Molecular Genetics and Metabolism Reports 1 (2014) 158–169

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Differential hexosamine biosynthetic pathway gene expression with type 2 diabetes

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ARTICLE INFO

Article history:
Received 19 March 2014
Accepted 19 March 2014
Available online 17 April 2014

Keywords:
Hexosamine biosynthetic pathway
Fasting blood glucose
Pre- and overt diabetes
Leukocytes
Gene expression

ABSTRACT

The hexosamine biosynthetic pathway (HBP) culminates in the attachment of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) onto serine/threonine residues of target proteins. The HBP is regulated by several modulators, i.e. *O*-linked β-N-acetylglucosaminyl transferase (OGT) and β-N-acetylglucosaminidase (OGA) catalyze the addition and removal of *O*-GlcNAc moieties, respectively; while flux is controlled by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFPT), transcribed by two genes, *GFPT1* and *GFPT2*. Since increased HBP flux is glucose-responsive and linked to insulin resistance/type 2 diabetes onset, we hypothesized that diabetic individuals exhibit differential expression of HBP regulatory genes. Volunteers (n = 60; n = 20 Mixed Ancestry, n = 40 Caucasian) were recruited from Stellenbosch and Paarl (Western Cape, South Africa) and classified as control, pre- or diabetic according to fasting plasma glucose and HbA1c levels, respectively. RNA was purified from leukocytes isolated from collected blood samples and *OGT*, *OGA*, *GFPT1* and *GFPT2* expressions determined by quantitative real-time PCR. The data reveal lower *OGA* expression in diabetic individuals ($P < 0.01$), while pre- and diabetic subjects displayed attenuated *OGT* expression vs. controls ($P < 0.01$ and $P < 0.001$, respectively). Moreover, *GFPT2* expression decreased in pre- and diabetic Caucasians vs. controls ($P < 0.05$ and $P < 0.01$, respectively). We also found ethnic differences, i.e. Mixed Ancestry individuals exhibited a 2.4-fold increase in *GFPT2* expression vs. Caucasians, despite diagnosis ($P < 0.01$). Gene expression of HBP regulators differs between diabetic and non-diabetic individuals, together with distinct ethnic-specific gene profiles. Thus differential HBP gene regulation

Abbreviations: HBP, hexosamine biosynthetic pathway; *O*-GlcNAc, *O*-linked β-*N*-acetylglucosamine; OGT, *O*-linked β-N-acetylglucosaminyl transferase; OGA, β-N-acetylglucosaminidase; GFPT, glutamine:fructose-6-phosphate amidotransferase.

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http://dx.doi.org/10.1016/j.ymgmr.2014.03.003

may offer diagnostic utility and provide candidate susceptibility genes for different ethnic groupings.

1. Introduction

The hexosamine biosynthetic pathway (HBP) culminates in the attachment of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) onto target proteins. *O*-GlcNAcylation is a dynamic and reversible post-translational modification where *O*-GlcNAc moieties are attached to the hydroxyl groups of serine/threonine residues of target cytoplasmic and nuclear proteins [1]. The HBP usually functions as a nutrient sensor under normal physiological conditions and plays a fundamental role in modulating intracellular signaling and gene transcription [2,3]. Crosstalk between phosphorylation and *O*-GlcNAcylation is abundant since there is a competition for similar binding sites on target proteins [3,4]. However, while phosphorylation is controlled by a multitude of kinases and phosphatases, *O*-GlcNAcylation is regulated by only two known enzymes, *O*-linked β-N-acetylglucosaminyl transferase (OGT) and β-N-acetylglucosaminidase (OGA) that catalyze the addition and removal of *O*-GlcNAc moieties, respectively [5,6]. Regulation of HBP flux is also controlled by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFPT; or sometimes referred to as GFAT) that catalyzes the reaction of glucosamine with fructose-6-phosphate to form glucosamine-6-phosphate [7,8]. There are two GFPT isoforms – *GFPT1* and *GFPT2* – that are transcribed by separate genes with differing tissue distributions [9] raising the possibility of distinct roles.

It is well established that enhanced HBP activation – by augmented intracellular oxidative stress – is robustly linked to hyperglycemia and insulin resistance, two hallmarks of type 2 diabetes [10,11]. In support, recent clinical studies linked greater HBP flux and subsequent upregulation of global *O*-GlcNAcylation to the onset of type 2 diabetes [12,13]. Furthermore, a single mutation in the *OGA* gene (also referred to as *MGEA5*) of a Mexican-American population resulted in the early termination of *OGA* translation leading to decreased expression and increased susceptibility to diabetes [14]. OGT dysregulation is also implicated in the onset of insulin resistance. For example, hepatic OGT overexpression impairs the expression of insulin-responsive genes and causes insulin resistance and dyslipidemia [15]. In support, OGT can trigger hepatic gluconeogenesis thus confirming the importance of the HBP in the development of glucose intolerance [16]. Since *O*-GlcNAcylation of target proteins occurs in a glucose-responsive manner [12,13] and persistently higher HBP flux is strongly associated with the development of insulin resistance/type 2 diabetes [11,17], diabetic individuals are likely to display differential HBP gene expression. However, there are very limited clinical studies that investigated gene expression of HBP regulators with the development of type 2 diabetes, and to our knowledge none that examined *OGT* and *OGA* mRNA levels within this context. As we previously found greater leukocyte *O*-GlcNAcylation with the onset of type 2 diabetes and since all the HBP regulatory genes are known to be expressed in white blood cells [13,18–20], we here hypothesized that the *OGA*, *OGT*, *GFPT1*, and *GFPT2* genes are differentially expressed in leukocytes isolated from pre-diabetic and diabetic individuals compared to matched controls. Thus the key objective of this study is to focus on gene expression analysis of various HBP modulators in order to determine whether any variability can be exploited to assist with type 2 diabetes detection.

2. Materials and methods

2.1. Participant recruitment

Study participants (n = 60; n = 20 Mixed Ancestry, n = 40 Caucasian) were recruited from two neighboring metropolitan regions, namely Stellenbosch and Paarl (Western Cape, South Africa). All recruited participants were personally informed about the study and were requested to sign a written consent form detailing the study aims and procedures. This study was approved by the Committee for Human Research at Stellenbosch University (reference number: S12/03/074) and was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, the Medical Research

Council Ethical Guidelines for Research in South Africa, and the South African Guidelines for Good Clinical Practice.

2.2. Characterization of participants

Participants were assigned to one of three groups (control, pre-diabetes, or diabetes) according to their fasting blood glucose and HbA1c levels, respectively. The participants for this study were distinct from our previously published study [13], i.e. they were newly recruited. Subject recruits were grouped based on the American Diabetes Association (ADA) guidelines stipulating: fasting plasma glucose levels <5.6 mmol/L (controls); 5.6–6.9 mmol/L (pre-diabetes); and >7 mmol/L (type 2 diabetes) [2]. The ADA also recognizes the use of HbA1c and specifies a range of <5.7% for controls, 5.7–6.5% for pre-diabetes, and >6.5% for diabetes [21]. The number of samples differs between groupings because of technical difficulties in measuring HbA1c levels, and due to methodological error certain samples were excluded from statistical analyses. Clinical information of recruited subjects is summarized in Table 1 (based on ADA fasting plasma glucose criteria) and was obtained by requesting volunteers to complete a detailed questionnaire including information regarding age, gender and ethnicity.

2.3. Sampling

Whole blood samples were collected from participants (under fasting conditions) through venipuncture into specified tubes provided by PathCare, Stellenbosch (Western Cape, South Africa). Clinical measurements included: fasting blood glucose (4-mL potassium oxalate/sodium fluoride tube), insulin (5-mL serum separating tube), and HbA1c (4-mL EDTA tube). For molecular studies, collected blood samples (4 mL EDTA tube) were transported and stored on ice. Leukocytes were subsequently isolated as is routinely performed in our laboratory [13] and total RNA was extracted within 3 h of sample collection.

2.4. RNA extraction and precipitation

Total RNA was extracted using the QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) together with the RNase-Free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's protocol. We determined the purity and concentrations of RNA samples by spectrophotometry (NanoDrop® ND-1000 spectrophotometer V3.0.1, NanoDrop Technologies, Wilmington DE). Samples were stored at −80 °C (in 3 volumes of 100% ethanol) until all 60 samples had been collected.

RNA precipitation was performed using a standard sodium acetate protocol. Samples were removed from −80 °C, allowed to thaw on ice, whereafter sodium acetate was directly added to a final concentration of 0.3 M. Afterwards samples were immediately placed at −80 °C for 20 min. The samples were subsequently centrifuged at 12, 000 *g* at 4 °C for 15 min, and the supernatant thereafter discarded. Subsequently, 500 μL of 75% ethanol was added and samples again centrifuged at 12, 000 *g* at 4 °C for 15 min. After discarding the supernatant, RNA pellets were air-dried and dissolved in RNase-free water (Qiagen, Hilden, Germany) to a final concentration of 200 ng/μL.

Table 1
Summary of patient details (based on ADA fasting plasma glucose criteria).

	Controls	Pre-diabetic	Diabetic
Sample size	30	14	16
Age (years)[a]	50 ± 2.3	55.4 ± 4	54.8 ± 2.4
Gender (M/F)	6/24	7/7	6/10
Ethnicity (MA/C)	7/23	6/8	7/9
FPG (mmol/L)[a]	5 ± 0.07	6.3 ± 0.1	10 ± 0.8
HbA1c (%)[a]	5.6 ± 0.08	6.6 ± 0.2	8.7 ± 0.5
Insulin (mIU/L)[a]	9.1 ± 1.1	14.3 ± 3.6	32.3 ± 11.5[b]

Abbreviations: C — Caucasian; FPG; fasting plasma glucose; F — female; M — male; MA — Mixed Ancestry.
[a] Values are expressed as mean ± SEM.
[b] The large SEM for insulin in the diabetic group is due to exogenous insulin treatment.

2.4.1. cDNA synthesis and quantitative real-time PCR

First strand cDNA synthesis was performed using the GoScript™ Reverse Transcription System (Promega, Fitchburg WI) according to the manufacturer's guidelines. A total of 2.5 μL of RNA (200 ng/μL) was transcribed. Synthesized cDNA samples were amplified using SYBR® Green I dye (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocols. All experiments (from cDNA synthesis to real-time PCR) were performed on two separate occasions, in triplicate, on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Each reaction contained a 10 μL reaction mixture consisting of 2 × LightCycler® 480 SYBR Green I Master, 0.5 pmol of each primer and 50 ng of the corresponding cDNA sample (refer to Table 2 for primer sequences for *OGT*, *OGA*, *GFPT1* and *GFPT2*). PCR cycles were as follows: pre-incubation for 10 min at 95 °C; amplification for 15 s at the annealing temperature (T_A) (refer to Table 2); and elongation for 20 s at 72 °C. Standard curves were generated using pooled samples. To measure gene expression, relative concentrations were used after being normalized to the relative concentration of the β-glucuronidase (*GUSB*) gene (refer to Table 2 for primer sequences). We also employed β-actin (*ACTB*) and ribosomal protein L37a (*RPL37A*) as additional reference genes that allowed for further confirmation of findings.

2.5. Statistical analysis

One-way ANOVA (GraphPad Software, Inc., San Diego CA) followed by a Bonferroni *post hoc* test [comparing all groups and comparing selected groups (denoted with capped lines between bars)] was used to calculate differences in *OGA*, *OGT*, *GFPT1* and *GFPT2* expressions between groups. Relative concentrations from experimental rounds 1 and 2 were averaged. All values are expressed as the mean ± SEM (values are shown as a percentage relative to that of the controls). P values < 0.05 were accepted as significant.

3. Results

3.1. Reduced OGA gene expression in diabetic individuals

We initially evaluated *OGA* mRNA expression levels in pre-diabetic and diabetic individuals compared to control subjects. When analyzing the data according to HbA1c levels, diabetic individuals displayed a 35.6 ± 6.3% decrease in *OGA* expression vs. control subjects (Fig. 1A). This difference was also statistically significant when compared to the pre-diabetic group. We confirmed these findings by normalizing the

Table 2
Quantitative real-time PCR conditions and primers.

Gene	Ensembl accession number	Primer	Sequence (5′–3′)	Cycle number	T_M (C)	T_A (C)	Product Size (bp)
ACTB	ENSG00000075624	F	ATT GCC GAC AGG ATG CAG AA	40	57.1	60	150
		R	GCT GAT CCA CAT CTG CTG GAA		57.4		
GFPT1[a]	ENSG00000198380	F	TGC TGT GCT GAG AGG CTA TGA TGT	40	60.2	65	358
		R	GTC CAG AAA TGC AAC ACC CAG CAT		60.2		
GFPT2[a]	ENSG00000131459	F	GGG CGA CAA GGC CGT GGA AT	40	63.1	60	107
		R	CAG CCA CTG CGG CGA TGT CA		63.1		
GUSB	ENSG00000169919	F	CTC ATT TGG AAT TTT GCC GAT T	40	57.3	55	80
		R	CCG AGT GAA GAT CCC CTT TTT A		59.9		
OGA[a]	ENSG00000198408	F	GCA GCA CCC TCT TTA AAT GCC ACA	40	60.2	60	368
		R	CCT GGC ACA AAC TGC TCC TTG TTT		60.3		
OGT[a]	ENSG00000147162	F	GCA ACG TGG CCG ACA GCA CA	40	63.7	62	117
		R	TGC AGT GTC TCT CAG CTG CCT CA		62.4		
RPL37A	ENSG00000145592	F	ATT GAA ATC AGC CAG CAC GC	40	56.6	60	203
		R	AGG AAC CAC AGT GCC AGA TCC		56.6		

Abbreviations: ACTB — β-actin; bp — base pairs; F — forward; GFPT1 — glutamine:fructose-6-phosphate amidotransferase 1; GFPT2 — glutamine:fructose-6-phosphate amidotransferase 2; GUSB — β-glucuronidase; OGA — O-GlcNAcase; OGT — O-GlcNAc transferase; R — reverse; RPL37A — ribosomal protein L37a; T_A — annealing temperature; T_M — melting temperature.

[a] Primers are gene-specific and not transcript-specific.

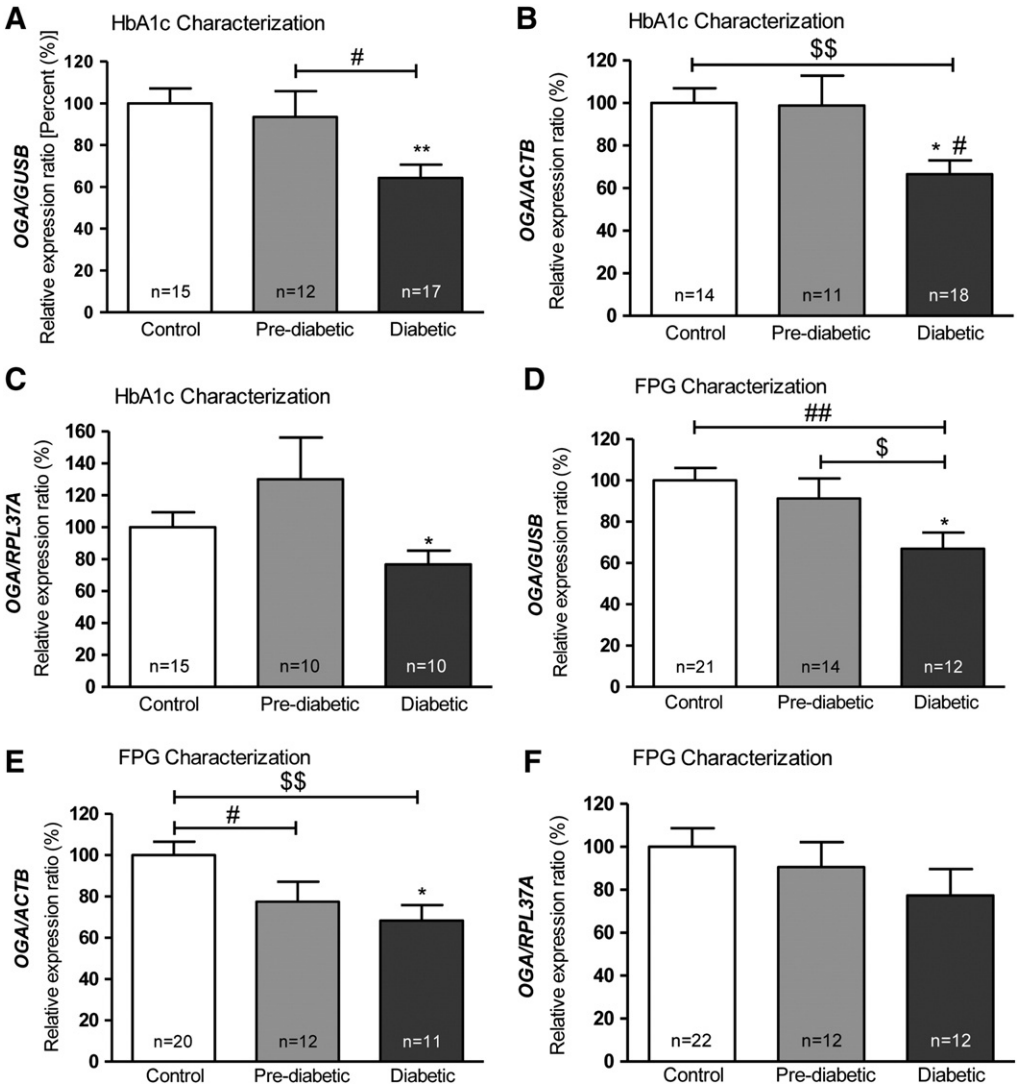

Fig. 1. Reduced *OGA* gene expression in diabetic individuals. Decreased *OGA/GUSB* gene expression in diabetic vs. pre-diabetic (#$P < 0.05$), and control subjects (**$P < 0.01$) (n = 44) (HbA1c characterization). B. Lower *OGA/ACTB* expression in diabetic vs. control (*$P < 0.05$; $$P < 0.01$), and pre-diabetic individuals (#$P < 0.05$) (n = 43) (HbA1c characterization). C. Attenuated *OGA/RPL37A* expression in diabetic vs. control individuals (*$P < 0.05$) (n = 35) (HbA1c characterization). D. Lower *OGA/GUSB* gene expression in diabetic subjects vs. pre-diabetic ($P < 0.05$) and control subjects (*$P < 0.05$; ##$P < 0.01$) (n = 47) (blood glucose characterization). E. Decreased *OGA/ACTB* levels in diabetic vs. control (*$P < 0.05$; $$P < 0.01$), and pre-diabetic vs. control subjects (#$P < 0.05$) (n = 43) (blood glucose characterization). F. No statistically significant differences in *OGA/RPL37A* expression between study groups (n = 46) (blood glucose characterization). Data are expressed as the mean ± SEM. Values are expressed as a relative percentage to controls (100%).

data to two additional reference genes, *ACTB* (Fig. 1B) and *RPL37A* (Fig. 1C). A similar pattern emerged when study participants were categorized according to fasting blood glucose levels, i.e. *OGA* expression was reduced by 33 ± 7.8% and 24.3 ± 9.8% vs. control and pre-diabetic subjects, respectively (Fig. 1D). The findings were corroborated by normalizing to *ACTB* (Fig. 1E) and *RPL37A* (Fig. 1F). Although normalizing to

three separate reference genes and different characterizations yielded varying degrees of sensitivity, the same fundamental trend was observed: decreased *OGA* expression in diabetic individuals compared to control and pre-diabetic individuals.

3.2. Decreased OGT expression with pre- and overt type 2 diabetes

Diabetic participants (HbA1c characterization) exhibited significantly lower *OGT* expression levels (by 23.6 ± 2.2%) vs. control individuals (Fig. 2A). As with *OGA*, there was also a significant difference compared to pre-diabetic individuals. These differences were validated by employing *ACTB* (Fig. 2B) and *RPL37A* (Fig. 2C) as additional reference genes. Of note, when data were analyzed by HbA1c criteria then the numbers for diabetic individuals increased to 18 (versus n = 16 listed in Table 1 [based on ADA fasting plasma glucose criteria]. To limit redundancy we did not include a second table for the HbA1c classification since values only show relatively minor changes when compared to Table 1. When study subjects were categorized according to fasting blood glucose levels, the diabetic group showed a decrease of 17.2 ± 2.3% vs. control participants (Fig. 2D) which was confirmed using *ACTB* (Fig. 2E) and *RPL37A* (Fig. 2F). Of note, *OGT* expression levels differed significantly between pre-diabetic and control groups (Fig. 2B, D and E).

3.3. Distinct GFPT isoform gene expression is associated with ethnicity

No significant differences were found (for all three groups) when evaluating gene expression levels of *GFPT1* and *GFPT2* according to HbA1c and fasting plasma glucose classifications (Fig. 3A–D). Since a previous study reported ethnic-related changes in *GFPT* gene expression [26], we decided to separately evaluate *GFPT* for the Caucasian and Mixed Ancestry participants. Due to the relatively small number of Mixed Ancestry participants we were unable to determine *GFPT* expression for this group alone; however, we were able to do so for the Caucasian population. Here there were no changes in *GFPT1* expression levels for control, pre-diabetic and diabetic individuals. However, we found attenuated *GFPT2* gene expression (by 54.7 ± 5.3%) for the Caucasian diabetic vs. control groups, and a 38.8 ± 12.2% decrease for the Caucasian pre-diabetic vs. control groups (Fig. 4A and B).

Closer examination of inter-individual *GFPT2* expression levels showed that these data varied more widely than any of the other genes investigated here, i.e. with the lowest and highest values differing by ~28-fold. Since *GFPT2* expression differed between the three study groups only when the two ethnic groups were separated, and not in combination, we next investigated whether there were any differences in *GFPT2* expression levels between Caucasian and Mixed Ancestry individuals, regardless of diagnosis. Our data reveal – after correcting for diabetic status – that *GFPT2* expression levels were 2.4-fold higher for the Mixed Ancestry vs. the Caucasian group (Fig. 4C). However, *GFPT1* expression levels were not significantly altered between Caucasian and Mixed Ancestry participants (Fig. 4D). The expression levels of *OGA* and *OGT* were also unaffected by ethnicity (Fig. 4E and F).

4. Discussion

Since increased HBP flux is linked to the onset of type 2 diabetes, we hypothesized that genes controlling key pathway regulators are differentially expressed within this context. This study evaluated *OGA*, *OGT*, *GFPT1*, and *GFPT2* gene expression in leukocytes isolated from control, pre-diabetes and type 2 diabetes participants (according to ADA criteria). The focus was on leukocytes since it is known that all genes here investigated are indeed expressed in these cells [13,18–20] and it can be relatively easily isolated from volunteers — if the aim is for it to eventually become a diagnostic gene test. The main findings of this study are: a) Downregulation of *OGA* and *OGT* gene expression with type 2 diabetes; and 2) distinct *GFPT2* gene expression associated with ethnicity.

4.1. Downregulation of OGA and OGT gene expression with type 2 diabetes

The finding of decreased *OGA* expression is consistent with previous clinical studies showing increased *O*-GlcNAcylation with type 2 diabetes [12,13]. Thus lower gene expression of the enzyme responsible for the removal of *O*-GlcNAc moieties in diabetic individuals may help explain the reported elevation in *O*-GlcNAcylation. In addition, others reported the manifestation of insulin resistance/type 2

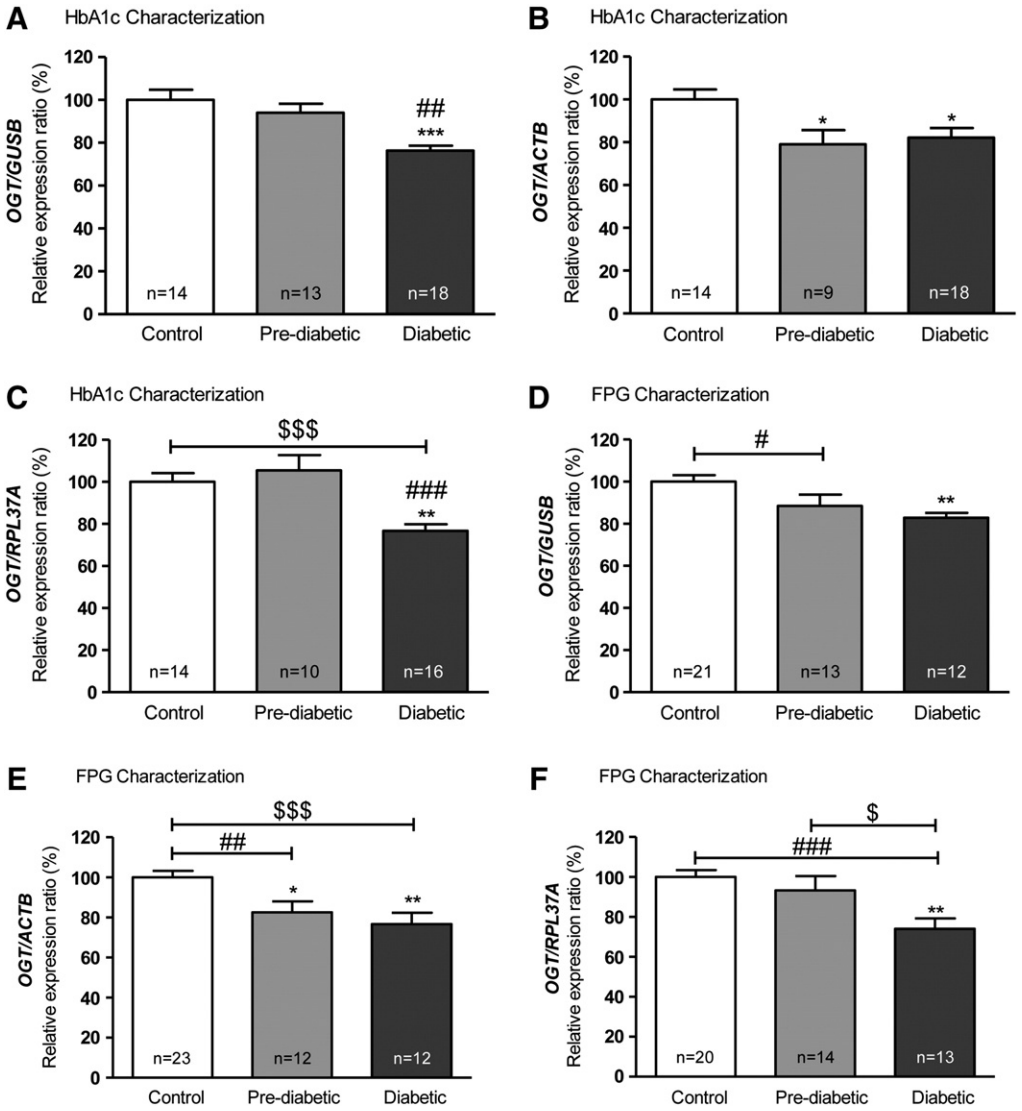

Fig. 2. Decreased *OGT* expression with pre- and overt type 2 diabetes. A. Attenuated *OGT/GUSB* gene expression levels in diabetic subjects vs. pre-diabetic (##$P < 0.01$) and control subjects (***$P < 0.001$) (n = 45) (HbA1c characterization). B. Decreased *OGT/ACTB* levels in diabetic (*$P < 0.05$) and pre-diabetic (*$P < 0.05$) individuals vs. controls (n = 41) (HbA1c characterization). C. Lower *OGT/RPL37A* expression in diabetic subjects compared to control (**$P < 0.01$; $$$$P < 0.001$) and pre-diabetic subjects (###$P < 0.001$) (n = 40) (HbA1c characterization). D. Decreased *OGT/GUSB* gene expression levels in diabetic subjects vs. control subjects (**$P < 0.01$) pre-diabetic vs. control individuals (#$P < 0.05$) (n = 46) (blood glucose characterization). E. Lower *OGT/ACTB* expression in diabetic vs. control subjects ($$$$P < 0.001$; **$P < 0.01$) and pre-diabetic vs. control subjects (##$P < 0.01$; *$P < 0.05$) (n = 47) (blood glucose characterization). F. Attenuated *OGT/RPL37A* levels in diabetic subjects vs. pre-diabetic ($$P < 0.05$) and control subjects (**$P < 0.01$; ###$P < 0.001$) (n = 47) (blood glucose characterization). Data are expressed as the mean ± SEM. Values are expressed as a relative percentage to controls (100%).

diabetes phenotype with the use of *O*-GlcNAcase inhibitors [22]. As far as we are aware, this is the first clinical study to determine *OGA* mRNA transcript levels for individuals diagnosed with type 2 diabetes.

Our *OGA* expression data are in agreement with research done on a Mexican-American population where a single mutation within the *OGA* gene causes early termination of its translation, thereby reducing

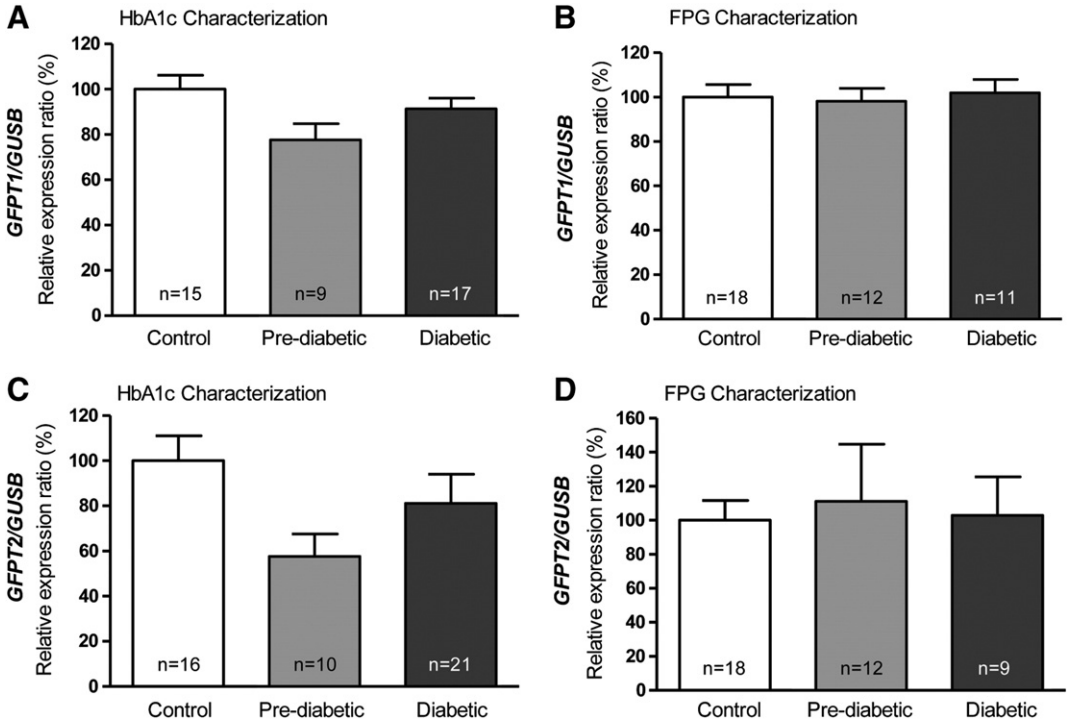

Fig. 3. Evaluation of *GFPT* isoform expression. A. *GFPT1* gene expression for the three study groups (n = 41) (HbA1c characterization). B. *GFPT1* gene expression for the three study groups (n = 41) (blood glucose characterization). C. *GFPT2* gene expression for the three study groups (n = 47) (HbA1c characterization). D. *GFPT2* gene expression for the three study groups (n = 39) (blood glucose characterization). Data are expressed as the mean ± SEM. Values are expressed as a relative percentage to controls (100%).

expression [14]. Furthermore, a study previously performed by our laboratory showed that diabetic participants (characterized by ADA HbA1c criteria) exhibited lower leukocyte OGA protein expression compared to matched controls [13]. Conversely, type 2 diabetic individuals can also display enhanced OGA protein expression [12,13]. For example, we previously found increased OGA protein levels in diabetic individuals when leukocytes were sub-typed into granulocytes and lymphocytes, respectively [13]. Likewise others have discovered greater OGA protein expression in erythrocytes with type 2 diabetes and proposed that this may be an adaptive response to attenuate high levels of *O*-GlcNAcylation and its subsequent damaging effects [12]. How can the variation between different studies and gene versus protein data be explained? We propose that such inconsistencies may be due to a variety of reasons, including a) the specific cell type examined (erythrocytes vs. total leukocytes vs.

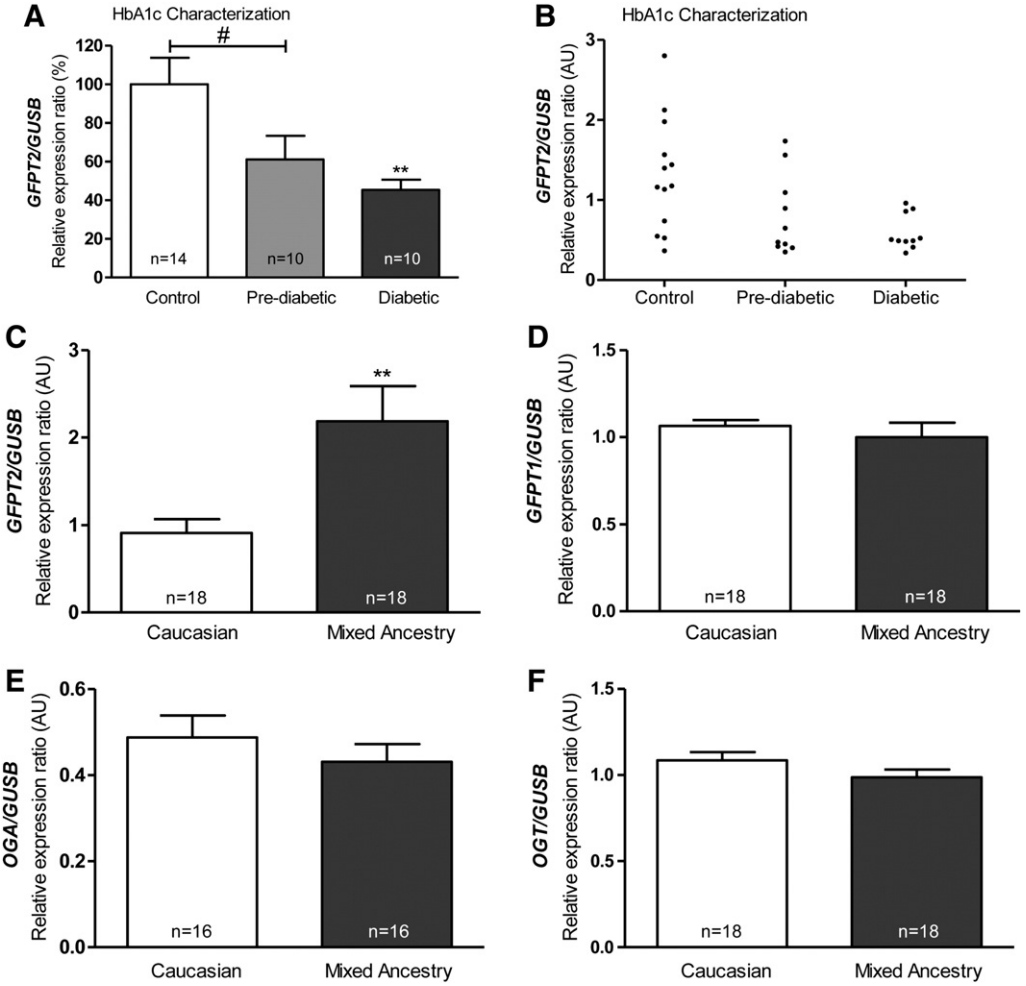

Fig. 4. Distinct *GFPT* isoform gene expression with ethnicity. A. Lower Caucasian *GFPT2/GUSB* gene expression levels in diabetic vs. control individuals (**$P < 0.01$) and pre-diabetic vs. control individuals (#$P < 0.05$) (n = 34) (HbA1c characterization). B. Scatter plot demonstrating the distribution of *GFPT2/GUSB* expression levels between study groups (n = 34) (HbA1c characterization). C. Comparison of *GFPT2/GUSB* gene expression levels for Caucasians versus Mixed Ancestry individuals (**$P < 0.01$; n = 36). D. Comparison of *GFPT1/GUSB* gene expression levels for Caucasians versus Mixed Ancestry individuals (n = 36). E. Comparison of *OGA/GUSB* gene expression levels for Caucasians versus Mixed Ancestry individuals (n = 32). F. Comparison of *OGT/GUSB* gene expression levels for Caucasians versus Mixed Ancestry individuals (n = 36). Note: to generate comparisons in C, D, E, and F. above, the 36 individuals were equally divided between the Caucasian and Mixed Ancestry groups for respective control, pre-diabetic and diabetic categories (with matched blood glucose levels).

granulocytes and lymphocytes) — that may contain more/less *O*-GlcNAc modification sites [13]; b) different cell types exhibiting distinct *O*-GlcNAc cycling rates [12]; c) differences in population admixture; and d) due to the complex regulation of *OGT* and *OGA*, mRNA levels may not necessarily correlate with protein expression [23]. However, further studies are required to investigate these possibilities, while the use of transcript specific primers targeting individual *OGA* isoforms may help elucidate observed discrepancies.

We also determined that type 2 diabetic individuals exhibited reduced *OGT* gene expression. Moreover, changes in *OGT* gene expression, unlike the *OGA* data, could distinguish between control and pre-diabetic subjects. However, this result was surprising and raises the question how to reconcile attenuated *OGT* expression with higher *O*-GlcNAcylation typically found with type 2 diabetes. We propose that the subtle, yet significant reduction in *OGT* gene expression at a relatively early stage of disease progression (pre-diabetes), may represent an attempt to lessen chronic HBP activation and higher *O*-GlcNAcylation under hyperglycemic conditions in an effort to avoiding subsequent harmful effects.

However, others found unchanged OGT protein expression in type 2 diabetic individuals [12,24]. It is our opinion that this incongruity is likely due to the highly complex nature of *OGT*, i.e. it can be alternatively spliced to produce a number of different isoforms containing variable tetratricopeptide (TPR) domains [25]. This allows each *OGT* isoform to modify/interact with a particular group of substrates [25], and also localize it to distinct intracellular compartments/tissues [24,25]. Moreover, OGT also undergoes post-translational modification that is regulated by a wide variety of kinases [18,24], and can itself be *O*-GlcNAcylated [18]. As a result of this complex regulation, changes at the mRNA expression level may not directly correlate with protein expression and/or enzyme activity [24]. Furthermore differences could stem from the phenotype of red vs. white blood cells, the distinct assays employed to measure expression levels (qPCR vs. blotting), and variation in the cohort. As before, an investigation of mRNA levels of distinct *OGT* isoforms could help to shed light on this discrepancy.

4.2. Distinct GFPT2 gene expression associated with ethnicity

The *GFPT* data showed no significant changes between the three study groups here investigated. Since others found ethnic-related changes in *GFPT* gene expression [26], we separately determined its expression for the Caucasian and Mixed Ancestry participants, respectively. Here Caucasian participants displayed a significantly lower *GFPT2* gene expression (when classified by HbA1c) with type 2 diabetes. Moreover, *GFPT2* gene expression could distinguish between control and pre-diabetic subjects. These findings are in disagreement with others that reported higher *GFPT2* mRNA expression in diabetic individuals from the Indian subcontinent [20]. However, the latter study employed semi-quantitative PCR for *GFPT2* gene expression analysis, which has several methodological limitations when compared to quantitative real-time PCR. Moreover, differences in expression may also occur depending on the white blood cell populations studied [13], e.g. we employed leukocytes versus lymphocytes used in the Indian study [20] and ethnic related differences.

Attenuated *GFPT2* levels in combination with decreased *OGT* expression may represent an adaptive response to hyperglycemia-mediated increases in *O*-GlcNAcylation, which may be detrimental to leukocyte function. Diminished *GFPT2* would slow the flux through the HBP resulting in less UDP-GlcNAc (the substrate for the *O*-GlcNAc modification), while decreases in *OGT* would be expected to attenuate the HBP terminal modification. However, it remains unclear whether enzyme activity and protein expression levels would mimic changes in gene expression in this instance.

Our findings show that *GFPT2* gene expression varies according to ethnicity. In support, Mixed Ancestry individuals displayed a 2.4-fold increase in *GFPT2* expression levels compared to Caucasians (after adjusting for diabetic status).

Increased *GFPT2* expression levels in the Mixed Ancestry population may help explain the relatively high prevalence of type 2 diabetes in this population residing within the greater Cape Town region (Western Cape, South Africa) [27]. These data are consistent with another study where it was established that African-Americans exhibit a 2-fold increase in *GFPT2* expression compared to Caucasian individuals [26]. Since both positive and negative correlations between GFPT expression/activity and type 2 diabetes were previously found [20,28], it is suggested that *GFPT* isoforms may also be post-translationally regulated [28,29].

4.3. Clinical relevance of HBP biomarkers (total leukocytes vs. subpopulations)

As mentioned, OGA and OGT protein expressions as well as total *O*-GlcNAc levels have already been studied in erythrocytes [12], leukocytes and leukocyte subpopulations (granulocytes and lymphocytes) [13]. While these studies found an increased OGA protein expression with diabetes progression (erythrocyte and leukocyte subpopulations), we discovered the converse. However, when OGA protein expression was measured in the total leukocyte population, the diabetic group then displayed decreased OGA levels [13], although not as sensitive and statistically significant as the current *OGA* gene expression data. Thus we are of the opinion that HBP regulation may be cell-specific; however more studies are required to fully elucidate this intriguing notion. To date, the most sensitive and statistically robust differences between controls, pre- and diabetic individuals were generated in granulocytes and this may prove true for gene expression as well [13]. Further studies examining HBP regulatory gene expression in this population should prove useful and may provide enhanced sensitivity.

While we agree that all of the above assays possess its own merits, *OGT* gene expression is the first HBP biomarker able to distinguish between all three study groups — albeit making use of varying classification criteria and different reference genes to normalize data. The current findings together with previous work earlier discussed strengthen the emerging hypothesis that HBP biomarkers may provide diagnostic utility in the future. Also, HBP regulatory protein and gene expression levels certainly appear to be significantly altered with diabetes progression regardless of discrepancies (cell-specific and gene vs. protein), and this provides impetus for further research.

4.4. Limitations

While the current study demonstrates promising findings it is not without limitations. Protein expression and enzyme activity data would have proven invaluable to help explain discrepancies between our gene data and previous studies that focused on the protein level.

In conclusion, the current pilot study found differential gene expression of HBP regulators with the onset of type 2 diabetes and provides further impetus for validation studies with a larger sample size. Despite the relatively small sample size and the potential for admixture, our data reveal significant decreases in *OGA* and *OGT* gene expression in diabetic individuals, while *GFPT2* expression exhibits ethnic-dependent regulation. We propose that the significant downregulation of HBP gene markers may in time offer potential diagnostic utility, while also providing candidate susceptibility genes for type 2 diabetes in different ethnic groupings.

Acknowledgments

M.C. helped conceived the idea, researched and analyzed the data, and wrote the manuscript. M.F.E. conceived the idea, analyzed the data, and wrote the manuscript. M.F.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. This work was supported by the South African National Research Foundation and Stellenbosch University (to M.F.E.).

No potential conflicts of interest relevant to this article were reported.

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