patients at five public primary health care facilities; this study showed that 56.6% of these patients had an HbA1c < 6.5%.[4] Another smaller study, with 132 patients, showed that more than half of patients had HbA1c levels >7.0%.[5]

In Trinidad and Tobago, a major challenge is the inherent inadequacies at the point-of-care in the public health care sector. The inability to consistently monitor blood glucose levels (HbA1c) contribute to treatment failure and Dhanoo and colleagues[6] found that "… only 20% had

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an HbA1c reported in the past year." Another report by Roopnarinesingh et al[7] concluded that "… health care professionals in our study reported significant barriers to achieving optimal diabetes care in Trinidad and Tobago."

Metformin is a first-line drug in patients newly diagnosed with diabetes. It decreases hepatic gluconeogenesis and increases both glucose utilization and insulin sensitivity in tissues.[8,9] Although most patients respond favorably to monotherapy, many are unresponsive and remain above target levels even at maximal doses. Some studies have found that non-response to metformin monotherapy could be as high as 35%.[10-12] A six-month follow-up study in newly diagnosed patients with diabetes started on metformin monotherapy found that about one-third had HbA1c >7%.[13] A recent systematic review showed that there is limited evidence regarding factors associated with clinical response to metformin.[14] Although no studies in Trinidad have specifically highlighted treatment failure on metformin monotherapy, the high rates of polypharmacy in our patients with diabetes point indirectly to failed metformin monotherapy.

Although most patients respond favorably to monotherapy, many are unresponsive and remain above target levels even at maximal doses.

Many factors contribute to non-response to treatment, including non-adherence to drug therapy and lifestyle changes. Additionally, polymorphisms in genes related to a drug's pharmacokinetics and pharmacodynamics also play an important role. Several genes are critical in the clinical response to metformin, such as those related to glucose transport and regulation. Specialized organic cation transporters (OCT1, OCT2 and OCT3) are critical for the absorption, distribution and excretion of metformin. OCT and other transporters (and their associated genes) involved in the pharmacokinetics of metformin are presented in Table 1. Metformin is excreted unchanged; therefore, these transporters must be functional to maintain therapeutic plasma levels.[15] A recent systematic review and meta-analysis of 45 studies in 10 ethnic groups identified 34 polymorphisms for OCT1.[16] Polymorphisms in these transporters alter the body's ability to achieve and maintain plasma levels sufficient to illicit a therapeutic response.

Several studies have identified single nucleotide polymorphisms (SNPs) associated with genes encoding for organic cation transporters (SLC22A1, SLC22A2, SLC22A3,) and other drug transporters (SLC47A1 and SLC29A4), which affect metformin pharmacokinetics.[17-19] Polymorphisms in non-transporter genes, such as the ATM gene (associated with DNA repair and cell cycle control), also affect the clinical response to metformin.[20-22]

Relevance of the Study

To our knowledge, this would be first study of its kind in the English-speaking Caribbean, and it would be clinically relevant to determine the frequencies of metformin-associated SNPs in a population where more than 95% have either African or Asian Indian or African/Asian Indian mixed ancestry. This ethnic composition is significantly different than those in North America, Europe and Asia and we would expect differences in SNP frequencies. However, we would expect similar correlation between presence of SNP and clinical response to metformin. It is hoped that these results will highlight the need for a more patient-centric or precision medicine approach[23] to care for patients with diabetes in Trinidad, where alternative treatment could be initiated early to prevent long-term complications.

There is a large community of patients with diabetes in Trinidad, with many being poorly controlled. The results of our study will be communicated to physicians at public primary health care institutions and a final report submitted to the regional health authority and the Ministry of Health. It is hoped that this information would be used by decision-makers at these institutions to guide health care policy. It is envisioned that patient-centric decisions would be made regarding alternative therapeutic options for patients who remain unresponsive to metformin monotherapy early in their treatment to prevent long-term complications.

The Diabetes Association of Trinidad and Tobago (DATT) is a patient-driven non-governmental

Table 1. Transporters involved in metformin pharmacokinetics

Site	Transporter	Gene
Gut (luminal)	Plasma monoamine transporter (PMAT)	SLC29A4
Gut (luminal)	Organic cation transporter (OCT3)	SCL22A3
Gut (basolateral)	OCT1	SLC22A1a
Liver	OCT1	SLC22A1a
	OCT3	SLC22A3
	Multidrug and toxin and extrusion (MATE1)	SLC47A1a
Bile/kidney (luminal)	MATE1	SLC47A1a
Kidney (basolateral)	OCT2	SLC22A2

a. Genes of interest in this study.

organization with the mission to educate patients on their well-being using education-based strategies. We envision that the general outcome of the study may be useful to DATT and will allow patients to ask their physicians questions about their clinical response to metformin therapy and open the dialogue for genetic testing and alternative patient-centered antidiabetic therapy.

Study Objectives

The primary aim of this study is to determine the allele and genotype frequencies of three of the most important SNPs associated with metformin response (ATM rs1121617; SLC22A1 rs594709 and SLC47A1 rs2289669) in drug-adherent patients with type 2 diabetes on metformin monotherapy. Our null hypothesis is that mean HbA1c in persons with polymorphisms would be equal to the mean HbA1c in persons without polymorphisms. We assume that these polymorphisms are random and occur independently of other variables predictive of HbA1c, such as lifestyle, obesity and genes associated with diabetes. Metformin has a maximal effect to decrease HbA1c by 1.5% and a similar difference in HbA1c between the two groups (persons with and without polymorphisms) would be considered clinically significant.

Methods

Study Design and Setting

The study will be cross-sectional in design and convenient sampling will be used to include 216 patients with type 2 diabetes and adherent with metformin monotherapy. Patients will be recruited from 10 chronic disease clinics at public primary health care facilities throughout the North Central Regional Health Authority (NCRHA) region in Trinidad. At these selected clinics patients will be approached in the waiting rooms to determine their eligibility. The study has IRB approval from the University of the West Indies St. Augustine Campus and the NCRHA Ethics Committees. All procedures will be in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent will be obtained from all participants included in the study.

Participants and Selection

Inclusion Criteria

Diagnosed with type 2 diabetes, adherent with metformin monotherapy for at least six months, aged > 18 years, and nationals of Trinidad and Tobago.

Exclusion Criteria

Type 1 DM, gestational diabetes, insulin or other oral anti-diabetic agents, anemia (Hb<12.0g/dL for women, Hb<13.5g/dL for men), eGFR<45ml/min/1.72m^2, liver cirrhosis, other endocrine disorders, malignancies, systemic inflammatory diseases, heart failure, concomitant glucocorticoid, aged <18 years.

Selection

Participants will be informed of the nature of the study and asked to voluntarily participate after satisfying the inclusion criteria and before signing their informed consent. Race, ethnicity and sex of recruited partici-

pants will reflect the demographic of selected clinics. Demographic data will be collected via an interviewer-administered questionnaire. Blood pressure, weight and height will be measured and body mass index (BMI) will be calculated. Patients' notes will be consulted to record history of FBG and HbA1c readings, current metformin dose, changes in metformin dose and duration of therapy.

Within one week of enrollment, recruited patients will return to have blood samples collected after a 12-hour overnight fast to measure fasting blood glucose, fasting insulin and HbA1c. DNA will also be extracted from whole blood using standard methodology to determine the presence of the three SNPs of interest. DNA will be stored for future sequencing studies to determine whether other mutations exist related to non-response to metformin for these three genes of interest.

Sample Size

According to published data, the frequencies of the minor alleles for SLC47A1 rs2289669, ATM rs11212617 and SLC22A1 rs594709 are 47.19%, 36.51% and 26.78% respectively.[24]

The sample size was calculated using findings from a recent study which showed the difference in allele frequency in responders vs non-responders as 44% to 27%.[25] The comparison of proportions calculator can be found at https://select-statistics.co.uk/calculators/sample-size-calculator-two-proportions/; it gives a sample of 108 in each group to determine this difference. We will, therefore, need 108 responders and 108 non-responders on metformin for the total sample size of 216.

PCR Methodology

DNA will be isolated using the QIAamp blood mini kit (Qiagen, CA, USA) that allows for rapid purification of at least 4 µg of total DNA from 200µL whole blood. Regions of interest will be amplified using 1 µg of DNA and the HotStarTaq Master Mix Kit (Qiagen, CA, USA) with appropriate primers (Biosynthesis Inc; TX, USA). We will initiate PCR for all genes of interest using the general manufacturer's instructions with an initial activation step at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds to 1 minute, annealing at 50–68°C (approximately 5°C below Tm of primer) for 30 seconds to 1 minute and extension at 72°C for 1 minutes, with a final extension at 72°C for 10 minutes. Amplified products will be digested with the appropriate restriction enzyme (New England Biolabs; MA, USA). Digest products will be electrophoresed on agarose gels alongside a DNA ladder to determine the sizes of the PCR product and digest fragments.

The primers for ATM gene (ataxia telangiectasia mutated) SNP rs11212617 are Forward - 5'-TGGGTTGCTTGTGGATAACATATAGTTGG- 3' and Reverse - 5'- GAGAAGGCAGTAAAGTGAAGGATACAGAG- 3'. The PCR product size would be 209bp; and restriction digest with *HpyCH4III* would produce 153bp and 56bp fragments (24). The primers for OCT1 (organic cation transporter1) SNP SLC22A1* rs594709 are: Forward - 5′GGCGCTTCCCACACTCAT3′ and Reverse: 5′GAGGAAAGCTCCACATGTAACC3′. The PCR product size would be 50bp; and restriction digest with *HaeII* would produce two 25bp fragments. The primers for MATE1 (Multidrug and toxin and extrusion) SNP SLC47A1* rs2289669 are Forward - 5'-TCAGTTTCCACAGTAGCGTCG-3'and Reverse - 5'-GACACTGGAAGCCACACTGAA-3'. The PCR product size will be 211bp; and restriction digest with *TaqI* will be product 21bp and 190 bp fragments.[26]

Statistical Analysis

Statistical analyses will be performed using SPSS, Version 21 (IBM, USA). Chi-squared analyses or Fisher's Exact tests will be used to detect any significant differences in the frequency of the SNPs of interest between responders and non-responders and between ethnic groups. Continuous variables would be reported as means and standard errors and categorical variables as proportions. Regression models, adjusted and unadjusted, will be used to determine continuous and categorical predictors of uncontrolled diabetes (HbA1c >7%). Unless otherwise stated, P=.05 will be considered significant.

Ethical Considerations and Protection of Human Subjects

The risk of participating in the study is minimal. There will be minimal risk of breach of patient confidentiality as questionnaires will be anonymized with the use of unique protocol numbers without identifiers that could be traced back to the patient. The research assistant/phlebotomist will request the use of a private room to allow for privacy at the health care facility. Most of the inter-

view questions will be health-related and not of a sensitive, private nature. However, patients will be informed that they may refuse to answer questions that make them uncomfortable and could withdraw from the study without penalty or change in the standard of care at the facility.

The completed anonymized questionnaires will be kept in a locked cabinet at the principal investigator's (PI) office. However, contact details will be kept on a separate sheet that will match the unique code on the data collection form. This information sheet will be kept in a separate location from the data collection forms in the secured PI's office and will be kept for up to three years after the completion of data collection. All data transcribed to the electronic database would be coded (without identifiers) and stored on a secure password-protected computer in the PI's office. Only members of the research team will have access to these and other confidential information.

The research assistant/phlebotomist will be trained and experienced in blood drawing and there should be minimal risk in this activity. The research sites for blood-drawing will be public health care facilities, so in the event of any adverse reactions emergency medical facilities will be at hand.

Blood samples will be collected, processed and the plasma stored at -80°C for batch analysis of glucose and insulin. Whole blood will be stored for DNA isolation and subsequent molecular biology and the detection of the three SNPs of interest. Bio-banking will be done for further study, which may include sequencing of the genes of interest, particularly SLC22A1 and SLC47A1, to determine whether yet unknown SNPs in these genes may also be predicting metformin response in the Trinidadian population. Only members of the research team will have access to these stored blood and DNA samples. All stored plasma and blood samples will be anonymized.

All included patients with diabetes will have been previously diagnosed and treated at the selected primary public health care facilities. The study parameters will be restricted to the measurement of fasting blood glucose, fasting insulin, HbA1c measurements and DNA extraction to determine incidence of the three SNPs associated with metformin response. Therefore, in this group we do not anticipate any incidental findings.

Study Limitations

Regional and international clinical guidelines are used in the management of patients with diabetes in the primary health care setting in Trinidad, but there are several challenges. A major challenge is the inconsistent and sparse use of HbA1c to assess control, largely due to economic constraints. Although fasting blood glucose is measured at each clinic visit as a surrogate, it is not sufficient to adequately determine disease control.

Metformin decreases HbA1c by a maximal of 1.5%, and we assume that in the challenged Trinidad setting, it is given as monotherapy for patients who are expected to reach the target of 7% (presumably based on initial FBS readings at the clinic). However, these are mere assumptions in the absence of baseline HbA1c readings. Therefore, it would be difficult to determine whether metformin monotherapy was effective in maximally reducing HbA1c levels in our sample of drug-adherent patients.

Additionally, lifestyle modifications also reduce HbA1c values, so it would be difficult to attribute HbA1c reduction solely to metformin. It is suspected that some patients with HbA1c >8.5% on diagnosis (although not measured) will be prescribed metformin monotherapy and lifestyle modifications with the expectation that both interventions would bring blood glucose levels to target (less than 7%).

We will attempt to recruit only patients who are adherent to metformin treatment to reduce the direct causal effect of non-adherence on clinical response. As SNPs are non-modifiable genetic risk factors, it would be reasonable to assume that their presence or absence would precede the outcome of interest, ie, glycemic response to metformin. But, in the absence of confirmatory associations with these three SNPs, there would be unknown confounding factors (including other SNPs) that may predict clinical response to metformin.

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Conflict of Interest

No conflicts of interest to report.

Author Contributions

Research concept and design: Clement, Singh, Motilal, Maharaj, Nunez-Smith; Acquisition of data: Singh, Nunez-Smith; Data analysis and interpretation: Nunez-Smith; Manuscript draft: Clement, Singh, Motilal, Maharaj; Statistical expertise: Clement, Singh, Nunez-Smith; Acquisition of funding: Clement, Singh, Motilal, Maharaj, Nunez-

Smith; Administrative: Clement, Singh, Motilal, Nunez-Smith; Supervision: Clement, Singh, Nunez-Smith

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