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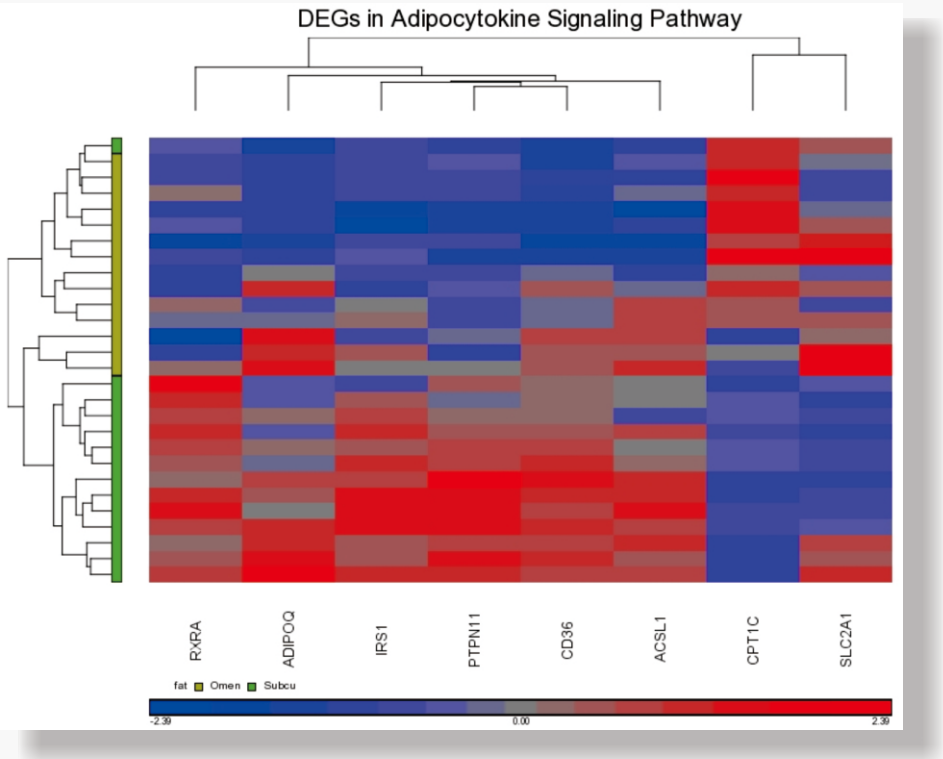

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The relation of flow-mediated vasodilatation and diastolic function in uncomplicated Type 2 diabetic patients*

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ABSTRACT

Objectives: To evaluate the association of diastolic function of the left ventricle with flow- mediated dilatation (FMD) in uncomplicated Type 2 diabetes mellitus patients. Methods: Eighty-two uncomplicated Type 2 diabetic patients were examined by pulse and tissue Doppler echocardiography and FMD of brachial artery. The patients were divided into 2 groups according to the size of the left ventricular relaxation parameter—E'. Results: The average age of the patients was 61 ± 6 years. FMD was 5.0 ± 1.8% in 41 patients with E' from 3 to 7.4 cm/s (mean 6 cm/s) comparing to 5.1 ± 1.9% (p = 0.96) in 41 patients with E' from 7.5 to 10.9 cm/s (mean 8.9 cm/s). E/E' was 11.2 ± 2.3 in the group with lower E' and 9.1 ± 1.6 in the group with higher E' (p < 0.001). Linear negative correlation was found between E/E' and FMD for the patients with E' from 3 to 7.4 cm/s (R^2 = 0.131; p = 0.025) but not for the group of patients with the higher E'. The significant association between FMD and E/E' was confirmed by multivariate analysis ((R^c)^2 = 0.233; p < 0.05). Conclusion: FMD has no impact on the left ventricular relaxation. However FMD is negatively associated with E/E' in Type 2 diabetic patients who have low E' as a sign of an impaired early relaxation.

Keywords: Flow-Mediated Vasodilatation; Tissue and Pulse Doppler Echocardiography; Type 2 Diabetes Mellitus; Diastolic Function; Left Ventricular Relaxation

1. INTRODUCTION

Endothelial dysfunction is independently associated with diastolic dysfunction in humans [1]. Flow-mediated dilatation (FMD) of brachial artery is recognized parameter of endothelial function and is associated with cardiovascular prognosis [2,3]. It is significantly more deteriorated in patients with Type 2 diabetes mellitus in comparison to nondiabetic subjects [4]. Diastolic heart failure and diastolic dysfunction are also frequent in patients with Type 2 diabetes mellitus [5,6].

However there are only limited data about the association of flow-mediated vasodilatation of brachial artery with diastolic function in patients suffering from Type 2 diabetes mellitus [7].

The aim of our study was to evaluate if FMD is associated with an early stage of diastolic dysfunction characterized by the incipient impairment of the left ventricular relaxation in patients with uncomplicated Type 2 diabetes mellitus without any previous cardiovascular accident, microalbuminuria and systolic dysfunction.

In order to answer this question the relation between FMD of brachial artery and echocardiography parameters of diastolic function examined by pulse and tissue Doppler echocardiography was evaluated.

2. SUBJECTS AND METHODS

Patients with Type 2 diabetes mellitus were examined at the Department of Medicine, University Hospital Prague Motol, Czech Republic. The diagnosis of Type 2 diabetes mellitus was based on the following: clinical findings, fasting blood glucose, C-peptide measurement and a negative finding for anti-beta-cell antibodies at least 1 year prior to enrolment in the study. The patients were only included in the study if their casual blood pressure was well controlled regardless of treatment for arterial hypertension—equal or below 135/85. Patients

*Conflict of interest: None declared.

with a history of cardiovascular or other heart diseases, presence of regional LV kinetic abnormalities or a global LV ejection fraction <55%, a history of severe GFR impairment (<0.5 ml/s/1.73 m^2) and/or microalbuminuria, or a prognosis of a life-limiting disease were excluded. The patients were divided into 2 equal groups according to size of E'—parameter of an early left ventricular relaxation.

The study was approved by the Ethics Committee of the University Hospital Motol and undertaken in accordance with the Declaration of Helsinki.

Transthoracic echocardiography, including pulse Doppler and tissue Doppler imaging (TDI) were performed using a Philips Sonos® 7500 cardiac ultrasound unit (Philips Healthcare, Andover, MA, USA).

Interventricular septal thickness (IVSd), posterior wall thickness (PWd), LV diastolic diameter (LVDd), LV systolic diameter (LVDs), and left atrial diameter were determined. Left ventricular mass (LVM) was calculated by the following equation: LVM (g) = 1.04 × [(IVSd + LVDd + PWd)^3 − LVDd^3] − 13.6. Left ventricular mass index (LVMI) was determined as LVM/body surface area. Ejection fraction of the left ventricle was evaluated according to Teicholz equation [8].

Mitral inflow velocity was traced and the following variables of diastolic function were measured and evaluated: peak velocities of early (E) and late (A) transmitral flow, the ratio E/A, and deceleration time (DT). The peak velocities of early (E') and late (A') diastolic mitral annular motion (average of septal and lateral values) were determined from TDI recordings, and mitral E/E' and E'/A' ratios were calculated. In our laboratory, approximately 4 weeks apart the mean intra-observer difference was 3.6% and inter-observer difference 4.6%.

Endothelial function was evaluated by measuring the FMD of the brachial artery using a standardized protocol. FMD of the brachial artery was examined with scanner CC-15M71-MA (Toshiba, Otawara, Japan).

Subjects were required to be fasting and not use any tobacco-containing products for 8 hours before the study. Subjects were placed in a supine position in a temperature-controlled room for 10 min before imaging. A blood pressure cuff was placed on the widest part of the proximal right forearm. Using a 10-MHz linear array vascular ultrasound transducer was located above the elbow and scanned in longitudinal sections. After recording baseline B-mode ultrasound images of the brachial artery, the cuff was inflated to 250 mm Hg for 5 min to induce reactive hyperaemia. Brachial artery images were obtained 60 and 90 seconds after deflation. Studies were recorded digitally. The primary outcome variable was the maximum FMD (in %), the largest percentage of change in the brachial artery diameter after reactive hyperaemia relative to the baseline diameter. The absolute maximum FMD (in cm), the absolute difference in brachial artery size after reactive hyperaemia compared with baseline, also was reported.

In our laboratory, approximately 4 weeks apart had an interscan FMD difference of only 0.36% and the median inter-reader variability was from −0.24% to +0.12%.

Biochemical examination of blood samples included glycosylated haemoglobin (HbA_{1c}), lipid analysis and creatinine. HbA_{1c} was measured using high-performance liquid chromatography and a spectrophotometer detection system (Tosoh G8 analyser; Tosoh Corporation, Tokyo, Japan) with a normal range of 2.8% - 4.5% (7 - 26 mmol/mol) according to the International Federation of Clinical Chemistry and Laboratory Medicine. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol were measured using a direct enzymatic method with catalase; triglycerides were measured using the Fossati enzymatic method and serum creatinine (SCr) was measured using an enzymatic method; all of which were performed by an ADVIA® 1800 Clinical Chemistry System (Siemens Medical Solutions, Tarrytown, NY, USA). Glomerular filtration (eGFR) was estimated using the abbreviated Modification of Diet in Renal Disease equation: $2.92 \times (\text{SCr} \times 0.011312)^{-1.154} \times \text{age}^{-0.203}$ (if female × 0.742).

3. STATISTICAL EVALUATION

Continuous variables are given as mean ± SD. Between-group comparisons of continuous parameters were performed by Mann-Whitney test. The x^2-test was used to compare categorical variables. Relationships between continuous variables were assessed using linear regression analysis. In order to detect the independent predictors of the parameters of diastolic function (E/A, E', E/E' and E'/A') multivariate regression analysis using the method of orthogonal projections to latent structure was evaluated. The following parameters were included into multivariate analysis: age, gender, BMI, history of hypertension, HbA_{1c}, LDL and HDL cholesterol, systolic and diastolic BP, eGFR, LVMI and FMD. A p-value < 0.05 was considered to be statistically significant. The value $(R^c)^2$ expresses the percentage variation of the matrix of the dependent variable explained by the independent variables. Data were analysed using the StatGraphics Centurion Data Analysis and Statistical Software, version XV (Statpoint Technologies Inc., Warrenton, VA, USA).

4. RESULTS

The baseline characteristics of 82 patients with Type 2 diabetes mellitus are presented in 1st column of **Table 1** and their echocardiography parameters are shown in the 1st column of **Table 2**.

Table 1. Demographic and clinical characteristics of all of the patients with Type 2 diabetes that participated in the study (n = 82) and two groups stratified according to their peak velocity of early diastolic mitral annular motion (E' in cm/s).

Parameter	Entire cohort	E' (3 - 7.4)	E' (7.5 - 10.9)	p
	n = 82	n = 41	n = 41	
Age (years)	61 ± 6	62 ± 6	61 ± 7	NS
Gender (number of female/%)	28 (34%)	14 (34%)	14 (34%)	NS
BMI (kg/m^2)	31 ± 3.7	30.7 ± 3.5	31.2 ± 3.9	NS
Diabetes duration (years)	11.2 ± 7.6	13.4 ± 8.1	9.0 ± 6.5	0.007
HbA_{1c} (%)	6.0 ± 1.6	6.4 ± 1.4	5.6 ± 1.7	0.023
C-peptide (nmol/l)	1.26 ± 0.53	1.33 ± 0.51	1.18 ± 0.54	NS
Diabetes treatment				
Diet only (number/%)	5 (6.1%)	1 (2.4%)	4 (9.7%)	NS
Oral agents (number/%)	61 (74.4%)	27 (65.6%)	34 (82.9%)	NS
Insulin (number/%)	4 (4.9%)	2 (4.9%)	2 (4.9%)	NS
Insulin and oral agents (number/%)	12 (14.6%)	8 (19.5%)	4 ((9.8%)	NS
LDL cholesterol (mmol/l)	2.8 ± 0.9	2.8 ± 1.0	2.9 ± 0.8	NS
HDL cholesterol (mmol/l)	1.2 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	NS
Triacylglycerols (mmol/l)	2.0 ± 0.9	2.2 ± 0.9	1.9 ± 0.8	NS
Hypolipidemic treatment	62 (75.6%)	32 (78%)	30 (73.2%)	NS
Statins (number/%)	52 (63.4%)	27 (65.9%)	25 (61%)	NS
Fibrates (number/%)	11 (13.4%)	6 (14.6%)	5 (12.2%)	NS
Systolic BP (mmHg)	133 ± 8	133 ± 9	132 ± 8	NS
Diastolic BP (mmHg)	82 ± 6	83 ± 6	82 ± 7	NS
Treated hypertension (number/%)	66 (80.5%)	33 (83%)	33(83%)	NS
ACE inhibitors, sartans (number/%)	56 (68.3%)	30 (73.2%)	26 (63.4%)	NS
Beta blockers (number/%)	28 (34.1%)	16 (39%)	12 (29.3%)	NS
Calcium antagonists (number/%)	22 (26.8%)	10 (24.4%)	12 (29.3%)	NS
Diuretics (number/%)	30 (36.6%)	16 (39%)	14 (34.1%)	NS
Other hypotensives (number/%)	9 (11%)	6 (14.6%)	3 (7.3%)	NS
Serum creatinine (umol/l)	85.9 ± 19.2	89.5 ± 20.8	89.5 ± 17.7	NS
eGFR (ml/s/1.73 m^2)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.4	NS

Data are presented as mean ± SD (range) or n (%). The difference between subgroups E' < 7.5 cm/s and E' ≥ 7.5 cm/s was analysed by Mann-Whitney test for continuous data and by x^2-test for categorical data. NS, not statistically significant ($P > 0.05$); HbA_{1c}, glycosylated haemoglobin; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; BP, blood pressure; BMI, body mass index.

The patients were divided into 2 groups depending on the size of E'. Forty-one patients with E' from 3 to 7.4 cm/s (mean 6 cm/s) form the 1st group while the patients with E' from 7.5 to 10.9 cm/s (mean 8.9 cm/s) were included into another one. The difference of E' between groups was highly significant ($p < 0.001$). The comparison of baseline characteristics of both groups of patients are summarised in 2nd and 3rd columns of **Table 1** and echocardiography parameters in 2nd and 3rd columns of **Table 2**.

Table 2. Echocardiography characteristics of all of the patients with Type 2 diabetes that participated in the study (n = 82) and two groups stratified according to their peak velocity of early diastolic mitral annular motion (E' in cm/s).

Parameter	Entire cohort	E' (3 - 7.4)	E'(7.5 - 10.9)	p
	n = 82	n = 41	n = 41	
E/A (ratio)	0.97 ± 0.28	0.89 ± 0.28	1.05 ± 0.26	0.006
E' (cm/s)	7.5 ± 1.7	6.0 ± 1.0	8.9 ± 0.9	<0.001
E/E' (ratio)	10.2 ± 2.2	11.2 ± 2.3	9.1 ± 1.6	<0.001
E'/A' (ratio)	0.70 ± 0.19	0.69 ± 0.19	0.81 ± 0.17	<0.001
DT (cm)	228 ± 46	231 ± 47	225 ± 44	NS
IVST (mm)	11.7 ± 0.7	11.8 ± 0.7	11.7 ± 0.7	NS
PWT (mm)	11.9 ± 0.8	11.8 ± 0.7	11.9 ± 0.8	NS
LVDd (mm)	46.0 ± 4.1	45.6 ± 4.5	46.4 ± 3.7	NS
LVDs (mm)	27.7 ± 2.7	27.1 ± 2.9	27.7 ± 2.4	NS
LAD (mm)	37.8 ± 2.9	37.9 ± 2.8	37.6 ± 2.9	NS
LAD/m^2 (mm)	18.3 ± 2.0	18.5 ± 2.1	18.0 ± 1.9	NS
FS (%)	40.2 ± 16.0	40.2 ± 1.6	40.0 ± 1.4	NS
LVEF (%)	61.0 ± 3.2	60.8 ± 3.3	61.2 ± 3.3	NS
LVM (g)	274 ± 44	233 ± 47	242 ± 41	NS
LVMI (g/m^2)	114 ± 18	113 ± 20	115 ± 16	NS

Data are presented as mean ± SD (range).The difference between subgroups E'< 7.5 cm/s and E' ≥ 7.5 cm/s was analysed by Mann/Whitney test NS, not statistically significant (P > 0.05); E, peak velocity of early transmitral flow; A, peak velocity of late transmitral flow; A', peak velocity of late diastolic mitral annular motion; DT, deceleration time of parameter E; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; LAD, left atrial diameter; FS, fractional shortening; LVEF, left ventricular ejection fraction; LVM, left ventricular mass; LVMI, left ventricular mass index.

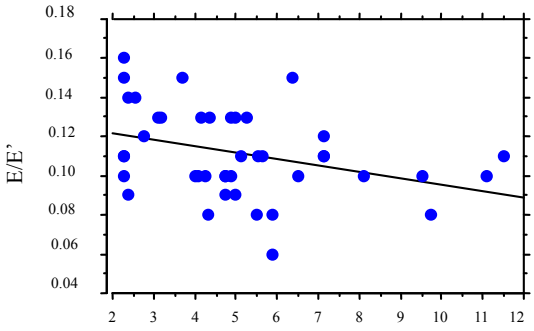
FMD of brachial artery was 5.0 ± 1.8 in the group with E' between 3 and 7.4 cm/sec and 5.1 ± 1.9% (N.S) in 41 patients with E' from 7.5 to 10.9 cm/s, p = 0.96.

The mean diameter of brachial artery before ischaemia in the group of patients with low E' was 0.44 ± 0.05 cm comparing to 0.44 ± 0.06 with high E' (p = 0.98) and after ischaemia in the group of patients with low E' was 0.46 ± 0.05 cm comparing to 0.46 ± 0.06 cm with high E' (p = 0.96)

The linear correlations of E', E/A, E'/A' and E/E' with FMD were not significant when evaluating all 82 subjects.

The range of E/E' was 6 to 15 in patients with low E' and 6 to 12 in the group with high E'.

The linear correlation between E/E' and FMD was negatively significant in the group of the patients with E' between 3 and 7.4 cm/sec (R^2 = 0.131; p = 0.025; **Figure 1**) but not in the group with E' between 7.5 and 10.9 cm/sec. No significant correlation was detected between another parameters of diastolic function—E', E/A, E'/A' with FMD in the groups of diabetic patients with the low or high E'.



y = 0.128 – 0.003 x, R^2 = 0.119, p = 0.025

Figure 1. Bivariate regression plot for E/E' and FMD in the group of patients with E' from 3 to 7.4 cm/s.

The result of multivariate analysis confirmed the significant association of FMD with E/E' (**Table 3**).

5. DISCUSSION

Diastolic dysfunction in Type 2 diabetic patients is

Table 3. Multivariate analysis.

		$(R^c)^2$	p
E’			
	HbA_{1c}	0.579	<0.01
	Duration of diabetes	0.217	<0.05
E/E’			
	Treatment for hypertension	0.150	<0.05
	FMD of brachial artery	0.233	<0.05
	eGFR	0.130	<0.05
E/A			
	Age	0.661	<0.01
E’/A’			
	Age	0.076	<0.05
	HbA_{1c}	0.515	<0.01
	Duration of diabetes	0.217	<0.05

The value (R^c) expresses the percent of variation of the matrix of the dependent variable explained by the independent variables; E’, early peak diastolic annular velocity of mitral valve; E/E’ ratio of early peak transmitral flow velocity to early peak diastolic annular velocity of mitral valve; E’/A’, ratio of early to late peak diastolic annular velocity of mitral valve; E/A, ratio of early to late peak diastolic transmitral flow velocities; HbA_{1c}, glycosylated haemoglobin; FMD, flow-mediated vasodilatation; eGFR, estimated glomerular filtration rate.

associated with the presence of hypertension and coronary artery disease [9,10]. In order to minimize the impact of these factors on parameters of diastolic function the patients with blood pressure exceeding 135/85 mm Hg, history of the previous cardiovascular disease, abnormal ECG, presence of the left ventricular systolic dysfunction on echocardiography examination and microalbuminuria were excluded from entering to this study.

No significant linear correlation between parameters of diastolic function (E/A, E’, E’/A’ and E/E’) and FMD in all 82 patients may be interpreted that in uncomplicated Type 2 diabetic patients there is no relationships between the incipient stage of diastolic dysfunction and flow-mediated vasodilatation.

However the impairment of left ventricular relaxation may be found often in diabetic patients without any clinical cardiovascular complications. Tissue Doppler parameter E’ is described as a marker of an early left ventricular relaxation relatively independent of haemodynamic changes [11-13]. The lower is E’ the higher is probability of abnormal left ventricular relaxation as a marker of the incipient stage of the left ventricular dysfunction [12].

Cut-off point of E’ for relaxation impairment depends on age. In patients between 50 and 70 years who were included into our study the mean value of E’ in healthy persons is 7.5 cm/s [14]. It was reason why the patients with E’ below 7.5 cm/s (mean 6 cm/s, range 3 - 7.4 cm/s) formed one group with high probability of the left ventricular relaxation impairment. The group of patients with normal left ventricular relaxation had E’ 7.5 cm/s and more (mean 8.5 cm/s, range 7.5 - 10.9 cm/s).

There was no significant difference in age, gender, BMI, the presence of hypertension, systolic and diastolic blood pressure, application of treatment for hypertension, lipid abnormalities and diabetes itself, index of mass of the left ventricle and FMD of brachial artery between the diabetic patients according to the size of E’. The patients with E’ from 3 to 7.4 cm/s have the worse diabetic control (HbA_{1c}) and longer duration of Type 2 diabetes. These findings are in agreement with some previous studies [15-17].

The parameter E/E’ correlates with the left ventricular end-diastolic pressure measured invasively [11,12]. Its value rises when the left ventricular relaxation is impaired and so it is also considered as a marker of diastolic function. However it may be changed significantly also by haemodynamic changes [11,12].

FMD in our patients significantly correlated with E/E’ only in the group of patients who presented low E’. This finding may be interpreted that improvement of flow-mediated vasodilatation leads to decrease of the left ventricular end-diastolic pressure in patients with the left ventricular relaxation impairment.

The presence of no significant difference of FMD between the patients with the low and high E’ means that the change of FMD does not lead to change of an early diastolic relaxation in uncomplicated Type 2 diabetic patients. However the impairment of an early diastolic relaxation triggers the relation that between another parameter of diastolic function E/E’ and flow-mediated dilatation.

Limitation of our study is mainly in assessment of cut-off point of E’ between normal and pathological LV early diastolic relaxation. Most likely cut-off point should be lower than 7.5 cm/s because if it was decreased (e.g. 6.5 cm/s) the correlation between E/E’ and FMD has become even more significant.

The relationship between endothelial function and an early stage of diastolic dysfunction is also supported by correlation of E/E’ with plasma adrenomedullin in Type 2 diabetic patients with low E’ [18].

6. CONCLUSIONS

The association between E/E’ and FMD of brachial artery found in patients with E’ from 3 to 7.4 cm/s supports the conclusion that the association of flow-mediated vasodilatation and diastolic function in uncompli-

cated Type 2 diabetic patients is present from an early stage of the left ventricular relaxation impairment.

By this way the improvement of flow-mediated vasodilatation (endothelial function) may have positive effect on haemodynamic situation already in uncomplicated Type 2 diabetic patients.

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Journal of Diabetes Mellitus

Comparison of glycemic variability between basal-bolus and premixed insulin therapy*

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ABSTRACT

Background: Several studies have shown that twice-daily injections of premixed insulin analogs (MIX) could achieve comparable HbA1c levels to basal-bolus (BB) therapy. However, HbA1c does not necessarily reflect short-term glycemic fluctuations that may contribute to the onset or progression of diabetic complications. Therefore, in this study, we compared MIX and BB therapies in terms of their effects on glycemic variability. Methods: We performed a cross-sectional observational study of patients attending our outpatient clinics to compare the effects of two insulin regimens on glycemic variability. We recruited patients treated with MIX or BB with HbA1c < 8.4%. A total of 27 patients (11 treated with BB and 16 treated with MIX) were enrolled and wore a continuous glucose monitor (CGM) for 72 h, while continuing their usual lifestyle and insulin doses. Results: No significant differences in CGM-determined glycemic markers were observed between the two groups. However, the post-lunch duration of glucose levels > 180 mg/dL (t > 180) was significantly shorter with BB therapy (88 ± 76 min) than with MIX therapy (145 ± 54 min; p < 0.05). After classification according to HbA1c levels, markers of glycemic variability were better in patients treated with BB than in those treated with MIX in better control group. Conclusion: These results suggest that BB therapy achieves better glucose profiles than MIX therapy in patients with type 2 diabetes, particularly after lunch.

Keywords: Glycemic Variability; Insulin Therapy; Diabetes; Continuous Glucose Monitoring

1. INTRODUCTION

The aim of diabetes treatment is to prevent diabetic complications by controlling blood glucose levels [1,2]. Insulin treatment effectively lowers blood glucose levels and improves glycemic control, and many patients with type 1 and type 2 diabetes require insulin therapy. Among various insulin regimens, basal-bolus (BB) therapy is thought to most closely imitate physiological insulin secretion by frequent injections of insulin [3]. On the other hand, to maintain the patients' motivation, attempts have been made to reduce the physical and mental burden of diabetes treatment [4]. To this end, several studies have shown that twice-daily injections of premixed insulin analogs (MIX therapy) can achieve HbA1c levels comparable with that achieved by BB therapy but with fewer injections [5-7]. Thus, MIX therapy is preferred by many patients with type 2 diabetes.

Glycemic fluctuations (*I.e.*, variability), as well as HbA1c, have been implicated in the onset and progression of diabetic complications [8-10]. Hence, it is necessary to evaluate glycemic control in terms of both HbA1c and glycemic variability.

We previously reported that oxidative stress markers, which may reflect glycemic variability, were not significantly different between BB and MIX therapies [11]. However, we could not reach a firm conclusion, particularly in patients with HbA1c < 7.4%.

*Author contributions: SY, RH and TY designed the study; RH performed statistical analyses; and GI, YT and KA coordinated the study. All authors have read and approved the final manuscript.
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Therefore, to expand on these earlier findings, we compared BB and MIX therapies in terms of glycemic variability using continuous glucose monitoring (CGM).

2. METHODS

2.1. Patients

Patients were recruited from the outpatient clinic of Kitasato Institute Hospital, between June and December 2009. Patients with type 2 diabetes, treated with BB or MIX insulin therapy for ≥4 months and HbA1c < 8.4% were eligible for this cross-sectional observational study. HbA1c < 8.4% in inclusion criteria is defined as "fair control" by Japanese Diabetes Society. We excluded patients with "poor control" whose HbA1c is 8.4% or more. Other exclusion criteria included severe liver failure (>3 × the upper limit of normal), severe renal failure (serum creatinine > 1.3 mg/dL), anemia (hemoglobin < 10.0 g/dL) or a change in HbA1c ≥ 1.0% during the 2-month observation period. All patients received an explanation of the procedures and possible disadvantages of participating in the study. All of the patients gave written informed consent before study entry. This study was approved by the Institutional Review Board of Kitasato Institute Hospital and was performed in accordance with the Declaration of Helsinki.

2.2. Treatments and Protocols

The patients continued with their original insulin regimen. BB therapy comprised basal-bolus therapy with insulin glargine (Lantus®; Sanofi-Aventis, Paris, France) or detemir (Levemir®; Novo Nordisk A/S, Bagsværd, Denmark) once daily in combination with preprandial insulin: insulin lispro (Humalog®; Eli Lilly, Indianapolis, IN, USA), insulin aspart (NovoRapid®; Novo Nordisk A/S) or human insulin (Novolin R®; Novo Nordisk A/S). MIX therapy comprised patients treated with twice-daily injections of 50/50 premixed insulin lispro (Humalog® MIX 50; Eli Lilly) or 30/70 premixed insulin aspart (NovoRapid® 30 MIX; Novo Nordisk A/S). The insulin regimen and dosage were not allowed to be changed unless severe hypoglycemia occurred.

In this study, we used the CGMS® Gold™ (Medtronic MiniMed, Northridge, CA, USA) for CGM. This system can determine interstitial glucose levels every 5 minutes for 72 h. Subjects measured their blood glucose values using a Medisafe Mini® glucose meter (Terumo, Tokyo, Japan) four times a day and inserted the values into the CGM for data calibration.

Patients were equipped with the CGM system when they visited the outpatient clinic of Kitasato Institute Hospital, and were monitored for 72 h as outpatients. During this period, patients were asked to continue their usual lifestyle.

2.3. Assessment of CGM Parameters

The following parameters were analyzed using data obtained during the middle 48 hours of wearing the CGM: mean glucose, standard deviation of mean blood glucose values (SD), M-value [12], mean amplitude of glycemic excursions (MAGE) [13], area under the curve of glucose levels > 180 mg/dL (AUC > 180), and duration of glucose levels > 180 mg/dL (t > 180) and < 70 mg/dL (t < 70). Next, we categorized the 48-hour data into postprandial (the 4-hour period from the start of each meal) and nocturnal (0:00 - 6:00) periods. Then we calculated the following parameters: mean glucose, AUCpp (area under the curve of glucose levels above the preprandial blood glucose levels), area under the glycemic fluctuation curve (area under the curve of glucose levels above and below the preprandial blood glucose levels), AUC > 180, t > 180, and t < 70 of postprandial period. We also calculated mean glucose, SD, AUC > 180, t > 180, and t < 70 for the nocturnal period.

Next, we evaluated the relationships between HbA1c and glycemic variability (SD, M-value and MAGE). Because Monnier *et al*. found that post-lunch plasma glucose (PG) and extended post-lunch PG values were significantly and independently correlated with HbA1c [14], we also analyzed the relationships between HbA1c and post-lunch glycemic variability (AUCpp and area under the glycemic fluctuation curve).

Finally, we classified patients according to HbA1c (< 7.4% and ≥ 7.4%) and compared glycemic variability (SD, M-value, MAGE, post-lunch AUCpp and area under the glycemic fluctuation curve) between these groups of patients. We used HbA1c < 7.4% or not as the cutoff value because this value is defined as "inadequate" by the Japan Diabetes Society (JDS) [15].

2.4. Statistical Analysis

All values are means ± standard deviation. Differences between the two groups of patients for CGM parameters were analyzed using unpaired *t*-tests. Pearson's univariate regression analysis was used to evaluate the relationships between HbA1c and CGM parameters. Values of $P < 0.05$ were considered to be statistically significant, except in the classified analysis, for which $P < 0.01$ was considered to be significant. SPSS software version 16.0J (SPSS Japan Inc. Tokyo, Japan) was used for statistical analyses.

3. RESULTS

3.1. Patient Characteristics

We registered 27 patients (BB, n = 11; MIX, n = 16).

CGM data were incomplete for one patient in the BB group. Two patients (one in each group), were excluded from the analyses of the postprandial periods because the meal start times were unclear. One patient in the MIX group was excluded from the analyses of the postprandial periods because he started eating dinner within 4 hours after starting lunch. The baseline characteristics of the patients in both groups are shown in **Table 1**. The baseline variables were equivalent in both groups.

3.2. CGM Parameters

The 24-hour glucose profiles for both groups are shown in **Figure 1** and the glycemic parameters are shown in **Table 2**. There were no significant differences between the two groups in any of the glycemic markers derived from the 48 hours of CGM data. Furthermore, there were no differences between the groups for any of the glycemic markers post-breakfast and post-dinner. However, post-lunch t > 180 was significantly higher in the MIX therapy group than in the BB therapy group (145 ± 54 min vs. 88 ± 76 min, respectively; $P < 0.05$). There were no significant differences between the two groups in terms of nocturnal glycemic markers.

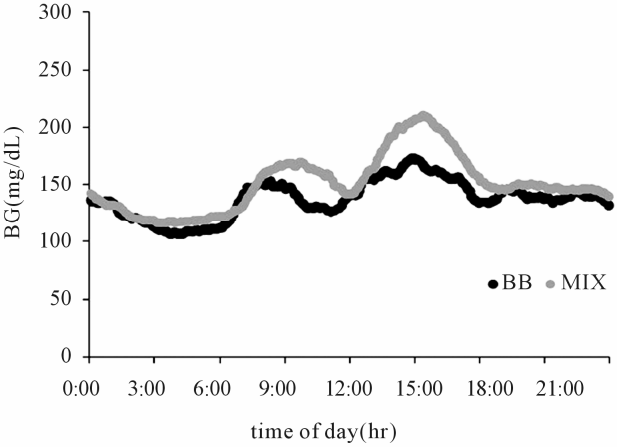

Figure 1. Twenty-four-hour glucose profile in patients treated with basal-bolus insulin (●) or twice-daily injections of premixed insulin analogs (●).

3.3. Correlations between HbA1c and Markers of Glycemic Variability

The correlation between HbA1c and M-value in both groups is shown in **Figure 2**. HbA1c was significantly correlated with the M-value in the BB therapy group (r =

Table 1. Patient characteristics.

	BB (n = 10)	MIX (n = 16)	P value
Sex (males/females)*	6/4	13/3	n.s.
Age (years)	62 ± 13	62 ± 8	n.s.
Duration of diabetes (years)	14 ± 9	17 ± 6	n.s.
Duration of insulin therapy (years)	3 ± 2	4 ± 2	n.s.
Body mass index (kg/m^2)	23.2 ± 4.1	24.0 ± 2.9	n.s.
Insulin dosage (U/kg/day)	0.43 ± 0.15	0.36 ± 0.22	n.s.
HbA1c (%) 2 month before	6.9 ± 0.6	7.4 ± 0.5	n.s.
HbA1c(%) at enrollment	7.0 ± 0.7	7.3 ± 0.5	n.s.
Serum C peptide (ng/mL)	1.69 ± 2.00	1.04 ± 0.79	n.s.
Systolic blood pressure (mmHg)	127 ± 17	127 ± 15	n.s.
Diastolic blood pressure (mmHg)	73 ± 13	72 ± 10	n.s.
Triglyceride (mg/dL)	100 ± 51	123 ± 71	n.s.
LDL cholesterol (mg/dL)	112 ± 36	115 ± 25	n.s.
HDL cholesterol (mg/dL)	61 ± 17	57 ± 13	n.s.
Retinopathy* (None/Non-proliferative/Proliferative)	5/5/0	5/9/2	n.s.
Nephropathy* (None/Albuminuria/Proteinuria)	3/6/1	11/5/0	n.s.
Neuropathy* (−/+/unknown)	5/3/2	9/6/1	n.s.

BB: basal-bolus therapy; MIX: twice-daily injections of premixed insulin analog therapy; n.s.: not significant. *Fisher's exact test; other variables were compared using Student's *t*-test or Welch's test.

Table 2. Comparison of glycemic parameters.

		BB (n = 10)	MIX (n = 16)	p-value
48-hour CGM data	mean glucose (mg/dL)	138 ± 20	151 ± 20	n.s.
	SD (mg/dL)	42 ± 13	47 ± 15	n.s.
	M-value (mg/dL)	13 ± 5	17 ± 6	n.s.
	MAGE (mg/dL)	108 ± 40	123 ± 37	n.s.
	AUC > 180 (mg·hr/dL)	153 ± 154	264 ± 177	n.s.
	t > 180 (min/day)	277 ± 213	392 ± 196	n.s.
	t < 70 (min/day)	32 ± 42	55 ± 71	n.s.
		BB (n = 9)**	MIX (n = 14)**	
post-breakfast	mean glucose (mg/dL)	145 ± 33	168 ± 39	n.s.
	AUCpp (mg·hr/dL)	119 ± 86	173 ± 113	n.s.
	area under the glycemic fluctuation curve (mg·hr/dL)	155 ± 65	204 ± 100	n.s.
	AUC > 180 (mg·hr/dL)	33 ± 38	71 ± 99	n.s.
	t > 180 (min)	63 ± 58	81 ± 74	n.s.
	t < 70 (min)	5 ± 10	5 ± 9	n.s.
		BB (n = 9)**	MIX (n = 14)**	
post-lunch	mean glucose (mg/dL)	169 ± 38	195 ± 29	n.s.
	AUCpp (mg·hr/dL)	189 ± 137	317 ± 182	n.s.
	area under the glycemic fluctuation curve (mg·hr/dL)	203 ± 128	332 ± 164	n.s.
	AUC > 180 (mg·hr/dL)	72 ± 68	134 ± 80	n.s.
	t > 180 (min)	88 ± 76	145 ± 54	0.045*
	t < 70 (min)	0 ± 0	4 ± 8	n.s.
		BB (n = 9)**	MIX (n = 14)**	
post-dinner	mean glucose (mg/dL)	146 ± 27	143 ± 32	n.s.
	AUCpp (mg·hr/dL)	108 ± 112	92 ± 91	n.s.
	area under the glycemic fluctuation curve (mg·hr/dL)	174 ± 118	141 ± 90	n.s.
	AUC > 180 (mg·hr/dL)	30 ± 36	29 ± 40	n.s.
	t > 180 (min)	73 ± 52	54 ± 60	n.s.
	t < 70 (min)	0 ± 0	14 ± 34	n.s.
		BB (n = 10)	MIX (n = 16)	
night time	mean glucose (mg/dL)	119 ± 22	122 ± 27	n.s.
	SD (mg/dL)	19 ± 10	15 ± 6	n.s.
	AUC > 180 (mg·hr/dL)	10 ± 22	7 ± 17	n.s.
	t > 180 (min)	22 ± 31	26 ± 50	n.s.
	t < 70 (min)	23 ± 39	10 ± 23	n.s.

Comparisons were made using Student's *t*-test. BB: basal-bolus therapy; MIX: twice-daily injections of premixed insulin analog therapy; n.s.: not significant. *Statistically significant at $P < 0.01$. **Three patients (BB, n = 1; MIX, n = 2) were excluded from the analysis because the meal start time was inappropriate for post-meal analysis.

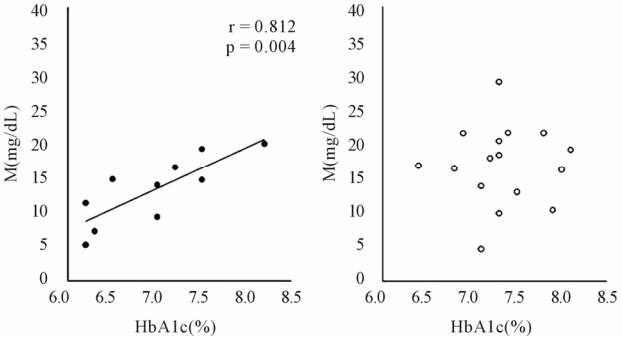

Figure 2. Correlations between HbA1c and M-value in patients treated with basal-bolus insulin (a) or twice-daily injections of premixed insulin analogs (b).

0.812, $P < 0.05$), but not in the the MIX therapy group. Similarly, there were significant correlations between HbA1c and SD, MAGE, post-lunch AUCpp and area under the glycemic fluctuation curve in the BB therapy group ($r = 0.772$, $P < 0.05$; $r = 0.706$, $P < 0.05$; $r = 0.830$, $P < 0.05$; and $r = 0.855$, $P < 0.05$, respectively), but not in the MIX therapy group.

3.4. Classification According to HbA1c

The results of the classified analysis are shown in **Table 3**. Among patients with HbA1c ≥ 7.4%, there were no significant differences in any of the parameters between the BB and MIX groups. In contrast, when we analyzed patients with HbA1c < 7.4%, post-lunch AUCpp and area under the glycemic fluctuation curve were significantly lower in the BB therapy group than in the MIX therapy group. Furthermore, MAGE was lower in the BB therapy group than in the MIX therapy group, although it was not statistically significant ($P = 0.033$).

4. DISCUSSION

In this study, we used CGM to compare glycemic variability between two insulin regimens—BB therapy and twice-daily MIX—in patients with type 2 diabetes.

Analyses of 48 hours of data showed no significant difference between the two therapies. However, SD, M-value and MAGE, markers of glycemic variability, may not necessarily reflect the acute glucose fluctuations, such as postprandial hyperglycemia. Thus, we focused on the postprandial and nocturnal periods and compared glycemic variability within these periods.

Comparison of glycemic control after breakfast and dinner, and during the night showed no significant differences between the two therapies. On the other hand, post-lunch t > 180 was significantly higher in the MIX therapy group. Because preprandial glucose levels were equivalent in both groups (data not shown), these results suggest that the blood glucose levels of patients treated with MIX increased rapidly after lunch, which exposed

Table 3. Classified analysis.

HbA1c < 7.4%				
		BB (n = 7)	MIX (n = 10)	p-value
48-hour CGM data	SD (mg/dL)	37 ± 11	49 ± 16	n.s.
	M-value (mg/dL)	11 ± 4	17 ± 7	n.s.
	MAGE (mg/dL)	91 ± 28	134 ± 41	0.033
		BB (n = 6)**	MIX (n = 9)**	p-value
post-lunch	AUCpp (mg·hr/dL)	115 ± 68	349 ± 189	0.006*
	area under the glycemic fluctuation curve (mg·hr/dL)	136 ± 62	353 ± 184	0.008*
HbA1c > 7.4%				
		BB (n = 3)	MIX (n = 6)	p-value
48-hour CGM data	SD (mg/dL)	54 ± 9	44 ± 14	n.s.
	M-value (mg/dL)	18 ± 3	17 ± 5	n.s.
	MAGE (mg/dL)	147 ± 38	106 ± 23	n.s.
		BB (n = 3)**	MIX (n = 5)**	p-value
post-lunch	AUCpp (mg·hr/dL)	336 ± 122	213 ± 171	n.s.
	area under the glycemic fluctuation curve (mg·hr/dL)	338 ± 120	245 ± 136	n.s.

Comparisons were made using Student's *t*-test. BB: basal-bolus therapy; MIX: twice-daily injections of premixed insulin analog therapy; n.s.: not significant. *Statistically significant at $P < 0.01$. **Three patients (BB, n = 1; MIX, n = 2) were excluded from the analysis because the meal start time was inappropriate for post-meal analysis.

these patients to hyperglycemia. Several large-scale trials have suggested that postprandial hyperglycemia is an independent risk factor for macrovascular diseases [16-19].

So the results of this study suggest that, at equivalent HbA1c levels, the risk for diabetes complications may be higher in patients of MIX therapy than BB therapy. This interpretation is supported by previous findings that the risk of retinopathy progression differed significantly between intensively and conventionally treated patients with comparable HbA1c levels [20].

Generally, as glycemic control deteriorates, glycemic variability increases. However, the difference in glycemic variability between BB therapy and twice-daily MIX therapy is not clear. In this study, we analyzed the correlations between HbA1c and markers of glycemic variability. As **Figure 2** showed, there was a positive correlation between HbA1c and glycemic variability such as M-value only in BB therapy. This suggests that patients in BB therapy with good control can reduce glycemic variability and may avoid hypoglycemia but not patients in MIX therapy with good control. The results of these analyses suggested that MIX therapy might not sufficiently suppress the fluctuations in blood glucose, even at lower HbA1c levels.

To exclude the influence of HbA1c levels, we divided the patients into groups based on HbA1c (*I.e.*, <7.4% vs. ≥7.4%). Similar to post-lunch AUCpp and area under the glycemic fluctuation curve, MAGE was lower in the BB therapy group than the in MIX therapy group, among patients with HbA1c < 7.4%. MAGE was designed to quantitate major glucose excursions and exclude minor ones [21], hence MIX therapy may be unable to suppress major excursions caused by postprandial hyperglycemia at lower HbA1c levels. Our previous study showed an insignificant difference in oxidative stress markers between BB and MIX therapies in patients with HbA1c < 7.4% [11]. Although the sample size of the study was small, BB therapy might suppress the postprandial hyperglycemia more effectively than does MIX therapy. Flexibility in dosage adjustment may also account for the differences in glycemic variability between the two therapies. During twice daily treatment with premixed insulin, some type 2 diabetic patients complain of hypoglycemia before lunch, necessitating a reduction in insulin dose at breakfast. Reducing the insulin dosage at breakfast, however, causes an increase in evening plasma glucose levels in some patients; indicating the limitation of this insulin regimen. Patients treated with BB, on the other hand, can adjust their insulin doses in response to fasting and postprandial glucose. Thus, BB therapy can suppress postprandial hyperglycemia and glycemic variability more appropriately without increasing hypoglycemia. Therefore, BB therapy may be a better regimen to achieve HbA1c goal and prevent diabetic complications. Indeed, a recent meta-analysis of randomized controlled trials suggests that the BB regimen is better at achieving the HbA1c goal compared with all other regimens, including basal, prandial and MIX [22]. Combining MIX therapy with α-glucosidase inhibitors or glinides may help to reduce postprandial hyperglycemia [23-26].

This study has several limitations: 1) as we mentioned before, the sample size was small; 2) this was a non-randomized observational study. Although the baseline characteristics were equivalent in both groups, other factors may have affected the results. Hence, a large-scale, randomized study is needed to confirm our results. Moreover, glucose monitoring was performed in outpatients. Therefore, CGM should also be performed in an inpatient setting to evaluate glycemic variability more precisely and exclude patient lifestyle factors.

In conclusion, BB therapy achieves better glucose levels, particularly after lunch, than twice-daily MIX in patients with type 2 diabetes. Twice-daily injections of MIX may be insufficient to suppress fluctuations in blood glucose and postprandial hyperglycemia, even at lower HbA1c levels.

5. ACKNOWLEDGMENTS

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Role of sphingosine kinases and sphingosine 1-phosphate in mediating adipogenesis

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ABSTRACT

Background: Recent development of obesity involves promotion of preadipocyte differrentiation. This study investigated the role that sphingosine kinases (SPHK) and ceramide-derived sphingosine 1-phosphate (S1P) play in adipocyte terminal differentiation. Materials and Methods: The mouse 3T3-L1 cell line was used as a model for adipogenesis. Cells were harvested at specific time points after initation of differentiation, and SPHK activity was measured. 3T3-L1 cells were treated with S1P and expression of early adipogenesis transcription markers was measured by real time PCR. The expression of S1P-receptors (S1PRs) during differentiation was measured. Results: SPHK activity is induced when 3T3-L1 cells are treated with insulin, dexamethasone, and isobutylmethylxanthine to induce differentiation. SPHK1 is active in preadipocytes and early in the differentiation process. Both SPHK1 and SPHK2 isozymes contribute to activity in differentiated adipocytes. Inhibition of SPHK1 attenuates adipocyte differentiation; however, extracellular S1P does not rescue the effect of SPHK1 inhibition on adipogenesis. Although treatment of preadipocytes with S1P induced message expression of the early adipogenesis transcription factor CC AAT/ binding protein-alpha, continued treatment did not fully support the development of differentiated adipocytes. Sphingosine 1-phosphate receptors (S1PRs) are expressed in preadipocytes and message expression declines markedly during adipocyte differentiation. Conclusion: These results demonstrate that the contribution of SPHK and S1P to adipogenesis is mediated primarily through biphasic activation of SPHK1 and 2 with extracellular S1P and S1PRs playing little role during preadipocyte differentiation.

Keywords: Adipocyte; Adipogenesis; Obesity; Sphingosine Kinase; 3T3-L1 Cells; Sphingosine 1-Phosphate; Sphingosine 1-Phosphate Receptor

1. INTRODUCTION

Adipogenesis involves the activation of a complex series of transcriptional pathways which induce the expression of proteins required for the development of lipid-laden adipocytes [1]. Differentiation is initiated when preadipocytes undergo growth arrest followed by mitotic clonal expansion, and then entry into the terminal differentiation pathway. Two transcription factors which play a predominant role in adipogenesis are CCAAT/ enhancer-binding protein-α (CEBP-α) and peroxisome proliferator-activated receptor-γ (PPAR-γ) [2]. The mouse 3T3-L1 cell line serves as a model for adipogenesis and is used as a system to identify additional signaling molecules that contribute to the terminal differentiation process.

Sphingosine 1-phosphate (S1P) is a novel bioactive phospholipid which has been demonstrated to participate in a wide array of cellular processes. S1P serves dual roles both as an intracellular signaling molecule and as an extracellular ligand for G protein-coupled S1P recaptors ($S1PR_{1-5}$) [3]. These cell surface receptors are ubiquitously, yet differentially, expressed in tissues, and they mediate diverse signal transduction pathways eliciting responses specific to the cell type [4,5]. S1P acting through plasma membrane S1PRs has been demonstrated to play a role in cell differentiation, modulating angiogenesis [6], promoting embryonic stem cell transition to cardiomyocytes [7], and inducing development of mast cells from hematopoietic precursors [8]. Intracellular S1P regulates calcium release [9] and serves as a second messenger promoting cell proliferation in opposition to apoptosis [10]. Finally, intranuclear S1P has been dem-

onstrated to alter histone acetylation, a major epigenetic factor in chromatin remodeling and gene expression [11].

Sphingosine kinases (SPHK) are responsible for S1P biosynthesis from sphingosine [12]. Two SPHK isoforms have been identified [12-14]. SPHK1 has been described primarily in growth-promoting and anti-apoptotic activities, while SPHK2 is reported to be involved in apoptosis induction and inhibition of DNA synthesis/cell growth [11,15,16]. Knockout studies in mice suggest that SPHK1 and SPHK2 have redundant functions since deletion of either isozyme alone does not have a specific phenotype, whereas deletion of both genes results in defects in neurogenesis and vascular development with embryonic lethality [17]. Both isoforms are expressed in adipocytes and at least one isoform (SPHK1) participates in adipogenesis [18].

Differentiated rat adipocytes express $S1PR_{1-3}$ and $S1PR_5$ [19]. G-protein coupled signal transduction pathways attributed to the receptors include inhibition of adenylyl cyclase ($S1PR_1$), activation of phospholipase C ($S1PR_3$), and upregulation of mitogen activated protein kinase and c-Jun N-terminal kinase activities ($S1PR_{2-5}$) (reviewed in [20]). Extracellular application of S1P to rat adipocytes elicits increases in intracellular calcium and inositol-1,4,5-trisphosphate (IP3) and cAMP generation that is dependent on S1PR activation. In addition, S1P inhibits insulin-induced leptin production and activates lipolysis in a cAMP-dependent manner [19]. The specific S1PRs which mediate these activities have not been delineated. While it has been reported that intracellular S1P levels increase during adipocyte differentiation, and knockdown of SPHK1 partially blocks accumulation of lipid within 3T3-L1 cells, the mechanism by which S1P participates in adipocyte differentiation is not clear [18]. In this study, the 3T3-L1 cell model is used to study the participation of S1P in adipocyte differentiation. While SPHK enzyme activity increases robustly early in the process of adipocyte differentiation, expression of three S1PRs decreases, and treatment of preadipocytes with S1P only partially supports adipogenesis. The results of this study indicate that participation of S1P in adipocyte differentiation is biphasic, with intracellular S1P generation necessary and sufficient for adipogenesis.

2. MATERIALS AND METHODS

2.1. Materials

S1P and sphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). [$^{32}\gamma$]Adenosine triphosphate (ATP) (3000 Ci/mmol) and [^{32}P]-orthophosphate (8500 - 9120 Ci/mmol) were from Perkin Elmer (Boston, MA). Fetal bovine serum (FBS) was from PAA Laboratories (Bedford, MA), and fetal calf serum (FCS) from ATCC (Manassas, VA). Culture media and molecular boilogy reagents were from Invitrogen (Grand Island, NY). SPHK1 and SPHK2 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SPHK-I2 was from Cayman Chemicals (Ann Arbor, MI). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell Culture

Mouse 3T3-L1 cells were obtained from ATCC (Manassas, VA). Fibroblasts were maintained in DMEM containing 4.5 g/L D-glucose, 1.5 g/L $NaHCO_3$, 110 mg/L sodium pyruvate, 100 U/L penicillin, 0.1 mg/L streptomycin, and 10% FCS (growth media) at 37˚C, 5% CO_2 environment. When cells reached confluence, media was changed to include 10% FBS instead of FCS for 24 hours. Differentiation was induced (Day 0) by addition of media supplemented with 10% FBS, isobutylmethylxanthine (IBMX), dexamethasone, and insulin (0.1 mM, 1 μM, and 1 μg/ml). After 72 hours, induction media was removed and replaced with media containing 10% FBS and 1 μg/ml insulin. Media was changed every 2 - 3 days. To assess whether S1P could support adipocyte differentiation, 3T3-L1 preadipocyte cultures were maintained in DMEM + 10% FBS lacking IBMX, dexamethasone, and insulin with or without S1P (1 μM). Media was changed as above with re-addition of S1P at each media change. Cells were harvested as indicated.

2.3. *In Vitro* Assay of Sphingosine Kinase Activity

Cells (10 × 30 mm dish) were harvested at times indicated in the text. Harvested cells were lysed by freeze-thawing in SPHK buffer [21]. Lysates were centrifuged at 15,000 × g for 20 min at 4˚C, and the protein concentration of the supernatant fraction was determined. SPHK activity assays included (unless otherwise indicated): 40 - 80 μg protein/reaction, 50 μM sphingosine complexed with FAF-BSA (4 mg/ml suspension), and [^{32}P]-ATP (2.5 μCi, 1 mM) containing $MgCl_2$ (10 mM) [12]. Reaction linearity was determined in preliminary assays. Biochemical characterization of SPHK isozyme activities was determined in the presence of 400 mM potassium chloride (KCl) or 1% Triton X-100 (TX-100), as described previously [13]. Labeled S1P was extracted [21] and separated by thin-layer chromatography (TLC) on heat-activated silica gel G plates using a solvent system containing 1-butanol/methanol/acetic acid/water (80/20/10/20, v/v). [^{32}P]S1P was quantitated and SPHK activity was expressed as picomoles of S1P formed/min/mg of total protein [22].

2.4. Immunoblot Analysis

Cells were harvested in total protein lysis buffer con-

taining 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, pH 7.4, 1 mM PMSF, 10 µg/ml aprotinin, and 1% TX-100. Equal amounts of whole-cell lysate were resolved by SDS-PAGE (12.5% gel) and transferred to PVDF membrane. Membranes were blocked using Tris-buffered saline with Tween 20 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.05% Tween (TBST) containing 5% milk for 1 hour, and then incubated 2 hours at room temperature with the indicated SPHK antibodies (1:200 dilution). After washing three times with TBST, the membranes were probed with donkey α-goat horseradish peroxidase-conjugated secondary antibody to detect bands via WestPico chemiluminescence reagent (Pierce Biotechnology, Rockford, IL).

2.5. Oil Red O Staining

3T3-L1 cells were differentiated in the presence of SPHK-I2 inhibitor (0.5 - 10 µM) with or without S1P (1 or 10 µM). Inhibitor and S1P were re-added to the cell cultures at each media change. Adipocytes were washed with PBS and then fixed with 3.8% formaldehyde for 1 hour. Cells were then washed and stained with Oil Red O (3 mg/ml) and isopropyl alcohol in water (60/40, v/v) for 30 minutes. To assess the degree of differentiation, stained cells were then washed 4x with water, and Oil Red O extracted with isopropyl alcohol. The resultant staining was quantified by measuring OD_{500nm}. Un-differentiated 3T3-L1 preadipocytes cultured in DMEM + 10% FBS for 15 days were stained and served as background control.

2.6. mRNA Quantitation

3T3-L1 cells were treated with S1P (1 µM) in the presence or absence of differentiation media. Levels of CEBP-α, adipocyte complement-related protein of 30 kDa (Acrp30) and glucose transporter type 4 (GLUT4) mRNA were determined by real-time QPCR [23]. Adipocyte RNA was extracted following treatment, as described previously [24]. QPCR was carried out using using 1 µg of total RNA with primers to CEBP-α: sense, 5'-TGGACAAGAACAGCAACGAG-3'; antisense, 5'-CCTTGACCAAGGAGCTCTCA-3' (225 bp), Acrp-30: sense, 5'-GTTGCAAGCTCTCCTGTTCC-3'; antisense, 5'TCTCCAGGAGTGCCATCTCT-3' (192 bp), and GLUT-4: sense, 5'TTCCTTCTATTTGCCGTCCTC-3'; antisense, 5'TGGCCCTAAGTATTCAAGTTCTG-3' (168 bp). To measure S1PR mRNA during adipogenesis, QPCR was carried out using 1 µg of RNA harvested from cells at indicated time with primers to $S1PR_1$: sense, 5'-AAGTCTCTGGCCTTGCTGAA-3'; antisense, 5'-GATGATGGGGTTGGTACCTG-3' (183 bp), $S1PR_2$: sense, 5'-CCTCATCACCACCATCCTCT-3'; antisense, 5'-CCTCATCACCACCATCCTCT-3' (227 bp), $S1PR_3$: sense, 5'-TCTCAGGGCATGTCACTCTG-3'; antisense, 5'-CAGCTTTTGTCACTGCCGTA-3' (163 bp). QPCR was carried out with the iQ SYBR Green Supermix. Reaction conditions were: initial denaturation at 95˚C for 3 min. followed by 50 cycles of 95˚C for 10 sec and 61˚C for 30 seconds. Specificity of the reaction was verified by melt curve analysis. In each reaction, gene expression was normalized to 18S rRNA level and calculated using the $2^{-\Delta\Delta CT}$ method. Expression of each adipocyte differentiation gene at day 0 (undifferentiated) served as the control.

2.7. Statistical Analysis

Values are means ± SE. Significant differences between treatment groups were determine by Student's *t* test (paired) or one-way ANOVA with post hoc analysis using the Student-Newman-Keuls multiple comparison test. For data expressed as percent of basal or fold increase in expression, two-tailed, one-sample hypothesis test was used. QPCR expression data for adipogenesis genes was log transformed to address variability in absolute fold expression between experiments. Values of $P \leq 0.05$ were accepted as significant. Data analysis was performed using Statmost 32 (Dataxiom Software, CA).

3. RESULTS

3.1. SPHK Activity Increases during Adipogenesis

3T3-L1 cells were differentiated and cytosolic SPHK activity was measured at various time points after initiation of differentiation. SPHK activity was detectable in undifferentiated cells and increased early in the differentiation process, with maximal activity at day 6 post-differentiation (**Figure 1(a)**). The level of SPHK activity declined in a time-dependent manner after day 6; however, in fully differentiated adipocytes (day 15) SPHK activity remained elevated by approximately 2-fold compared to undifferentiated cells (**Figure 1(b)**).

SPHK activity did not correlate directly with SPHK isoform protein levels. For SPHK1 protein, although there were modest but significant increases in protein expression as early as day 6 post-differentiation, there were continued increases in protein levels on days 9 and 12, and maximal expression identified by day 15 with an approximately 7-fold change in SPHK1 compared to undifferentiated cells (**Figure 2(a)**). In contrast, SPHK2 protein increased maximally at day 6 post-differentiation and remained stable throughout the differentiation process (**Figure 2(b)**).

3.2. SPHK Isozyme Characterization during Adipocyte Differentiation

The contribution of isozymes SPHK1 and SPHK2 to

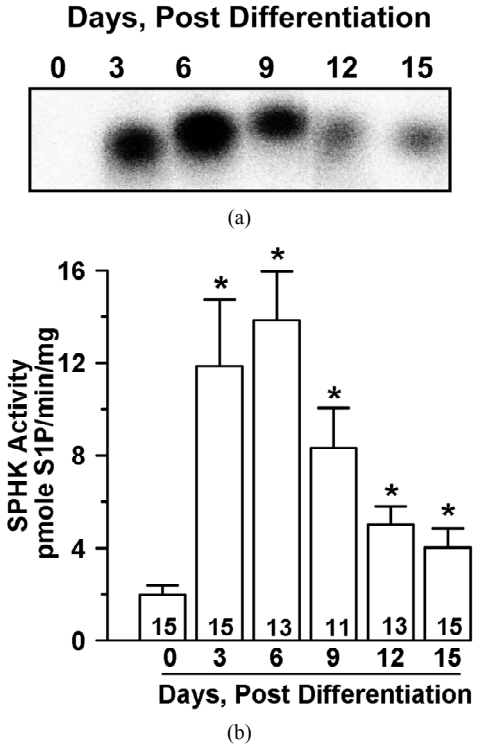


Figure 1. SPHK activity is induced during adipoctye differentiation. 3T3-L1 cells 2 days post-confluence were induced to differentiate for three days, followed by culture in media containing 1 µg/ml insulin for up to 15 days. Undifferentiated (Day 0) or differentiated cells were harvested as indicated. Cell cytosolic fractions (60 µg) were analyzed for SPHK activity in the presence of sphingosine (50 µM)/FAF-BSA (4 mg/ml) complex. (a) Representative phosphoimage of S1P produced, extracted, and isolated by thin-layer chromatography. (b) SPHK activity is expressed as pmole S1P/mg/min and values are mean ± SE for the number of experiments shown at the base of each bar. $^{*}p < 0.01$ vs. day 0 for all samples. P values determined by one-way ANOVA and multiple comparison test.

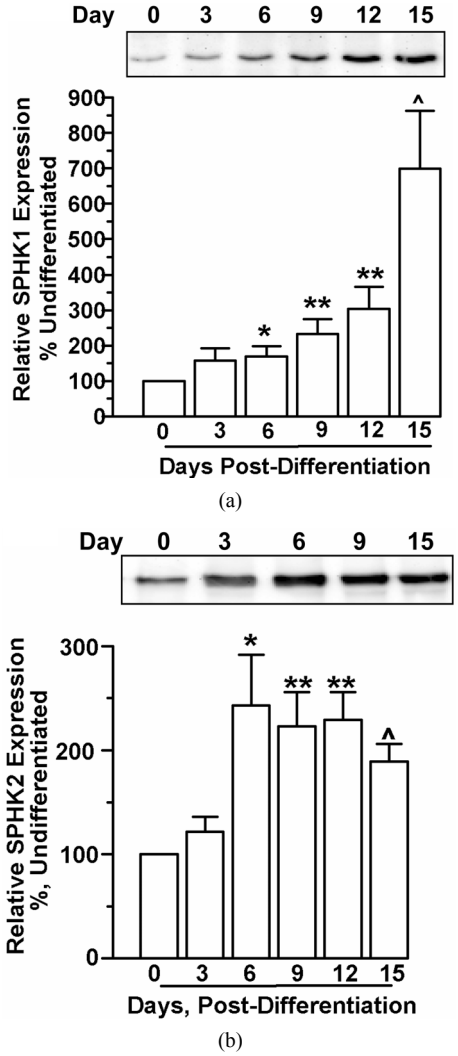


Figure 2. SPHK protein level expression does not correlate with activity during adipocyte differentiation. 3T3-L1 cells were induced to differentiate and cells were harvested on indicated days post-differentiation. Total protein extract (40 µg) was subjected to immunoblot analysis with either (a) SPHK1 or (b) SPHK2 specific antibodies. Representative immunoblots are shown. Quantitative analysis of the Western blots for 8-10 individual experiments showed that (a) SPHK1 levels and (b) SPHK2 levels increased following differentiation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\wedge}p < 0.005$ vs. day 0 (preadipocytes) as determined by one-way ANOVA and multiple comparison test.

total SPHK activity in differentiating adipocytes was determined by comparison of their activities in the presence of high salt (KCl) and detergent (TX-100) [13]. SPHK1 activity is inhibited by high salt and activated by TX-100, whereas SPHK2 behaves in a reciprocal manner. SPHK activity in preadipocytes was low and was activated by TX-100 indicating that SPHK1 activity predominates in preadipocytes (**Figure 3**). During the early phase of preadipocyte differentiation, total SPHK activity increases by approximately 4-fold on day 3 (**Figures 1** and **3**). However, neither KCl nor TX-100 treatment resulted in complete inhibition of SPHK activity (**Figure 3**), suggesting that both isozymes contribute to S1P production during adipogenesis. On the other hand, a significant 1.9-fold activation by TX-100 compared to the total activity on day 3 suggests that SPHK1 predominates at this stage. SPHK activity in fully differentiated adipocytes (day 15) was neither significantly inhibited nor activated in response to treatment with KCl or TX-100.

To further address the contribution that SPHK plays in adipocyte differentiation, 3T3-L1 cells were treated with

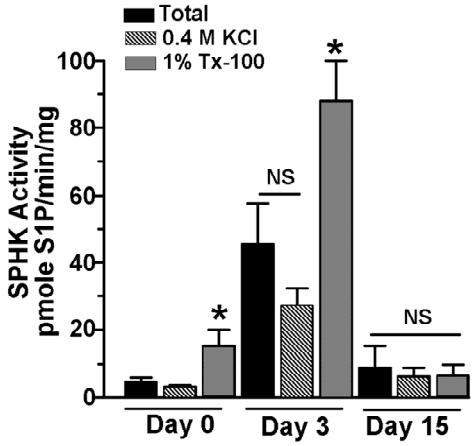


Figure 3. SPHK1 and SPHK2 isozymes are active during adipocyte differentiation. 3T3-L1 cells were induced to differentiate and cells were harvested on day 0, 3, and 15 post-differentiation. SPHK activity was determined in cytosolic fraction (60 µg) in the absence or presence of 0.4 M KCl and 1% Triton X-100 (TX-100), as indicated. Values are means ± SE for 7 independent experiments. P values were determined by one-way ANOVA with multiple comparison test. $^{*}p < 0.05$ vs. corresponding day total SPHK activity.

the SPHK1 specific inhibitor SPHK-I2 from the onset of differentiation. SPHK-I2 (10 µM) partially inhibited adipocyte differentiation as measured by the accumulation of lipid measured by Oil Red O staining on day 15 (**Figure 4**).

3.3. Effect of Extracellular S1P and SPHK1 Inhibition on Adipocyte Differentiation

In order to determine whether extracellular S1P affected adipogenesis or could rescue the partial block of adipocyte differentiation by biochemical inhibition of SPHK1, 3T3-L1 cells were differentiated for 15 days in the presence of SPHK-I2 (10 µM) with or without exogenous S1P. While undifferentiated preadipocytes had a low level of Oil Red O staining, treatment with S1P did induce an approximately 2-fold increase in lipid accumulation (**Figure 5(a)**). In the presence of differentiation media, cells had a 5-fold increase in Oil Red O staining vs. undifferentiated cells. However, the addition of S1P with differentiation media to cells significantly reduced Oil Red O staining by about 25% vs. cells treated with differentiation media only (**Figure 5(a)**). Co-culture of differentiating adipocytes with SPHK-I2 significantly inhibited lipid accumulation in the cells vs. cells treated with differentiation media only (**Figure 5(b)**). Treatment of cells with S1P (1 µM or 10 µM) did not compensate for the inhibition of SPHK1 and S1P biosynthesis by SPHK-I2, and lipid levels remained significantly lower than differentiated control cells (**Figure 5(b)**).

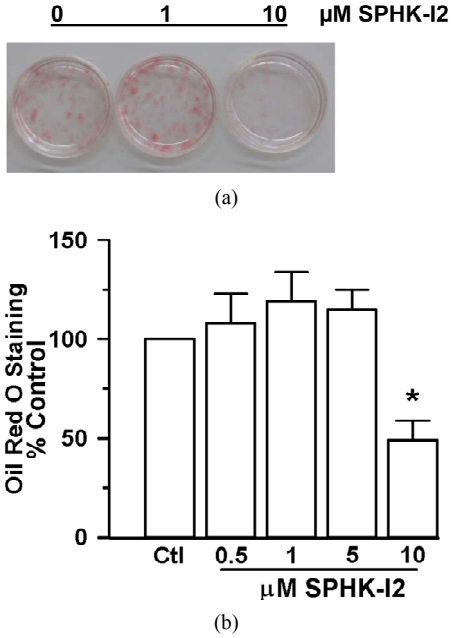
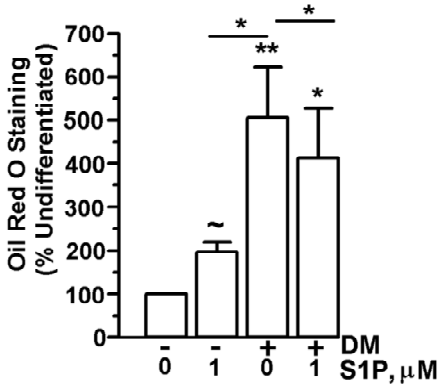


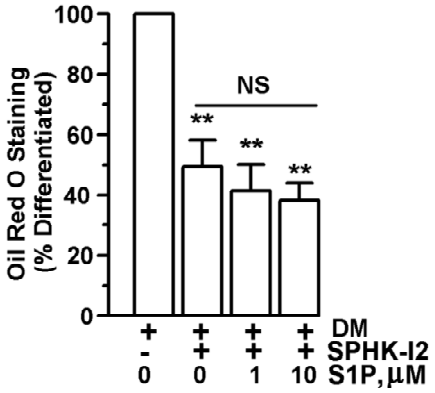
Figure 4. Pharmacologic inhibition of SPHK1 blocks lipid accumulation in adipocytes. 3T3-L1 cells were induced to differentiate in the presence of DMSO (vehicle control) or SPHK1 specific inhibitor SPHK-I2 at concentrations 0.5 - 10 µM. Media was changed every 2 - 3 days, and inhibitor was included throughout the entire differentiation period. On day 15, cells were subjected to Oil Red O staining. (a) Representative photograph of stained adipocytes differentiated in the presence or absence of SPHK-I2. (b) The degree of Oil Red O staining was expressed as a percent of the A_{500nm} compared to the vehicle-treated cells. Values are means ± SE for 3 independent experiments. P value was determined by two-tailed, one-sample hypothesis test. $^{*}p < 0.05$ vs. vehicle-treated adipocytes.

3.4. Extracellular S1P Effects on Early Adipocyte Differentiation Markers

Since treatment of preadipocytes with S1P resulted in significant accumulation of lipid compared to untreated preadipocytes (**Figure 5(a)**), S1P might serve as an early differentiation stimulus. Expression of CEBPα mRNA, an early indicator of commitment to adipogenesis [25], as well as expression of downstream adipocyte specific genes, Acrp30 and GLUT4, increased significantly in a time-dependent manner from days 3 to15 in 3T3-L1 cells cultured in differentiation media (**Figure 6(a)**). To address whether early adipogenesis activators are expressed in response to extracellular S1P, preadipocytes were treated with S1P (1 µM) in the absence of differentiation media for up to 15 days. Cells treated with S1P demonstrated an increase in CEBPα message expression by day 15 of the differentiation process compared to un-

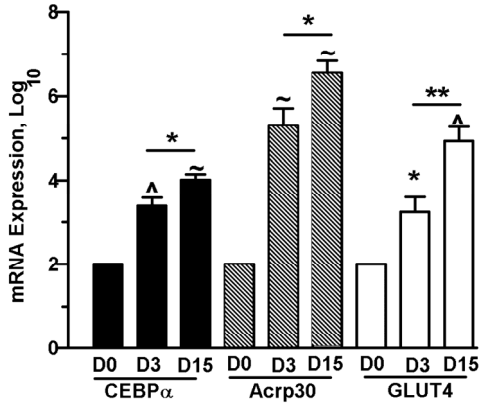


(a)



(b)

Figure 5. S1P does not fully support adipocyte differentiation. 3T3-L1 cells were induced to differentiate in the presence of DMSO (vehicle control), SPHK-I2 (10 μM), with (+) or without (−) the addition of S1P (1 or 10 μM). Control cells were treated with DMEM + 10% FBS lacking differentiation cocktail. Media was changed every 2 - 3 days, and cells were retreated with inhibitor and S1P throughout the entire experiment. Cells were subjected to Oil Red O staining. (a) Oil Red O staining expressed as a percent of A_{500nm} compared to undifferentiated cells cultured in DMEM + 10% FBS. Values are means ± SE for 6 independent experiments. (b) Oil Red O staining expressed as a percent of A_{500nm} compared to differentiated cells not treated with either S1P or SPHK-I2. Values are means ± SE for 4 independent experiments. P value was determined by two-tailed, one-sample hypothesis test. $^{*}p < 0.05$, $^{**}p < 0.025$, $^{\sim}p < 0.01$.



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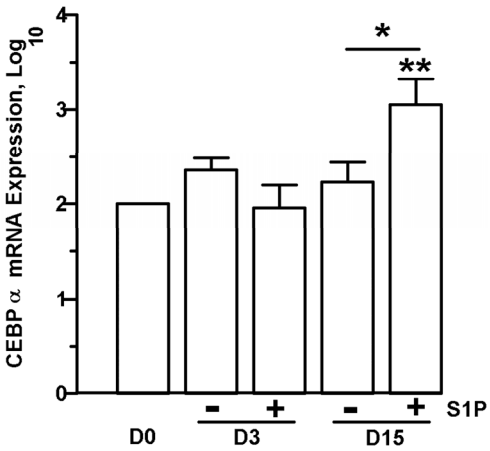

(b)

Figure 6. Prolonged culture of 3T3-L1 cells with S1P increases CEBP*α* mRNA expression. 3T3-L1 were induced to differentiate or were cultured in DMEM + 10% FBS with (+) or without (−) the addition of S1P (1 μM). Cells were harvested on days (D) 0, 3 or 15, and mRNA expression of CEBP*α*, Acrp30, and GLUT 4 relative to 18S rRNA were determined by qPCR. (a) mRNA expression in differenti- ated 3T3-L1 cells. (b) CEBP*α* m RNA expression in 3T3-L1 cells treated with S1P in the absence of differentiation media. Fold increase values over day 0 cells were log transformed and means ± SE were calculated for 4 - 7 ex- periments. P values were determined by two- tailed, one-sample hypothesis test (vs. D0) or two-tailed, two-sample hypothesis test (D3 vs. D15). $^{*}p \le 0.05$, $^{**}p \le 0.025$, $^{\wedge}p \le 0.005$, $^{\sim}p \le 0.0005$.

treated cells (**Figure 6(b)**). While Acrp30 and GLUT4 mRNA levels did increase (Acrp30, 437 fold ± 357 fold; $p < 0.025$; GLUT4, 293 fold ± 187 fold; $p < 0.0.08$) in day 15 cells treated with S1P compared to undifferentiated (day 0) cells, the increase was not statistically significant compared to cells cultured for 15 days in the absence of S1P (data not shown).

3.5. S1PR Expression during Adipocyte Differentiation

S1PRs are expressed in both primary rat preadipocytes and adipocytes [19], however, the expression pattern of S1PRs in the 3T3-L1 model has not been examined. One explanation for the observation that extracellular S1P does not fully support adipogenesis is that expression of

S1PRs in the undifferentiated state is low, thereby limiting S1P-mediated signaling events. Gene expression for three S1PRs—$S1PR_1$, $S1PR_3$, and $S1PR_2$—was measured during the differentiation process by real-time PCR. For all three receptors, mRNA levels were highest in the preadipocytes and declined significantly in a time-dependent manner during the 15 days of differentiation (**Figure 7**). Message expression during differentiation declined by 50% or more at day 3 for all S1PRs analyzed. By day 15, all three S1PR mRNA levels were markedly reduced, with $S1PR_1$ mRNA levels as low as 12 ± 1.4% of preadipocyte levels.

4. DISCUSSION

S1P acts both as an extracellular receptor ligand and intracellular signaling molecule in multiple cell types. A physiologic role for S1P has been demonstrated in mature adipocytes where it signals through S1P-receptors to stimulate lipolysis and modulate leptin secretion [19]. In addition, SPHK isoforms play a role in adipocyte differentiation [18]. The present study demonstrates that SPHK activity increases rapidly and remains elevated in 3T3-L1 cells undergoing terminal differentiation. Total SPHK activity in differentiating adipocytes increased by 6-fold at day 3 and 2-fold at day 15 compared to undifferentiated cells. However, the rapid increase in SPHK activity did not correlate directly with accumulation of SPHK isoform expression. The peak protein levels occurred later than peak enzyme activities and were evident at day 15 for SPHK1 and at day 6 for SPHK2. The early increase in SPHK activity confirms the observations of Hashimoto *et al*. [18] in which intracellular S1P levels increased approximately 3-fold by day 3 post-differentiation, preceding increases in SPHK message and protein. These results indicate that activation of endogenous enzyme is a more important contributor than new enzyme biosynthesis in the early/acute induction of SPHK during adipocyte differentiation. The question of what mediates the activation of SPHK during adipogenesis points to a likely involvement of cAMP in the process. Intracellular cAMP activates SPHK in osteoblast cells [26]. One of the earliest regulators of adipocyte differentiation are the CEBPs [25]. The expression of these transcriptional activators is at least partially mediated by cAMP [27], which increases in preadipocytes treated with differentiation cocktail containing the phosphordiesterase inhibitor IBMX. Treatment of rat adipocytes with S1P (30 μM) also increases cAMP production via S1PR mediated signaling [19], and in the present study, extracellular S1P partially supported adipocyte differentiation. Another regulatory pathway involves protein kinase C (PKC), since phorbol esters induce phosphorylation of SPHK1 [28] through activation of PKC and extracellular signal-regulated kinase 1/2 (ERK)-mediated phosphorylation [29]. Transient activation of the ERK pathway promotes the early stages of adipocyte differentiation [30]. Thus, both cAMP and the ERK pathway may contribute to the increase in SPHK activity seen with initiation of adipogenesis.

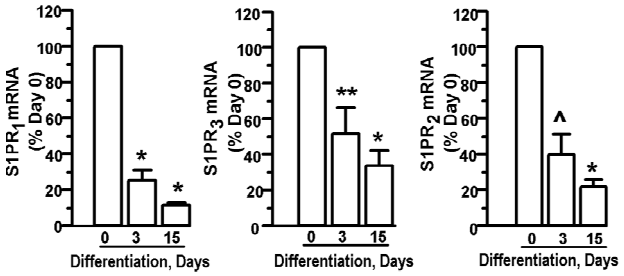

Figure 7. S1P receptor expression declines during adipocyte differentiation: 3T3-L1 cells were induced to differentiate. Cells were harvested at day 0 (preadipocytes), and day 3 and day 15 after initiation of differentiation. Adipocyte levels of S1PR mRNA relative to 18S rRNA for $S1PR_1$, $S1PR_3$, and $S1PR_2$, were determined. Data is expressed as percent of day 0 expression and values are means ± SE for 7 independent experiments. P value was determined by two-tailed, one-sample hypothesis test. $^{\wedge}p < 0.025$, $^{*}p < 0.001$, $^{**}p < 0.0025$.

While activation of endogenous enzyme likely represents the mechanism by which S1P levels rise early in the adipocyte differentiation process, SPHK message and protein accumulate during the differentiation process as well [18]. SPHK activity is regulated both acutely and chronically. Agents which have been demonstrated induce acute activation of SPHK include cytokines, glucose, phorbol esters, and G-protein coupled receptor ligands [22,31-33]. Chronic regulation of SPHK at the level of both transcription and translation has also been demonstrated. Transcriptional regulation of human SPHK1 by phorbol 12-myristate 13-acetate (PMA) in the human leukemia cell line MEG-O1 maps to the 5' promoter region just upstream of the first exon [34]. Promoter mapping identified sequence containing a candidate activator protein-2 (AP-2) site that is essential for PMA regulation of hSPHK1. AP-2 is induced by both PMA and cAMP participating in the activation of PMA- and cAMP-inducible genes [35]. In dopaminergic neuronal cells, neurotrophic factors increase SPHK1 transcription through CEBPs [36]. Initiation of adipocyte differentiation is dependent on accumulation of cAMP; thus, the activtion of SPHK during adipogenesis at the transcriptional level may be mediated by a similar transcriptional regulatory element. In addition, computer analysis using the TFSEARCH program (http://www.cbrc.fp/research/db/TFSEARCH.html) reveals a putative CEBPα transcription factor binding site upstream of the murine SPHK1 transcription start site that may be involved in the chronic regulation of SPHK1 message and protein during differentiation.

Several SPHKs have been reported for species including human, rat, mouse, yeast, and plants [14]. The contribution of individual SPHK isoforms to cellular function appears to be at least partially redundant as evidenced by the fact that specific phenotypes are demonstrated only when both SPHK isoforms are deleted [17]. In the present study, Western blot analysis of cytosolic lysates demonstrated that both SPHK1 and SPHK2 protein are induced in differentiating adipocytes. SPHK2 increased maximally early in the differentiation process, whereas SPHK1 increased more gradually with a maximum by 15 days. Such differences may be due to translation rates of the different genes, or to half-lives of the proteins themselves. We have previously demonstrated in insulinoma cells that the both SPHK isoforms are very stable, suggesting that upregulation of translation is the predominant mechanism for enzyme accumulation during adipocyte differentiation [33]. Moreover, since neither KCl nor TX-100 completely inhibited SPHK activity, both isozymes contribute to the total SPHK activity in preadipocytes and during adipocyte differentiation. SPHK activity was significantly activated by TX-100, which has been demonstrated to stimulate SPHK1 activity in cellular lysates. These results suggest that both SPHK1 and SPHK2 are expressed and active during adipocyte differentiation, with SPHK1 contributing more significantly to overall activity early in the differentiation process. The data also show that SPHK activity is inactivated or desensitized at the terminal stage of differentiation, even though SPHK1 and 2 protein levels are significantly elevated.

The observation that SPHK1 isozyme contributes significantly to the overall activity induced early in the differentiation process is confirmed by treatment with a specific pharmacologic SPHK1 inhibitor, SPHK-I2, which resulted in a partial inhibition of adipogenesis, mirroring inhibition of adipogenesis via genetic down-regulation of SPHK1 activity [18]. In human cancer cell lines overexpressing SPHK1, SPHK-I2 inhibits proliferation at lower concentrations than used in this study (IC_{50} 1 - 4.6 μM) [37]. However, the finding that differentiation was not completely inhibited by a SPHK1 inhibitor supports previous evidence that SPHK2 can partially compensate for SPHK1 activity during development and differentiation [17]. Alternatively, while extracellular S1P did not restore adipogenesis in the face of SPHK1 inhibition by SPHK-I2, S1P in this compartment may initiate adipogenesis.

The process of adipocyte differentiation is characterized by growth arrest, synchronous mitotic clonal expansion, followed by terminal differentiation [1]. Stimulation of adipogenesis leads to increases in intracellular S1P production, correlating with SPHK activation (**Figure 1**). However, it is not clear whether S1P participates in adipocyte differentiation via intracellular or extracellular signaling events. Extracellular S1P-mediated signal transduction occurs through the family of S1PRs which participate in cell growth, differentiation, and protection from apoptosis. Treatment of preadipocytes with micromolar concentrations of S1P leads to cAMP generation [19], an essential step in the promotion of adipogenesis. In this study, we tested whether extracellular S1P was sufficient to support adipogenesis. Although treatment of preadipocytes with S1P did result in a two-fold increase in lipid accumulation, overall, the degree of differentiation was significantly less than that with standard differentiation media. In fact, co-treatment of preadipocytes with DM and S1P attenuated lipid accumulation. In addition, while culture of preadipoctyes with S1P alone resulted in accumulation of CEBPα mRNA, the degree (~30% stimulation) and time-course of activation (as late as day 15) were significantly lower and later than that seen with differentiation media. S1P did promote adipogenesis as evidenced by Oil Red O staining, although the magnitude of the response was about half that of differentiation media alone. The failure of extracellular S1P to fully support adipogenesis may be in part explained by S1PR expression. Although expression of $S1PR_{1\text{-}3}$ mRNA is actually highest in undifferentiated cells, the mRNA levels of each receptor precipitously decline throughout the course of differentiation. If receptor protein parallels the mRNA expression levels, then S1P signal transduction could be severely impacted as differentiation proceeds. Alternatively, since S1P has growth-promoting effects, treatment of preadipocytes with S1P may interfere with the ability of the cells to undergo growth arrest, which is essential for commitment to adipocyte differentiation.

In summary, the evidence strongly suggests that intracellular SPHK activity and S1P are important contributors to adipogenesis. SPHK is active in differentiating adipocytes, with enzyme activation preceding changes in protein levels. Both SPHK1 and 2 are expressed, and enzyme activity is modulated during initiation of differentiation. Moreover, pharmacologic inhibition of SPHK1 negatively impacts adipogenesis. However, the evidence argues against a major role for extracellular S1P in modulating adipogenesis. Treatment of preadipocytes with extracellular S1P induced a modest accumulation of lipid that was not additive with and even reduced the effectiveness of differentiation media. Moreover, the sphingolipid did not rescue adipogenesis from the inhibition of SPHK1 by SPHK-I2. Rapid declines in S1PR message during differentiation may explain why S1P does not fully support adipogenesis. Alternatively, the loss of these receptors may represent a biologic transition from a rapidly dividing cell line to a growth arrested lineage capable of differentiating.

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Healthcare-transition risk assessment for emerging adults with diabetes type 1*

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ABSTRACT

AIM: To identify potential risk indicators for emerging adults transitioning to adult health care. BACKGROUND: Without maturity, independence, knowledge and motivation to manage their diabetes and successfully transition to adult healthcare, a proportion of emerging adults will struggle, leaving themselves vulnerable to diabetes-related complications. METHODS: Fif- ty-three emerging adults (aged 17 - 19 years) recently transitioned from pediatric to adult healthcare were recruited. Data included demographic, glycated haemoglobin, Body Mass Index, base-line and 12-month data from four psychosocial measures: Problem Areas in Diabetes, Diabetes Empowerment Scale, Hospital Anxiety and Depression Scale and dichotomous questions from the Eating Attitude Test. Missed appointments were obtained from hospital records. RESULTS: No significant differences in age, gender, BMI and individual survey scales between three appointment groups. Median HbA1c in the Sat-1 group was 7% (53 mmol/mol), compared with 8.6% (70 mmol/mol) and 8.5% (69 mmol/mol) respectively in the Sat-2 and Unsatisfactory groups. A ROC curve analysis and classification tree analysis identified optimal threshold values for the survey scales and their linear combinations. These values were used to make comparisons across two appointment and three HbA1c status groups. The unsatisfactory appointment group was characterised by statistically higher proportions of participants with (1) a low score for empowerment alone or in combination (less) with perceived problems and (2) statistically higher perceived problems and anxiety scores. CONCLUSIONS: Findings suggest that diabetes self-care confidence, less perception of diabetes as a problem, could be a useful indicator of future appointment attendance. Similarly, depression levels could be a useful predictor of better metabolic control following transition. RELEVANCE TO CLINICAL PRACTICE: This study offers an innovative use of existing metrics to identify at-risk emerging adults in a busy clinic. Rather than the emphasis being solely on blood glucose control, it may be time to consider including psychosocial measures to identify at-risk individuals at the first appointment in adult healthcare.

Keywords: Diabetes; Healthcare Transition; Emerging Adults; Risk Assessment

1. BACKGROUND

Health care transition for some young adults with diabetes is a difficult time with high rates of drop-out, poor attendance and sub-optimal glycemic control potentially putting individuals at higher risk of future diabetes-related complications [1-3]. Diabetic ketoacidosis (DKA) is the most immediate life-threatening complication. In addition to loss-to-follow-up [4,5] and infection [6,7], the commonest DKA precipitants are problems related to self-management including insulin omission [8,9], emotional stress and distress, or lack of application of sick-day rules [7,10,11]. Whilst many emerging adults [12] have attained the maturity, independence, knowledge and motivation to manage in a changing environment and will achieve successful transition, a proportion will struggle at this time, leaving themselves vulnerable to the problems of poor control and complications. Since "each 1% fall in HbA1c concentration leads to an estimated fall

*Conflict of interest: The authors declare they have no conflict of interest.

of 30% in the risk of microvascular complications" [2, p. 260], there is concern for this "at-risk" group who make up a disproportionately large percentage of those who ultimately fail to achieve effective metabolic control after transition.

Although several transition models exist to facilitate transition by improving the emerging adult's engagement with adult healthcare [5,13,14], risk assessment is also needed to identify who is most in need. In general there is agreement in the literature that measures to maintain three to four appointments per year are necessary as fewer appointments after transition is associated with higher HbA1c [5]. Hence, effective identification of those "at-risk" of eating disorders [1], missing appointments or disengaging with adult healthcare is needed. The aim of this study was to evaluate the use of four psychosocial variables, alone or in combination, in identifying these emerging adults at the first appointment after transition to adult health care.

2. METHODS

2.1. Research Design and Data Collection

A prospective cohort study design was used to capture consecutive new referrals with diabetes type 1 (DT1) over two years. The location was a dedicated Young Adult Diabetes Transition Clinic at a large metropolitan teaching hospital. Written, informed consent was obtained from all participants and the hospital's Human Research Ethics Committee approved the study. Demographic variables, biochemistry and four measures of psychosocial functioning: Problem Areas in Diabetes (PAID) questionnaire [15], Diabetes Empowerment Scale (DES) [16], Hospital Anxiety and Depression Scale (HADS) [17] and dichotomous questions from the Eating Attitude Test (EAT) [18]) were collected at baseline (T_0) and after twelve months (T_{12}). All participants were offered three-monthly follow-up visits as per clinic protocol. Missed or rescheduled appointments were obtained from hospital records.

2.2. Instrument Reliability

Each of the four psychosocial measures has been validated independently in similar populations previously and chosen for their psychometric strengths. With the SPSS reliability analyses set to pair-wise deletion of missing data, tests for this study were found to be consistent with extant literature. For example, the Cronbach's internal reliability alpha for PAID in the current sample was 0.93 (n = 53) and 0.94 (n = 37) at T_0 and T_{12} respectively. This 20-item five-point Likert-scale covers a range of emotional states frequently reported in diabetes and recommended for screening both depression and emotional problems [19]. Scores reflect the degree to which the item is perceived as being problematic (0 = no problem to 4 = serious problem). Total score is multiplied by 1.25 (range 0 - 100) as recommended [20, p. 70]. Higher scores indicate greater emotional distress.

The short-form of the DES-SF [21] measures diabetes related psychosocial self-efficacy or confidence to manage diabetes regimen, readiness to change behaviour, and perceived ability to set goals. The developers reported that both DES-SF and HbA1c levels changed in positive direction but were not correlated, suggesting that the two measures varied independently. The eight-item scale provides a brief overall assessment of diabetes related psychological self-efficacy. It has a five-point Likert scale and score range of 8 - 40. The reliability of the DES-SF tested against the original data set collected for the DES demonstrated an alpha of 0.85 and 0.84 when retested with a further 229 subjects. Similarly, in the current study, DES-SF demonstrated an alpha of 0.83 (n = 52) and 0.88 (n = 37) at T_0 and T_{12} respectively.

The four-point, 14-item, self-report HADS is based on the symptoms of anxiety and depression rather than emotional and physical disorders. Scores for each item range from zero to three with total scores for both seven-item subscales greater than 11 out of 21 equating to increased anxiety or depression. Over the last two decades the HADS has been used to quantify anxiety and depression within many specialities that use outpatient clinics in a similar manner to the subjects of interest in this study, including a diabetes clinic [22]. Reliability testing of the HADS subscales for the current sample indicated Cronbach's alpha scores for the depression subscale were the lowest at 0.77 and 0.66 at T_0 and T_{12} respectively, while scores for the anxiety subscale were 0.85 and 0.81 at T_0 and T_{12} respectively.

The EAT has been used as an outcome measure in many treatment studies and as a screening instrument for eating disorder risk. The four dichotomous questions in the EAT-26 focus on whether or not participants experienced eating binges, made themselves sick (vomited) to control weight or shape, used laxatives, diet pills or diuretics to control weight or shape, or had been treated for an eating disorder. A diagnostic interview is recommended if a "yes" response is given to any of the questions [23]. Given the literature on insulin abuse, an insulin-related question was also added: *Have you recently thought of, or withheld insulin to control your weight or shape*?

2.3. Analysis

Statistical analyses were completed using the IBM SPSS statistics version 19 package and in the R statistical computing environment, version R 2.13.1. Where missing data existed, analyses were carried out on the restricted data set: Imputation methods were not used. The

study group were classified firstly according to whether they had attended a satisfactory (≥3) number of appointments or not (<3 unsatisfactory). They were also categorised by HbA1c status in two ways. A three-category HbA1c status denoted a satisfactory classification as either HbA1c of 7.5% (59 mmol/mol) or less at T_0 that was maintained at or below 7.5% at T_{12} (Sat-1), or, in the case of baseline HbA1c being greater than 7.5%, the change in HbA1c from T_0 to T_{12} was less than 0.5% (Sat-2). The remaining participants had an HbA1c greater than 7.5% at T_0 and their change in HbA1c from T_0 to T_{12} was 0.5% or more (Unsat). The 2-category satisfactory HbA1c status combined Sat-1 and Sat-2 to form one satisfactory group to compare with the unsatisfactory HbA1c status group.

Descriptive statistics included median (LQ = lower quartile, UQ = upper quartile) for continuous variables and counts (%) for categorical variables. Distributions of the variables of interest were compared between appointment groups and also by HbA1c status, using the Mann-Whitney or Kruskal-Wallis test and χ^2 or Fisher's Exact test, as appropriate. Statistical significance was set at 5%. Cronbach's alpha was used to test internal reliability of measurements.

As well as the continuous survey scale variables, we evaluated 1) linear combinations of PAID and DES, and Anxiety and Depression scales; 2) threshold levels for survey scale variables; and 3) threshold levels for linear combinations of survey scale values. Survey scores were scaled to the same range before pairing, so that one scale did not dominate the other. To locate suitable threshold values, we used receiver operating characteristic (ROC) curve analysis and the partitioning methods of regression trees. The resulting threshold values were used to create binary predictors.

The predictive value of the survey scales was evaluated by constructing reclassification tables, and calculating the Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement (IDI) measures, following the method of Pencina *et al*. [24]. The NRI summarises the proportional changes in correct classifications due to introducing a predictor into a model. The IDI assesses improvement in case discrimination, by estimating the change in the difference in the mean predicted probabilities of the outcome between those with and without the outcome in question. The better model is characterised by a greater difference in IDI than observed in a comparatively poor prognostic model. Each survey scale, linear combination or threshold variable identified in the previous section was assessed after being added sequentially to a base model adjusted for age, gender and baseline BMI and HbA1c. Then a final enriched model was compared to the base model to evaluate the maximum possible improvement in predictive ability afforded by the addition of all significant variables.

3. RESULTS

Over the two-year study, the Young Adults with Diabetes (YADs) transition clinic at the study hospital received 72 new referrals from the local paediatric hospital. Of these, 58 attended at least one appointment and 53 (91%) volunteered for the study. Baseline demographic and biochemical features are shown in **Table 1**. Thirty-nine participants attended three or more appointments over the 12-month study (classified as satisfactory atten-

Table 1. Summary statistics at T_0 for all participants, participants classified by appointment attendance and summary statistics at T_0 and T_{12} for the 37 participants attending at T_{12}. Values are median (quartiles) for continuous data and count (%) for categorical.

	All (N = 53) appointment attendance				37 participants attending at T_{12}	
	T_0 values	Satisfactory ≥ 3:39 (74%)	Unsatisfactory < 3:14 (26%)	*P* value	T_0	T_{12}
Male	20 (38%)	13 (33%)	7 (50%)	0.341		11 (30%)
Age	18 (17.3, 19)	18 (18, 19)	18 (17.3, 19)	0.948		18 (18, 19)
HbA1c %	8.4 (7.7, 9.4)	8.2 (7.3, 9.1)	9.2 (8.4, 10.3)	0.056	8.2 (7.7, 9.2)	8.6 (7.1, 9.9)
mmol/mol	68 (61, 79)	66 (56, 76)	77 (68, 89)	0.056	66 (61, 77)	70 (54, 85)
BMI	24.3 (22.1, 26.7)	24.6 (22, 27)	23.8 (23, 25.6)	0.896	24.7 (22.1, 27.2)	23 (21, 27)
PAID	26.3 (15, 38.8)	23.8 (13.1, 33.8)	33.8 (20.3, 50)	0.120	23.8 (15, 33.8)	25.6 (15, 39.1)
DES	30 (27, 33)	30 (28, 33.5)	29 (24, 32.3)	0.159	31 (28, 34)	30 (24.8, 34.3)
Depression	2 (1, 3)	2 (1, 3)	2 (1, 4)	0.539	2 (1, 3)	2 (1, 4)
Anxiety	5 (4, 8)	5 (3, 7)	5 (4, 10.8)	0.417	5 (4, 7)	6 (4, 9)
EAT	0 (0, 1)	0 (0, 1)	0 (0, 1)	0.789	0 (0, 1)	0 (0, 1)

dance) and 14 attended two or less appointments (unsatisfactory attendance). Four participants in the satisfactory appointment category did not have a T_{12} appointment or HbA1c data. The majority of participants (35/37) for whom biochemical data were available at T_0 and T_{12} were also in the satisfactory appointment group (**Table 1**).

The median age of the sample was 18 years (range 16 - 24) and 38% were male. At baseline, median BMI was 24 (22, 27) kg/m^2 and HbA1c was 8.4 (7.7, 9.4)% or 68 (61, 79) mmol/mol. For the 37 participants with baseline and T_{12} data (**Table 2**), the median increase was 0.2%, however individual variations were as large as 4% (20 mmol/mol) above and below T_0 HbA1c. Likewise, the differences between T_0 and T_{12} for BMI and all the survey scales were not statistically significant (all $P > 0.2$).

3.1. Appointment Attendance

There were no significant differences in age, gender, BMI and the survey scales between the satisfactory and unsatisfactory appointment groups (all $P > 0.1$; **Table 1**). However, the data suggest a trend towards higher baseline HbA1c in unsatisfactory attendees (median 9.2 vs 8.2 in satisfactory attendees, $P = 0.056$), and towards higher PAID score (median 33.8 vs 23.8, $P = 0.120$). The change in HbA1c (ΔHbA1c) from T_0 to T_{12} could not be compared between the 2 appointment groups, since unsatisfactory attendees were largely absent at T_{12}. While EAT score did not significantly distinguish between appointment attendance categories, there was a trend towards higher scores among female participants ($P = 0.055$): overall more than 25% of females scored above zero compared to 5% of males. For unsatisfactory attendees, EAT scores were similar across genders, while in the satisfactory attendance group all males scored zero or one, but more than 30% of females scored above one on the test.

3.2. HbA1c Status

None of age, gender, BMI or T_0 survey scales was significantly different between satisfactory and unsatisfactory HbA1c groups (all $P > 0.1$). When the Sat-1 and Sat-2 sub-categories were evaluated, results were similarly not significant in comparisons across the 3 groups (see **Table 2**). By definition, HbA1c status reflects information about T_0 HbA1c as well as ΔHbA1c (Δ = change) from baseline to T_{12}. Median HbA1c in the Sat-1 group was 7% (mmol/mol), compared with 8.6% (70 mmol/mol) and 8.5% (69 mmol/mol) respectively in the Sat-2 and Unsatisfactory groups ($P < 0.005$ for the comparison between Sat-1 and Sat-2 or unsatisfactory groups). Median ΔHbA1c was 2% in the unsatisfactory

Table 2. Summary statistics by HbA1c status group at T_{12} (n = 37): median (LQ, UQ) for continuous variables, counts (%) for categorical variables.

	Satisfactory		Unsatisfactory	
HbA1c Status	Sat-1	Sat-2		*P* value
n (%)	7 (19%)	16 (43%)	14 (38%)	
Gender (Male)	2 (29%)	5 (31%)	4 (29%)	1
Age	18 (17.5, 18)	18 (18, 19)	18 (18, 18.8)	0.576
T_0 HbA1c %	7 (6.2, 7.3)	8.6 (8.2, 10.4)[a]	8.5 (7.7, 9.3)[ab]	<0.001
T_0 HbA1c mmol/mol	53 (44, 56)	70 (66, 90)	69 (61, 78)	<0.001
ΔHbA1c $T_{12}-T_0$:				
%	−0.1 (−0.4, 0.2)	−0.7 (−1.5, −0.3)	2.0 (1.1, 2.7)	<0.001
mmol/mol	−1.1 (−3.8, 1.6)	−7.1 (−16.1, −3.0)	21.3 (12.029.2)	<0.001
BMI	25.6 (23.4, 30.7)	25.4 (22, 27.1)	22.4 (20.9, 25.7)	0.350
PAID	28.8 (20.6, 48.1)	20.6 (11.9, 30.3)	29.4 (19.4, 33.8)	0.228
DES	30 (29, 32)	32 (28.3, 35)	30 (27.3, 32.8)	0.661
Depression	3 (2, 3)	2 (1, 3.3)	2 (1, 2)	0.273
Anxiety	5 (2.5, 10)	5.5 (4, 7)	5 (4, 6)	0.908
EAT	1 (0, 2)	0 (0, 1)	0 (0, 1)	0.596

Key: Δ = change, [a]$P < 0.005$ comparison with Sat-1 group, [b]$P > 0.05$ comparison between Sat-2 and Unsatisfactory group. The *P*-values for the mmol/mol and % versions of HbA1c are identical because the former is a linear transformation of the latter.

group, and overall showed increases of 0.7% up to a maximum of 4% (mmol/mol). The Sat-1 participants were those who remained stable, with change confined to within ± 1% of T_0 HbA1c. In Sat-2 participants, HbA1c increase was by definition less than 0.5%, but notably 75% of these participants demonstrated a decrease in HbA1c by T_{12}. When we examined ΔHbA1c as a continuous variable, there were no significant predictors among individual survey scales (all $P > 0.1$), though we observed trends for ΔHbA1c to increase as depression and EAT scores decreased ($P = 0.134$, 0.135). That is, there was a trend for participants who had lower depression or EAT scores at T_0 to also have higher HbA1c values a year later.

3.3. Threshold and Linear Combinations of Survey Scales

We explored the predictive value of linear combinations of PAID and DES, and anxiety and depression subscales. After scaling each pair to the same value range, we found that there was a trend for DES-PAID to be about 20 points lower among unsatisfactory attendees ($P = 0.055$, **Table 3**). The same combination was not statistically significant in distinguishing HbA1c status, but we note that median DES-PAID in the HbA1c Sat-2 participants was 10 to 20 points higher than in the Sat-1 or Unsatisfactory HbA1c participants ($P = 0.257$). Anxiety/Depression combinations were not significant for any of the responses.

From ROC curve analysis and classification tree analysis, we identified optimal threshold values for the survey scales and their linear combinations, and used these values to make comparisons across attendance and HbA1c status groups (**Table 3**). We found that the unsatisfactory appointment group was characterised by higher proportions of participants with low DES-PAID and DES ($P = 0.005$, 0.003) and higher PAID and Anxiety scores ($P = 0.046$, 0.049). For example: 50% have PAID scores greater than 36, compared with 21% of satisfactory attendees; 79% have DES-PAID below 38, compared with only 31% of satisfactory attendees.

Among the 37 participants assessed for HbA1c status, we found that, while a threshold DES-PAID variable did not distinguish HbA1c satisfactory or unsatisfactory status, the Sat-2 group was characterised by a higher proportion with high DES-PAID (≥57) than either the Sat-1 or unsatisfactory groups ($P = 0.026$). Participants with unsatisfactory HbA1c were more likely to have a low score on the *depression* subscale than those with satisfactory HbA1c (93% vs 48%; $P = 0.011$ for unsatisfactory vs all satisfactory; $P = 0.020$ for test over 3 categories).

3.4. Predictive Ability of Survey Scales

The NRI and IDI results in **Tables 4(a)** and **(b)** are illustrated for variables found to be significant or of interest in the previous sections. We found the variables EAT (stratified by gender), anxiety less than 10 and DES-PAID less than 38 were significant in increasingly accurate prediction of appointment attendance when added sequentially to a model already adjusted for age, gender and T_0 BMI and HbA1c. Threshold DES-PAID was chosen ahead of either scale separately, as it provided more accurate predictions in the final model. The full enriched model showed a NRI of 1.25 and IDI of 0.43 (both $P < 0.001$), and was 91% accurate in predicting appointment group. Among the 14 study participants who defaulted on appointments, reclassification was more accurate when these variables were included (71% correctly classified with risk greater than 50%, compared to 29% in the base model). Among the 39 satisfactory Attendees, reclassification was slightly less accurate

Table 3. Potential risk indicators: Median (LQ, UQ) DES-PAID and counts (%) for threshold variables by Appointment Group and the 3-category HbA1c status.

		Appointment attendance			HbA1c status			
T_0 variable	Threshold	Satisfactory	Unsatisfactory	*P* value	Sat-1	Sat-2	Unsatisfactory	*P* value
DES-PAID	-	49.4 (23.1, 65.9)	28.4 (19.5, 36.3)	0.055	49.4 (17.5, 50.0)	59.7 (34.7, 67.3)	41.6 (24.8, 53.0)	0.257
DES-PAID	<38	12 (31%)	11 (79%)	0.005	-	-	-	
DES-PAID	<57	-	-	-	6 (85%)	7 (44%)	12 (86%)	0.026
DES	<25	2 (5%)	6 (43%)	0.003	-	-	-	-
PAID	<36	31 (79%)	7 (50%)	0.046	-	-	-	-
Anxiety	<10	34 (87%)	8 (57%)	0.049	-	-	-	-
Depression†	<3	-	-		3 (43%)	8 (50%)	13 (93%)	0.020

Key: P-values from Kruskal-Wallis or $\chi 2$ test, † significant for both 2 and 3-category HbA1c status.

Table 4. (a) Reclassification table of appointment attendance predictions from enriched models when compared to base model with predictors gender, age, T_0 BMI and T_0 HbA1c. Net Reclassification and Integrated Discrimination Improvements (NRI/IDI) shown with misclassification rate; (b) Reclassification table of HbA1c predictions from enriched models when compared to base model with predictors gender, age, BMI and T_0 HbA1c. Net Reclassification and Integrated Discrimination Improvements (NRI/IDI) shown with misclassification rate.

(a)

Base Model: gender + age + T_0 BMI + HbA1c App't attendance		NRI/IDI	*P* value		Risk	Enriched model				*P* value
					≥3 App'ts	Risk ≤ 50%	Risk > 50%			
Variables added	DES-PAID < 38	0.96/0.15	0.002/0.007	Under Base Model	≤50%	38 (97%)	1 (3%)	Misclassification rate 9%		
	EAT[#]	0.23/0.09	0.459/0.038		>50%	0	0			
	Anxiety < 10	0.62/ 0.07	0.047/0.040		<3 App'ts			NRI	1.25	<0.001
					≤50%	4 (29%)	7 (50%)	IDI	0.43	<0.001
					>50%	0	3 (21%)			

Key: [#]EAT stratified by gender.

(b)

Base Model: gender + age + BMI + T_0 HbA1c		NRI/IDI			Risk	Enriched model				*P* value
HbA1c Status			*P* value		Satis HbA1c	Risk ≤ 50%	Risk > 50%			
Variables added	DES-PAID < 57	0.67/0.15	0.048/0.002	Under Base Model	≤50%	16 (70%)	4 (17%)			
	Depression < 3	0.90/0.17	0.008/0.010		>50%	2 (9%)	1 (4%)			
					Unsatis HbA1c			NRI	1.14	<0.001
					≤50%	3 (21%)	7 (50%)	IDI	0.33	<0.001
					>50%	0	4 (29%)			

when these variables were added (3% erroneously classified with risk more than 50%, compared to 0% in the base model).

The inclusion of a DES-PAID score less than 57 and a Depression score less than three in the model for HbA1c status provided a NRI of 1.14 and IDI of 0.33 (both $P < 0.001$). The prediction accuracy of HbA1c status in the full model was 78%. Among the 14 participants with unsatisfactory HbA1c, this improved reclassification by more than 50% (79% correctly classified with risk more than 50%, compared with 21% in the base model). Among the 23 participants with satisfactory HbA1c, reclassification was slightly less accurate when these variables were added (79% correctly classified with risk less than 50%, compared with 87% in the base model).

4. DISCUSSION

Healthcare transition is a difficult time with potentially poor outcomes for significant numbers of the emerging adult cohort with diabetes type 1. Our findings offer an innovative use of existing metrics to identify those at-risk in a busy clinical environment—particularly when each is unknown to the other, when we know that it can be a time of worsening glycemic control, and that psychosocial functioning will continue to decline into adulthood if adjustment problems are not remedied early [1, 25].

While three of the four psychosocial scales performed as predicted, alone they were unable to predict metabolic control or clinic attendance in this sample. Nevertheless, our findings are consistent with the prevailing view that more frequent appointments after transition are linked to improved HbA1c [2,5,26,27]. Similarly, the EAT scale, consistent with other studies [1,28,29], re-enforces the high frequency of potential eating disorders in females of this age-group—a useful initial metric for the diabetes educator.

In relation to composite measures, at least one (DES-PAID) could be a useful predictor of who might neglect their self-management during this vulnerable transition period. This novel use of two standard psychosocial measures suggests that a person's level of diabetes self-care confidence, less their perception of diabetes as a problem, could be a useful indicator of future appointment attendance. Similarly, the use of DES-PAID with a depression score of less than three could be a useful predictor of better metabolic control following transition. Glycated haemoglobin levels are known to be related to diabe-

tes-regimen confidence [30-32]. Diabetes-related confidence can also mediate the link between HbA1c and depression [33].

While the nexus between depression and DT1 is not consistent [34], assessment for emotional vulnerability that may impact on metabolic control [35] and or clinic attendance remains an important focus for future research. Understandably, perceptions of ineffective control over one's health play a role in the development of depression [33]. Yet the significant difference in depression scores for participants in this study is counterintuitive but could be interpreted using Thomas Gray's concept—"where ignorance is bliss, tis folly to be wise". Participants are not sufficiently worried about the effects of high HbA1c levels on their body to be anxious or depressed. This notion is also consistent with the finding for pump patients [36]—higher anxiety scores being associated with better control. Therefore, further work to extend the results of this study is needed. The interaction of diabetes-management confidence, problem-focused perceptions about diabetes, anxiety and depression for at-risk emerging adults at the time of transition is unknown. Certainly, others have recommended routine depression screening after transition [3,37,38].

This study has several limitations, the most obvious being the small volunteer sample from one hospital and missing values. The missing data were mainly due to participants' missed appointments, the phenomenon at the heart of the study itself. This potential for biased study results requires cautious use of the findings. Apart from this, the study used four psychometrically reliable and valid self-report measures with three of the measures achieving acceptable reliability scores for this sample. Whilst the longitudinal data provide temporal relationships for participants, non-participants are either confident about their self-management and that appointments are unnecessary, or they are using avoidance as a coping tactic. This study is unable to test either assumption.

5. CONCLUSION

Life for emerging adults is more than glucose control. The concomitant and potentially dissonant need to maintain glucose control as well as peer-relationships requires choices. Short-term social rewards that strengthen peer-group bonds mean that risks associated with being different, as in overt diabetes-related activities, are to be avoided [39]. Hence, there may be little consideration by the adolescent of short or long-term health consequences of missed appointments or other decisions made for social rewards; especially when the emerging adult feels "bullet-proof". Rather than the health professionals' emphasis being solely on blood glucose control, it may be time to consider including psychosocial measures to identify at-risk adolescents at the first appointment in adult healthcare.

5.1. Relevance to Clinical Practice

Whilst at-risk individuals are in particular need of the recommended three-to-four appointment per year for two years post-transition [3,27,40], the importance of their first appointment cannot be underestimated; clinically or generally. As part of the important early assessment of self-management skills around insulin management, eating behaviours and exercise [41], data from this study suggest it is time to consider including psychosocial measures to identify at-risk adolescents. An initial appointment process within an expanded diabetes educator role could help identify at-risk individuals who could benefit from targeted education or referral. A low cost initiative could be to give more attention to first appointment individuals with high HbA1c who see diabetes as a problem and or have low diabetes-related self-management confidence.

5.2. Contributions

Study design (PR, JK, GB), Data Collection (PR, GB), Manuscript preparation (PR, JK, GB, SB).

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Downregulation of Nrf2 and HO-1 expression contributes to oxidative stress in type 2 diabetes mellitus: A study in Juana Koslay City, San Luis, Argentina

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ABSTRACT

Oxidative stress is associated with diabetes mellitus, a condition characterized by increased prevalence and progression rate of cardiovascular disease. NFE2-related factor 2 (Nrf2) is a master regulator of cellular detoxification responses and redox status. The aim of this study was to examine associations between type 2 Diabetes Mellitus (T2DM), oxidative stress and the expression of NFE2-related factor 2 (Nrf2) in a population of diabetic patients living in Juana Koslay City, San Luis, Argentina. In addition, we evaluated the functional relevance of Nrf2 by measuring the HO-1 expression among persons with type 2 diabetes. We measured clinical and biochemical parameters related to lipid metabolism and oxidative stress in a population of Type 2 Diabetes Mellitus patients (T2DM, n = 40) and controls (Co, n = 30). Compared to Co, T2DM patients had higher fasting serum glucose, glycated hemoglobin, triglycerides, total cholesterol, low-density lipoprotein cholesterol, and thiobarbituric acid reactive substances and lower high-density lipoprotein cholesterol. T2DM individuals had also higher atherogenic index and body mass index than controls. We also founded that HO-1 mRNA in whole blood was lower in T2DM than controls, suggesting that T2DM may have an altered antioxidant response to oxidative stress. Interestingly, we found reduced Nrf2 mRNA in whole blood from T2DM compared to Co. The results from this study provide novel evidence that genes associated to antioxidant defense mechanisms are markedly reduced in patients with type 2 diabetes, and that the reduction in the expression of these genes could be associated to hyperglycemia and increased levels of MDA. Linear regression analysis revealed that there was a strong and positive correlation between the changes of Nrf2 and HO-1 expression levels.

Keywords: Type 2 Diabetes Mellitus; HO-1; Nrf2 and TBAR'S

1. INTRODUCTION

The pathogenesis of Type 2 Diabetes Mellitus (T2DM) has not been fully elucidated; however, there is growing evidence linking this disease to oxidative stress [1-3]. Oxidative stress, resulting from increased production or decreased removal of reactive oxygen species (ROS), plays a key role in the pathogenesis of late diabetes complications [2] and insulin—stimulated glucose uptake [4].

Increasing evidence indicates that increased production of reactive oxygen or nitrogen species (ROS or RNS) and/or impaired endogenously protective mechanism is the major factor responsible for the development and progression of vascular complications in diabetic patients,

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although several other mechanisms were also proposed [5-7].

One of the most important antioxidant machineries is the Nrf2 system, with the transcription factor Nrf2 as the central component [8]. The transcriptional factor NFE2-related factor 2 (Nrf2), a member of the cap'n'collar family, is a master regulator of cellular detoxification responses and redox status [9]. Under physiological conditions Nrf2 locates in the cytoplasm and binds to its inhibitor kelch-like ECH-associated protein 1 (KEAP1) [10]. KEAP1 could mediate a rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome [10]. Upon exposure of cells to oxidative stress or electrophilic compounds, Nrf2 is released from KEAP1 and translocates into the nucleus. There, it binds to antioxidant-responsive elements (ARE) in the genes encoding antioxidant enzymes such as NADPH quinoneoxidoreductase (NQO1), heme oxygenase-1 (HO-1), glutathione S transferase, superoxide dismutase (SOD), catalase, and γ-glutamylcysteine synthetase, increasing their expression to play a role in the detoxification, antioxidant, and anti-inflammatory processes [9-11].

Among a panel of potential candidate genes related to oxidative stress, the heme oxygenase-1 (HO-1) gene has drawn much attention with its potent antiinflammatory, antioxidant, and antiproliferative effects [12]. Heme oxygenase is a microsomal rate-limiting enzyme responsible for the oxidation and degradation of heme into biologically active metabolites—biliverdin, which is rapidly reduced to bilirubin by biliverdin reductase, carbon monoxide, and iron [13]. Three mammalian heme oxygennase isoforms have been identified. Among them, the inducible isoform HO-1 is a stress-responsive protein ubiquitously distributed in mammalian tissues and can be induced by various oxidative agents [14,15]. The induction of HO-1 has been considered an adaptive cellular defense response protecting cells or tissues against injuries in pathophysiological states, attributed to the antioxidant properties of bilirubin and biliverdin, the extrusion and sequestration of cellular free iron by ferritin, and the antiapoptotic and anti-inflammatory effects of carbon monoxide [12,14-16]. The beneficial role of HO-1 in diabetes has been reported in animal models and in *in vitro* assays involving exposure to glucose [12,17], but little information on humans is available.

Our aim in this study was to examine associations between type 2 Diabetes Mellitus (T2DM), oxidative stress and the expression of NFE2-related factor 2 (Nrf2) in a population of diabetic patients living in Juana Koslay City, San Luis, Argentina. In addition, we evaluated the functional relevance of Nrf2 by measuring the HO-1 expression among persons with type 2 diabetes.

2. RESEARCH DESIGN AND METHODS

2.1. Subjects

The present study was carried out in accordance to the guidelines of the Helsinki Declaration. A total of 70 volunteers (40 patients with type 2 diabetes and 30 healthy age-matched controls) participated in this investigation. Criteria published by the American Diabetes Association were used to diagnose T2DM [18]. These patients reside in Juana Koslay, San Luis, Argentina. The protocol for this study was approved by the local Institutional Review Board, and a written informed consent was obtained from each patient to be enrolled. During an initial interview with each patient, they were asked for diseases, medication and smoking histories. Exclusion criteria included liver, kidney and thyroid diseases, as well as the use of anti-lipemic drugs.

2.2. Anthropometric and Clinical Data

For each subject enrolled, height (meters) and weight (Kg) measurements were acquired. Height and weight were measured to the nearest 0.5 cm and 0.1 Kg, respectively. The body mass index (BMI) was calculated as weight divided by height squared (Kg/m^2). Those patients with a BMI equal or greater than 30.00 Kg/m^2 were considered as obese.

2.3. Blood Sampling

Blood samples were obtained from patients that had fasted overnight for a minimum of 12 h and were drawn from the antecubital vein between 08:00 am and 09:30 am. Blood sample were obtained with or without anticoagulants to obtain plasma and serum, respectively. After separation of plasma, cell pellets were used to extract RNA (see below).

2.4. Biochemical Measurement

Fasting plasma glucose (FPG) was measured by using a glucose oxidase method with a commercial enzymatic kit (Wiener Lab, Rosario, Argentina) and Glycated hemoglobin (HbA1c) concentration was measured with a coupled ionic-exchange chromatography/spectrophotometric assay (BioSystems, Barcelona, Spain) in a Bayer Express Plus Chemistry Analyzer (Bayer Diagnostics, Siemens, Germany). Total cholesterol (TC), triglycerides (TG) and HDL-c concentrations were measured using commercial kits by following manufacturer's instructions (Wiener Lab, Rosario, Argentina) in a Bayer Express Plus Chemistry Analyzer (Bayer Diagnostics, Siemens, Germany). Low density lipoprotein-cholesterol (LDL-c) was calculated with the Friedewald formula: LDL-c = total cholesterol (mg/dL) − HDL-c (mg/dL) – triglycerides (mg/dL)/5 [19].

2.5. Lipid Peroxidation Assay

Plasma lipid peroxidation was determined as described by Jentzsch *et al*. (1969). Plasma protein was precipitated in trichloroacetic acid (TCA). MDA produced during lipid peroxidation, reacts with thiobarbituric acid (TBA) and generas a pink-colored complex. After some further steps, the absorbance of the supernatant was measured spectrophotometrically at 532 nm (Bayer Diagnostics, Siemens, Germany) using 1,1,3,3-Tetra ethoxypropane (Sigma Chemical, St Louis, MO) as standard. TBARS concentration was determined in triplicate and all results are presented in M.

2.6. Measurement of Gene Expression

Total RNA was isolated from cell pellets using the TRIzol reagent following manufacturer's instructions (Life Technologies, Carlsbad, CA). Agarose gel electrophoresis and ethidium bromide staining confirmed the purity and integrity of isolated RNA. RNA concentration was assessed by spectrophotometric measurements at 260/280 nm. Then, ten micrograms of total RNA were reverse-transcribed with 200 units of moloney-murine leukemia virus (MMLV) reverse transcriptase (Promega) using random hexamers as primers in 20 µL reaction mixture following the instructions provided by the manufacturer. The reverse transcription reactions were performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) with conditions at 65˚C for 10 min, 37˚C for 60 min, and 90˚C for 5 min. PCR was used to quantify human Nrf2, HO-1 and Cyclophiline A expression. PCRs were carried out in 35 µl reactions. Each amplification reaction included 20 ng of cDNA, 20 pmol of each PCR primer and 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Waltham, MA). These reactions were performed in a buffer 1X containing 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), and 200 pmol/L of each deoxynucleotide triphosphate. cDNA and control preparations were amplified using the following conditions: 95˚C for 2 min followed by 40 cycles of: denaturation for 1 min at 95˚C, primer annealing for 1 min at 60˚C, and extension for 1 min at 72˚C; followed by a final extension at 72˚C for 5 minutes. Deoxy-oligonucleotide primers were constructed from the published cDNA sequence of HO-1, Nrf2 and Cyclophiline A, and the PCR products resulted in 323, 201 and 166 bp fragments, respectively. Cyclophiline A controls were performed for all PCRs. The primers sequences are presented in **Table 1**.

The PCR products were electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) ethidium bromide. The image was visualized and photographed under UV transillumination. The intensity of each band was measured using NIH *Scion Image* 1.6.3 software and reported as the values of band intensity units.

2.7. Statistics

All results are presented as mean ± SD. Student's t-test was used for the analysis of data with a Gaussian distribution. The Pearson's product moment and Spearman's correlation coefficients were used to determine the relationships between the studied parameters. Statistical significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Subject Characteristics

Seventy individuals were included in this study, 40 of them were diabetic and 30 were controls. **Table 2** shows the demographic characteristics of both groups. 54.3% were women and 45.7% were men, age distribution was not different between the groups with a media age of 56.3 years old. The average value of BMI was 27.1 kg/m^2 for the control group and 30 kg/m^2 for the diabetic one. There was a significant difference in weight and BMI between both groups. Diabetic female subjects were more obese than diabetic male subjects.

As shown in **Table 3**, fasting plasma glucose and HbA_{1c} concentrations were higher in the diabetic group when compared with age-matched control subjects. Total cholesterol, TG and LDL-c were increased in the diabetic group, while HDL-c was significantly lower in these patients, when compared to controls. LDL cholesterol was lower in diabetic female than in diabetic male subjects. The atherogenic index (AI) was two fold above the control value in the diabetic patients, what suggests a higher risk for cardiovascular diseases in these patients.

Abbreviations used: TG, triglycerides; TC, total cholesterol; LDL, low density lipoprotein; HDL, high den-

Table 1. Primers sequences for RT-PCR.

Gen/Gen Bank access	*Primers*	*Fragment size*
Nfr2 (*Homo sapiens NFE2- related factor* 2) NM_006164.3	*Forward* 5'AGATTCACAGGCCTTTCTCG 3' *Reverse* 5' CAGCTCTCCCTACCGTTGAG 3'	201 bp
HO-1 (*Homo sapiens heme oxigenase* 1) NM_002133.2	*Forward* 5'AGAGCTGCACCGCAAGGCTG' 3 *Reverse* 5' GGGAGTTCATGCGGGAGCGG' 3	323 bp
Cyclophiline A (*Homo sapiens peptidylprolyl isomerase A*) NM_021130.3	*Forward* 5' TCCTAAAGCATACGGGTCCTGGCAT 3' *Reverse* 5' CGCTCCATGGCCTCCACAATATTCA 3'	166 bp

Table 2. Anthropometric characteristics in control and T2DM patients.

	Control (n = 30)	T2DM (n = 40)	*p*
Gender (F/M)	23/7	15/25	
Age (years)	54.63 ± 10.63	58.13 ± 10.96	0.186
Weight (kg)	73.20 ± 16.08	85.98 ± 18.65	0.004
Height (m)	1.65 ± 0.08	1.67 ± 0.08	0.204
BMI (kg/m^2)	27.13 ± 5,29	30.00 ± 4.94	0.023

Data are shown as mean ± SD; Abbreviations used: BMI, body mass index.

Table 3. Biochemical characteristics in control and T2DM patients.

	Control (n = 30)	DMT2 (n = 40)	*p*
FPG (mg/dL)	85.10 ± 13.37	149.25 ± 51.74	<0.000 1
HbA1c (%)	5.51 ± 0.74	7.74 ± 2.04	<0.000 1
TC (mg/dL)	172.30 ± 30.28	233.55 ± 38.70	0.001
HDL-c (mg/dL)	41.60 ± 4.00	34.8 ± 6.30	0.04
LDL-c (mg/dL)	110.80 ± 26.20	142.1 ± 28.20	0.001
TG (mg/dl)	127.20 ± 28.16	190.68 ± 56.45	0.001
AI	3.41 ± 0.65	6.30 ± 1.82	<0.000 1

Data are shown as mean ± SD.

sity lipoprotein; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; AI: TC/HDL-c

3.2. Prooxidant Status

The malon-dialdehyde (MDA) concentration was assessed to evaluate the oxidative stress using the thiobarbituric acid-reactive substances (TBAR'S) method. The results obtained are consistent, with a significant increase in TBAR'S levels in the diabetic group, when compared to age-matched control subjects (**Figure 1(a)**). TBAR'S levels were lower in diabetic female than in diabetic male subjects (**Figure 1(b)**).

3.3. mRNA Expression

As shown in **Figure 2**, Nfr2 gene expression was significantly higher (0.90 ± 0.04 Nrf2/Cyclophiline A gene expression ratio) in leucocytes from controls when compared to type 2 diabetic patients (0.86 ± 0.04, $p < 0.0001$). Similarly, HO-1 gene expression was significantly higher in controls (0.80 ± 0.03 HO-1/Cyclophiline A gene expression ratio) when compared to the T2DM patients (0.74 ± 0.07, $p < 0.003$) (**Figure 3**). Nfr2 and HO-1 gene expression were not different between genders in both groups.

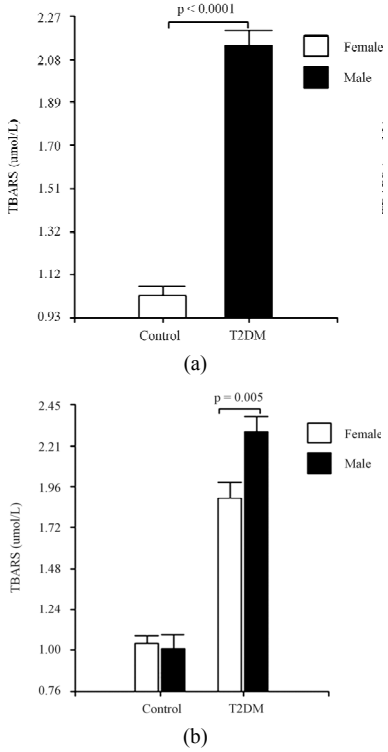

Figure 1. (a) Lipid peroxidation among healthy controls and diabetic subjects. (b) Lipid peroxidation among female and male in diabetic subjects. Data are expressed as mean ± SEM.

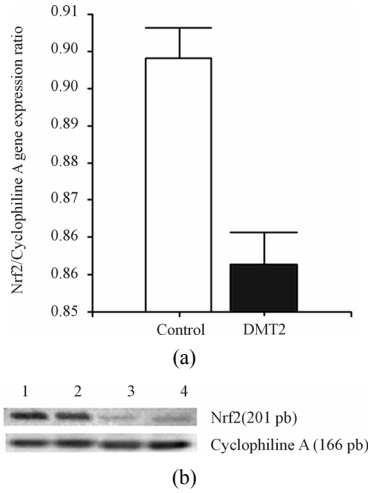

Figure 2. Expression of Nrf2. (a) The bar graphs represent the densitometric analysis of Nrf2 gene expression in leucocytes of controls (lanes 1 and 2) and type 2 diabetic patients (lane 3 and 4). (b) The expression of Nrf2 was assessed by RT PCR, using specific primers, and it was normalized against the expression of the housekeeping gene Cyclophiline A, as reported in *Material and Methods*. Data are expressed as mean ± SEM.

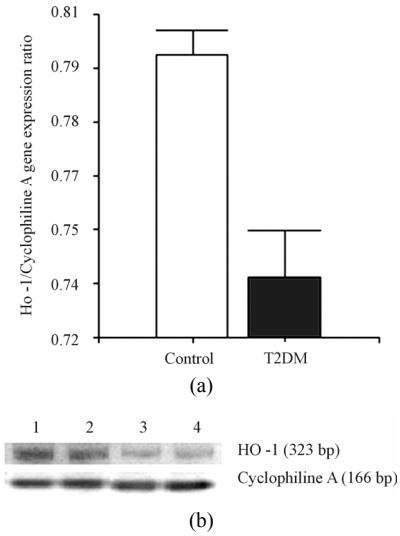

Figure 3. Expression of HO-1. (a) The bar graphs represent the densitometric analysis of HO-1 gene expression in leucocytes of controls (lanes 1 and 2) and type 2 diabetic patients (lane 3 and 4). (b) The expression of HO-1 was assessed by RT PCR, using specific primers, and it was normalized against the expression of the housekeeping gene Cyclophiline A, as reported in *Material and Methods*. Data are expressed as mean ± SEM.

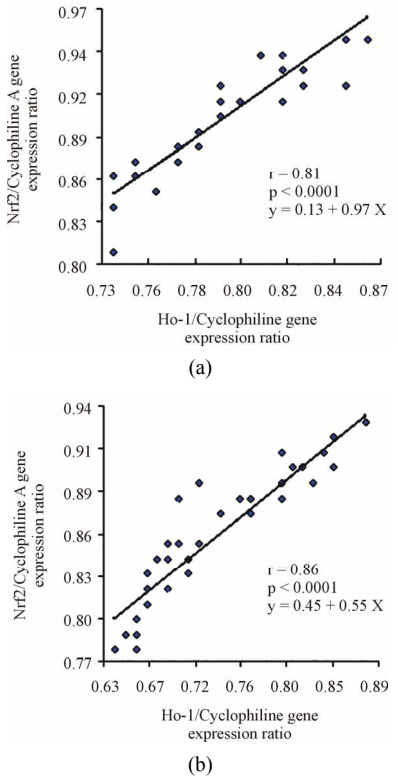

Figure 4. (a) Linear regression between Nrf2 and HO-1 in the control group. (b) Lineal regression between Nrf2 and HO-1 in T2DM.

3.4. Correlation and Lineal Regression Studies

Because both Nrf2 and HO-1 mRNA were reduced in the diabetic group, we performed correlations to determine whether this reduction was associated with metabolic factors that are important for insulin resistance. When the data from all diabetic patients were pooled together, HbA1c was positively correlated with FPG and BMI ($r = 0.484$, $p = 0.001$; $r = 0.307$, $p = 0.04$, respectively).

A subsequent linear regression analysis revealed that there was a significant and positive correlation between the changes of the Nrf2 and HO-1 expression levels, independent of the changes of the other measured factors. **Figure 4** shows the linear regression analysis between the expression of Nrf2 and HO-1 in both groups.

4. DISCUSSION

Here we measured some anthropometrical, clinical and biochemical parameters in a population of men and women diagnosed to be T2DM-patients, which reside in Juana Koslay city, San Luis—a state located in the central region of Argentina. In this study we report for the first time that T2DM patients have lower Nrf2 and HO-1 mRNAs in blood, when compared to healthy controls. This change was accompanied by an increased plasma concentration of TBAR'S.

Type 2 Diabetes Mellitus (T2DM) is common and is characterized by hyperglycemia, insulin resistance, and a relative impairment in insulin secretion. Abdominal obesity, specifically, is associated with resistance to the effects of insulin on the utilization of fatty acids and on peripheral glucose levels. Insulin resistance is integral in the pathogenesis of T2DM and is frequently accompanied by hypertension, high serum LDL, low serum high-density lipoprotein, and high serum triglyceride levels, which promote the development of atherosclerotic cardiovascular disease [20].

Hyperglycemia is a major factor in the development of diabetic complications, although the mechanisms of how increased glucose levels contribute to these changes have not been fully elucidated. Adverse biochemical changes associated to hyperglycemia include increased flux of glucose through the polyol pathway, enhanced nonenzymatic glycation and activation of the diacylglycerol-protein kinase C pathway. Hyperglycemia may also result in increased production of ROS within numerous biochemical pathways that have the potential to initiate adverse changes in the endothelial function [21]. Oxygen-free radicals had been suggested to be a contributory

factor in complications of diabetes mellitus. There are many reports that indicate that the changes in biochemical parameters are due to diabetes-induced oxidative stress.

Our results indicate the presence of higher levels of MDA in plasma of type 2 patients as compared to healthy controls. Increased levels of MDA in diabetic subjects are indication of lipid peroxidation. Increased production of MDA has also been demonstrated in the erythrocyte membranes of diabetic patients [22,23] and high-lipid peroxides levels are also observed in diabetic rats [24].

Oxidative stress is a consequence of an unbalance between the production of oxidants and antioxidants, which affects the structure, function and turnover of macromolecules leading to tissue dysfunction [25]. Oxidative stress has been suggested to be an important mechanism in the development of type 2 diabetes [1,2]. Nrf2 maintain the balances controlling the antioxidant defense in the body which are at stake in a variety of conditions including diabetes and diabetic neuropathy. In response to oxidative stress, Nrf2 escapes ubiquitination by dissociating from its negative regulator KEAP1 (Kelch-like ECH-associated protein 1), translocates to the nucleus, and activates the genes involved in the synthesis of antioxidant enzymes [26]. These genes carry a special Nrf2-binding site, called the antioxidant response element (ARE), in their promoter region [27]. Nrf2 itself contains an ARE in its promoter.

Lipid peroxidation leads to the formation of a number of short-length electrophilic fatty acid-derivatives and lipid peroxides, which are known to trigger Nrf-2 pathway activation and synthesis of antioxidant proteins and other proteins involved in the removal of electrophilic compounds [28,29]. Although acute activation of Nrf2 occurs *in vivo* in response to oxidized phospholipids' signaling, increased ROS production, hyperglycemia, and shear stress [30-32], in chronic disease states the antioxidant response is often insufficient to maintain redox balance and prevent disease progression [33,34]. Our T2DM patients have increased lipid peroxidation and lower Nrf2 mRNA in blood than controls.

Like many other antioxidant genes, HO-1 gene expression is triggered by the binding of Nrf2 to the antioxidant response element in its gene's promoter. In the anti-oxidative system, HO-1 acts as a key factor in mechanisms to mitigate oxidative stress because of its potent anti-inflammatory, antioxidant, and anti-proliferative actions [12]. In addition, impaired HO-1 protein expression/activity may be related to the pathogenic process induced by oxidative stress. Although accumulating evidence from animal studies supports a beneficial role of HO-1 in the diabetic state [12,17], it is still not clear whether HO-1 exerts a similar biologic effect in humans. We studied HO-1 expression in leucocytes from control and T2DM patients. As expected, decreased levels of HO-1 mRNA expression were observed in diabetic patients when compared to healthy controls. These findings suggest that reduced HO-1 expression may contribute to the development of type 2 diabetes, consistent with the results of several small clinical studies which found decreased mRNA expression of HO-1 in peripheral blood mononuclear cells [35], skeletal muscle [36], and retinal pigment epithelium [37] among patients with type 2 diabetes.

Taken together our data indicate that the decreased expression of HO-1 and increased levels of TBAR'S observed in T2DM could be a consequence of a reduced expression of Nrf2. Continuous oxidative stress may be a consequence of a reduced capacity of T2DM patients to trigger Nrf2-dependent antioxidant response against a positive energy balance.

The results from this study provide novel evidence that genes associated to antioxidant defense mechanisms are markedly reduced in patients with type 2 diabetes, and that the reduction in the expression of these genes could be associated to hyperglycemia and increased levels of MDA. The linear regression analysis revealed that there is a strong and positive correlation between the changes of the Nrf2 and HO-1 expression levels.

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Use of pocket pulse oximeters for detecting peripheral arterial disease in patients with diabetes mellitus*

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ABSTRACT

Recent Aims: New diagnostic methods are needed to detect peripheral arterial disease easier than using the ankle-brachial index measured by Doppler devices. We investigated whether the use of pocket pulse oximeters could meet sensitivity and specificity criteria as screening method to detect significant peripheral arterial perfusion deficits. Methods: We measured oxygen saturation (SaO_2) at index fingers and great toes (on horizontal and elevated 30˚) by a pocket pulse oximeter in 250 subjects with diabetes mellitus attending the outpatient clinic. A finger-to-toe SaO_2 gradient greater than 2% was considered abnormal. Ankle-brachial index was measured by a hand held Doppler device. Peripheral arterial disease was defined as an ankle-brachial index less than 0.9. Results: A total of 1392 (93%) valid SaO_2 readings were obtained. Twenty-seven (11%) patients were excluded due to not having measurable SaO_2 finger-to-toe gradients. A total of 223 patients were analyzed. Peripheral arterial disease was detected in 47 (21%) patients. A finger-to-toe SaO_2 gradient greater than 2% had sensitivity 42.6% (95% CI 30.0% - 55.3%), specificity 79.1% (95% CI 75.7% - 82.6%), positive predictive value 35.7% (95% CI 25.2% - 46.4%), negative predictive value 83.4% (95% CI 79.8 - 87.1), positive likelihood ratio 2.03 (95% CI 1.23 - 3.17) and negative likelihood ratio 0.73 (95% CI 0.54 - 0.93) to detect peripheral arterial disease. The area under the receiving operating characteristic curve was 0.69 (95% CI 0.62 - 0.77). Conclusion: Pocket pulse oximeters showed insufficient sensitivity as screening method for detecting peripheral arterial disease in patients with diabetes mellitus.

Keywords: Ankle-Brachial Pressure Index; Doppler Ultrasound; Pulse Oximetry; Comparative Study; Sensitivity; Specificity; Likelihood Ratio

1. INTRODUCTION

Peripheral arterial disease is a leading cause of limb amputations in patients with diabetes [1]. Early identification of at-risk patients consists on foot examination and screening for neuropathy and peripheral arterial disease [2]. The ankle-brachial index is considered the screening method in the evaluation at office for peripheral arterial disease [3,4]. Measuring an ankle-brachial index requires a continuous-wave Doppler machine, ultrasonic gel, and a sphygmomanometer with a blood-pressure cuff. Systolic blood pressure is recorded at both brachial arteries and at both dorsalis pedis and posterior tibial arteries. While the methods for calculating the ankle-brachial index can vary, one commonly accepted calculation is the ratio of the highest ankle systolic pressure divided by the highest brachial systolic pressure.

Although ankle-brachial index is considered as the standard method for the diagnosis of lower extremity peripheral arterial disease in field epidemiological surveys, in vascular laboratories, and in office practice, the procedure is cumbersome since it needs a dedicated device, it is time consuming and it requires technical skills.

*Conflict of interest: None.

These shortcomings may explain why peripheral arterial disease remains largely underdiagnosed in general practice [5]. Additional limitations include inaccurate measurements as a result of calcified or incompressible vessels (which would produce falsely elevated readings) and the presence of a subclavian-artery stenosis (which could also falsely elevate the ankle-brachial index on the side of the stenosis) [6].

Pulse oximetry has been developed as a non invasive screening method to detect low oxygen haemoglobin saturation in finger and toe tips. The rationale for using fingertip pulse oximetry as screening for peripheral arterial disease is based on the hypothesis that there would be a gradient of oxygen saturation between upper and lower limbs in patients with significant arterial perfusion defects [7]. Previous studies have shown conflicting results when this method is applied in different clinical settings. Therefore we aimed to assess the performance of pocket fingertip pulse oximeters to detect peripheral vascular disease in patients with diabetes.

2. MATERIALS AND METHODS

The study was carried out and reported according to the Standards for Reporting of Diagnostic Accuracy criteria (STARD Initiative) [8].

2.1. Study Population

Patients with diabetes mellitus were considered for enrollment in the study. Eligibility criteria included: 1) age equal or greater than 50 years old and diagnosed of diabetes mellitus; 2) able to walk; 3) given informed consent. The exclusion criteria used were: 1) obese individual requiring special cuffs; 2) presence of painful inflammatory processes, wounds, phlebitis or extreme edema; 3) presence of revascularization procedures or amputation in any of the limbs. Patients were prospectively enrolled during times the investigators were available. Data collection started in February 2011 and finished in December 2011.

2.2. Data Collection

Index test and reference standard were carried out sequentially during the same observation period after resting in supine position for 5 min in a room at 24˚C. Nail polish was removed before pulse oximetry was carried-out. Data collection was planned before the index test and reference standard was performed. Data included age, sex, duration of diabetes mellitus since diagnosis, smoking habit, diagnosis of hypertension or hyperlipidemia and past history of coronary artery disease or cerebrovascular disease. Laboratory data obtained were fasting blood glucose, hemoglobin$_{A1c}$, total cholesterol, HDL cholesterol, triglycerides, creatinine, glomerular filtration rate estimated by means of Modification of Diet in Renal Disease (MDRD-4) formula. In addition, patients were assessed for symptomatic peripheral artery disease by means of the Edinburgh claudication questionnaire [9].

2.3. Index Test

We used the Oxym6000 pocket-size fingertip pulse oximeter (Quirumed Health & Care, Beijing, China). These devices have a measurement range from 70 to 99%, and an accuracy of ± 2% on the stage of 70% - 99%. Pulse oximetry of the toes was considered abnormal if there was a decrease of more than 2% in arterial oxygen saturation (SaO_2) at the toe from the finger or a decrease of more than 2% on elevation of the foot by 30 cm at each side (SaO_2 gradient > 2%).

2.4. Reference Standard

The ankle-brachial index was used as the reference standard to identify patients with peripheral artery disease. Ankle-brachial index (ABI) was calculated in every patient after collection of all data. We used the ratio of the highest registered measurement of ankle and brachial blood pressure.

We used the definitions of normal and abnormal ABI values provided by the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines last review [4]. This includes a normal ABI range of 1.00 to 1.40, and abnormal values continue to be defined as those less than 0.90. ABI values of 0.91 to 0.99 are considered “borderline” and values greater than 1.40 indicate noncompressible arteries.

A handheld Doppler device with an 8 MHz continuous wave probe (Minidop ES-100 VX, Hadeco Inc., Japan) was used to assess the systolic blood pressure at the brachial, dorsalis pedis and posterior tibial arteries at each limb. Once the pulse sound was located by the handheld Doppler probe, a 24 - 32 cm. cuff of an aneroid sphygmomanometer (Riester Minimus III, Germany) was inflated until the signal disappeared. The cuff was then slowly deflated and the pressure at which the signal reappeared was recorded. Examination was repeated up to three times if no recording was obtained.

2.5. Sample Size Estimation

We would recommend pulse oximetry as an alternative diagnostic test if there were 80% certainty (power = 0.80) that its sensitivity was not more than 20% less than that for the ankle-brachial pressure index measured by handheld Doppler. On the basis of literature data, the estimated sensitivity for ankle-brachial index measured by Doppler is 95% [10]; therefore the expected sensitivity of pulse oximetry was set at 75% or more. We used as

rejection limit for type I error 0.05 (one-tailed). According to these assumptions, a total of 38 consecutive patients with peripheral vascular disease were needed to test our hypothesis [11].

2.6. Statistical Analysis

Continuous variables are summarized as mean (standard deviation) when normally distributed and median (interquartile range) when asymmetrically distributed. Categorical variables are presented as numbers (percentage). We analyzed the data using the handheld Doppler as the reference standard. Sensitivity, specificity, likelihood ratios and area under the receiving operating characteristic curve were derived for abnormal pulse oximetry with 95% confidence intervals (CIs). An analysis of receiving operating characteristic curve was used to select the SaO_2 gradient that maximized sensitivity without compromising specificity (MedCalc Software version 12.3.0, Mariakerke, Belgium). Statistical analysis was carried out using 2-way contingency table analysis and paired T-test (SPSS version 15.0, Chicago, USA).

3. RESULTS

3.1. Clinical and Demographic Characteristics

We screened a total of 250 patients. Twenty-seven (11%) patients were excluded due to not having measurable SaO_2 finger-to-toe gradients. A total of 223 patients were analyzed. Patients entering the study had a median age of 65 years (interquartile range from 59 to 71). Fifty-nine percent of patients were male. The median time since the diagnosis of diabetes was 10 years (interquartile range from 5 to 17 years). There was no difference between paired systolic blood pressure measurements in right arm and left arm (136.37 ± 24.28 mmHg vs. 136.16 ± 22.90 mmHg; $P = 0.79$).

As shown in **Table 1**, among 223 patients, 171 (76.7%) had hypertension, 176 (78.9%) hyperlipidemia, 55 (24.7%) were smokers, and 86 (38.6%) had a previous cardiovascular event. A total of 48 (21.5%) patients had a reduction of the estimated glomerular filtration rate below 60 mL/min/1.73 m^2.

A total of 47 (21.0%) patients had ankle-brachial index values less than 0.90, with a mean (SD) value of 0.70 (0.10). According Edinburgh questionnaire 27 (57.4%) had symptomatic peripheral arterial disease.

3.2. Pulse Oximetry Values

A total of 1392 (93%) valid SaO_2 readings were obtained. Patients with ankle-brachial index less than 0.90 showed statistically significant reductions in SaO_2 values taken at feet when they were compared to patients with normal ankle-brachial index (**Table 2**). We did not observe significant differences in SaO_2 values taken at feet compared to those taken at fingers within the ankle-brachial index categories considered (low, normal, or high). There were no significant differences in SaO_2 values taken at feet on horizontal or after 30˚ elevation. **Table 3** shows the distribution of finger-to-toe gradients according several intervals of ankle-brachial index. There was a significant association ($P = 0.013$) among ankle-brachial index intervals and finger-to-toe gradient categories.

Table 1. Characteristics of the patients entering the study ($N = 223$).

Characteristic	
Age (years), median [IQR]	65 [59 - 71]
Sex, *N* (%) Male Female	 133 (59.6) 90 (40.4)
Time since diagnosis of diabetes (years), median [IQR]	10 [5 - 17]
Patients with hypertension, *N* (%)	171 (76.7)
Patients with hyperlipidemia, *N* (%)	176 (78.9)
Patients with previous cardiovascular event, *N* (%)	86 (38.6)
Tobacco smoking status, *N* (%) Current	55 (24.7)
Fasting plasma glucose (mg/dL), median [IQR]	147 [119 - 189]
Glycated haemoglobin level (%), median [IQR] Glycated haemoglobin level (mmol/mol), median [IQR]	7.4 [6.5 - 8.4] 57 [48 - 68]
Total cholesterol (mg/dL), median [IQR] HDL cholesterol (mg/dL), median [IQR]	165 [136 - 189] 44 [37 - 53]
Triglycerides (mg/dL), median [IQR]	131 [93 - 178]
Creatinine (mg/dL), median [IQR]	0.9 [0.8 - 1.1]
Estimated glomerular filtration rate, *N* (%) <30 ml/min/1.73 m^2 30 - 59 ml/min/1.73 m^2 >59 ml/min/1.73 m^2	 5 (2.3) 43 (19.2) 175 (78.5)
Symptomatic intermittent claudication, *N* (%)	27 (12.1)

Hypertension was defined as current treatment for hypertension or a blood pressure of 140/90 mmHg or more. Hyperlipidemia was defined as current treatment for hypercholesterolemia or hypertriglyceridemia, or a non-high density lipoprotein cholesterol concentration greater than 130 mg/dL or triglycerides greater than 150 mg/dL. Symptomatic intermittent claudication defined by Edinburgh questionnaire.

3.3. Overall Performance of Fingertip Pulse Oximetry

Figure 1 described the flowchart of patients entering the study. Twenty-seven patients had not measure SaO_2 at toes, among them 17 (63%) patients had ankle-brachial index less than 0.90, 8 (30%) had normal ankle-brachial index values and, 2 (7%) had ankle-brachial

Table 2. SaO_2 at finger- and toe-tips according ankle-brachial index categories.

	Ankle-brachial index category				
	Group A: ABI <0.9 (*N* = 47)	Comparison: Group A vs. Group B *P*	Group B: ABI 0.9 - 1.4 (*N* = 172)	Comparison: Group B vs. Group C *P*	Group C: ABI > 1.4 (*N* = 4)
SaO_2 (%) Right arm, mean (SD)	97.07 (1.34)	0.264	97.37 (1.31)	0.570	97.75 (0.50)
SaO_2 (%) Left arm, mean (SD)	96.84 (1.57)	0.341	97.17 (1.69)	0.490	97.75 (0.50)
SaO_2 (%) Left leg 0˚ elevation, mean (SD)	95.97 (2.96)	0.029	97.26 (2.07)	0.636	97.75 (0.50)
SaO_2 (%) Left leg 30˚ elevation, mean (SD)	96.06 (1.88)	0.033	97.36 (2.06)	0.541	98.00 (0.00)
SaO_2 (%) Right leg 0˚ elevation, mean (SD)	95.93 (3.75)	0.035	97.49 (1.23)	0.409	98.00 (0.00)
SaO_2 (%) Right leg 30˚ elevation, mean (SD)	96.45 (3.03)	0.043	97.67 (1.22)	0.577	98.00 (0.00)

Table 3. Distribution of finger-to-toe gradient greater than 2% according different groups of ankle-brachial index values.

	Ankle-brachial index interval					
	Less than 0.50 (%)	0.50 - 0.79 (%)	0.80 - 0.89 (%)	0.90 - 0.99 (%)	1.00 - 1.40 (%)	Greater than 1.40 (%)
Finger-to-toe SaO_2 > 2% (abnormal)	0 (0)	13 (50)	7 (35)	10 (31)	26 (19)	0 (0)
Finger-to-toe $SaO_2 \leq 2\%$ (normal)	1 (100)	13 (50)	13 (65)	22 (69)	114 (81)	4 (80)

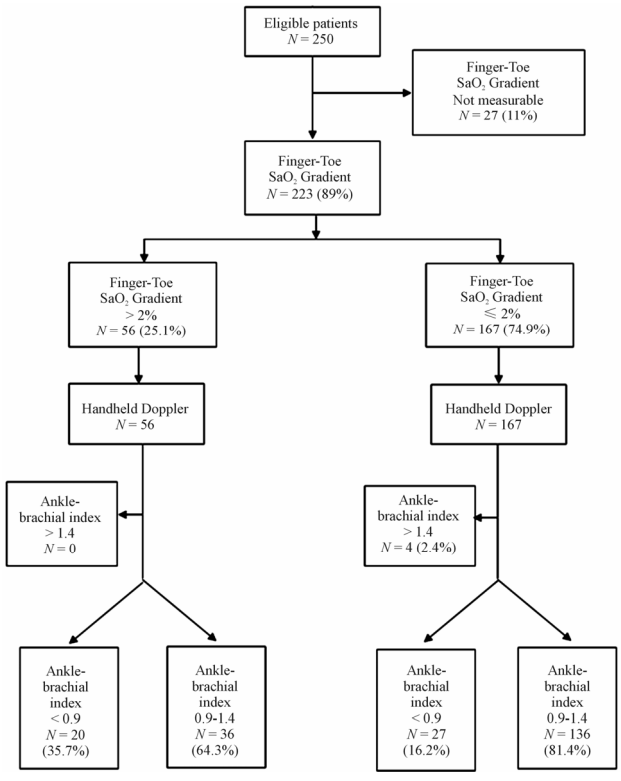

Figure 1. Flow diagram of the finger-toe pulse oximetry accu racy compared with handheld Doppler to estimate the presence of peripheral arterial disease.

values greater than 1.40.

Among 223 patients analyzed, 56 (25.1%) had a finger-to-toe SaO_2 gradient greater than 2% suggesting peripheral arterial disease. In this group, 20 (35.7%) patients had true positive results with a mean (± SD) ankle-brachial index 0.66 (± 0.14), and 36 (64.3%) patients had false positive results with a mean ankle-brachial index 1.07 (± 0.12) ($P < 0.001$).

A total of 167 patients (74.9%) had normal values of finger-to-toe SaO_2 gradient, suggesting absence of peripheral arterial disease. In this group, 136 (81.4%) patients had true negative results with mean (± SD) ankle-brachial index 1.08 (± 0.11) and 31 (18.6%) patients had false negative results with mean (± SD) ankle-brachial index 0.78 (± 0.22) ($P < 0.001$).

The overall performance of fingertip pulse oximetry was: sensitivity 42.6% (95% CI 30.0% - 55.3%), specificity 79.1% (95% CI 75.7% - 82.6%), positive predictive value 35.7% (95% CI 25.2% - 46.4%), negative predictive value 83.4% (95% CI 79.8 - 87.1), positive likelihood ratio of 2.03 (95% CI 1.23 - 3.17) and, a negative likelihood ratio of 0.73 (95% CI 0.54 - 0.93) (**Table 2**).

The area under the receiving operating characteristic (ROC) curve was 0.69 (95% CI 0.62 to 0.77) (**Figure 2**). In order to assess the consistency of the results, we carried out a subgroup analysis. The subgroups analyzed were: sex, male vs. female; time since diagnosis of dia-

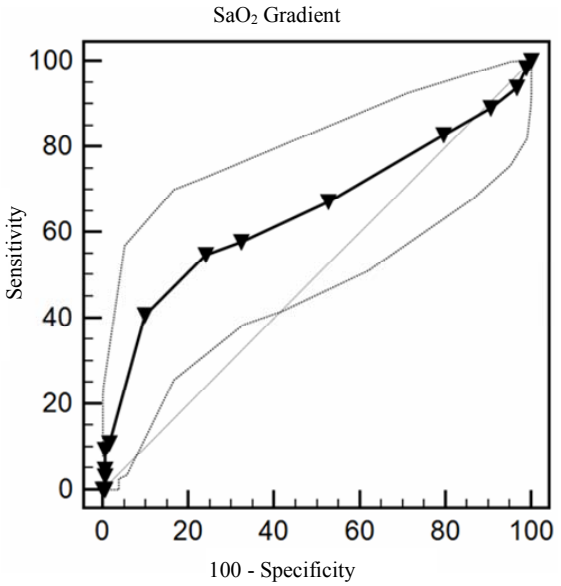

Figure 2. Area under the receiver operator characteristic curve and 95% confidence intervals for SaO_2 gradient measured by pocket pulse oximeter for the diagnosis of peripheral arterial disease.

betes, <10 years vs. ≥10 years; presence of intermittent claudication, yes vs. no; past history of cardiovascular disease yes vs. no. Subgroup analysis showed a poor sensitivity and fair specificity of the pocket pulse oximetry for every category of patients considered (**Table 4**).

4. DISCUSSION

We confirmed the hypothesis that patients with ankle-brachial index less than 0.90 showed significant decrease in SaO_2 values taken at feet when they were compared to those obtained from patients with normal ankle-brachial index. However, the discriminative power of finger-to-toe SaO_2 to diagnose peripheral arterial disease was poor. Moreover, in 11% of patients the fingertip device was not able to assess SaO_2 at toes.

Our study population accumulated significant cardiovascular risk factors with a high proportion of hypertension, hyperlipidemia and a long duration of diabetes. The prevalence of peripheral arterial disease defined by an ankle-brachial index less than 0.90 was 21.6%, a value similar than that reported in other population studies of patients with diabetes that varied between 19% and 31% [12,13].

In our experience the use of fingertip pulse oximetry as screening tool for peripheral arterial disease had a sensitivity value of 42.6%, with a positive predictive value of 37.5%. On the other hand, pulse oximetry showed a specificity of 77.2% with a negative predictive value of 83.4%, meaning that it had better performance for ruling out the disease.

The use of fingertip pulse oximetry for detecting peripheral arterial disease has been evaluated in a number of studies. Parameswaran *el al*. studied 57 asymptomatic patients with mean age 63 years and 9 years of known duration of diabetes in whom peripheral arterial disease was confirmed in 31% by Doppler waveform analysis [7]. The study showed that pulse oximetry had a sensitiveity of 77% and a specificity of 97%. Joyce *et al*. [14] and Ignjatović *et al.* [15] used pulse oximetry to evaluate degree of limb ischemia in patients attending two surgi

Table 4. Results for the pulse oximetry test according different categories of risk.

Test results	Sensitivity (95% CI), %	Specificity (95% CI), %	Positive likelihood ratio (95% CI)	Negative likelihood ratio (95% CI)
Sex				
Male	43.55 (25.31- 68.63)	74.44 (69.82 - 79.32)	1.70 (0.84 - 3.21)	0.76 (0.47 - 1.07)
Female	45.52 (19.42 - 72.4)	85.90 (81.56 - 90.67)	3.23 (1.04 - 7.66)	0.63 (0.31 - 0.99)
Time since diagnosis of diabetes				
Less than 10 years	50.10 (21.42 - 78.09)	82.83 (78.31 - 87.23)	2.90 (0.99 - 6.09)	0.60 (0.25 - 1.00)
Equal or greater than 10 years	36.83 (18.33 - 58.04)	77.62 (73.05 - 82.97)	1.64 (0.68 - 3.40)	0.81 (0.51 - 1.12)
Unknown				
Intermittent claudication				
Yes	50.03 (32.53 - 65.50)	75.03 (63.05 - 85.66)	2.00 (0.88 - 4.56)	0.67 (0.40 - 1.07)
No	33.32 (9.22 - 67.78)	81.21 (79.34 - 83.88)	1.77 (0.45 - 4.19)	0.82 (0.38 - 1.14)
Past history of cardiovascular disease				
Yes	38.12 (20.51 - 56.84)	76.4 (69.72 - 83.59)	1.61 (0.68 - 3.45)	0.81 (0.52 - 1.14)
No	60.07 (28.62 - 85.71)	81.3 (77.94 - 84.14)	3.21 (1.29 - 5.40)	0.49 (0.17 - 0.91)

cal vascular units. They found that SaO_2 assessed by pulse oximetry was useful to determine the stage of functional ischemia and the success of revascularization.

On the contrary, a poor sensitivity of pulse oximetry has been observed in some other studies. Jawahar *et al*. [16] studied a total of 51 legs with moderate and severe peripheral arterial disease and the sensitivity was 53%. Couse *et al*. [17] analyzed arterial oxygen saturation at the big toe in fourteen symptomatic patients with exercise induced leg pain, seven elderly men admitted to the hospital for a variety of reasons unrelated to vascular disease and six young men with no symptoms. Surprisingly, arterial oxygen saturation at rest was similar among three groups. They did not evaluate the performance characteristics of the test. Reasons for such discrepancies in test performance lie mainly in the type of population examined. Patients with symptoms of peripheral arterial disease or those suffering revascularization procedures are those with greater arterial perfusion defects, thus increasing the sensitivity of diagnostics tests. On the other hand, patients entering screening programs have lower probability of finding significant perfusion defects, and will require more sensitive tests to detect the disease. It had been argued that ankle-brachial index may not be the best reference method for diagnosis of peripheral vascular disease in patients with longer duration of diabetes mellitus or suffering from chronic kidney disease. The reasons are related with falsely elevated values of ankle-brachial index due to vascular calcification and noncompressible vessels [18,19]. A recent study confirmed the association between diabetes, regular hemodialysis and presence of arterial calcification in 269 patients with critical limb ischemia, but neither ankle systolic blood pressure nor ankle-brachial index were affected by the presence of vessel calcification [20]. Regarding our data, if long standing diabetes would have produced falsely elevated values of ankle-brachial index it should have been expected that pulse oximetry had greater sensitivity to detect significant arterial perfusion defects. From our data, and the literature reviewed pulse oximetry showed better performance to rule out significant peripheral arterial disease.

Our study included as strengths 1) a large sample size, big enough to assess test performance; 2) comparison with a known reference standard for office diagnosis; and 3) appropriate spectrum of patients in which the diagnostic test should apply in clinical practice. Although we found the use of pulse oximeter devices useful to rule out significant peripheral arterial disease, our study had some limitations: 1) we did not evaluate the interobserver variability, nevertheless all physicians had a training period before performing the test; 2) the sequence of measurements, pulse oximetry followed by ankle-brachial index may have biased the results, but the bias should have increase the agreement between two methods; and 3) there was no confirmatory test such as lower limb angiography to assess the presence of peripheral arterial disease, however, several societies consider that resting ankle-brachial index has enough sensitivity and specificity to be used a reference method [3,4].

In conclusion, pocket pulse oximeters showed insufficient sensitivity as screening method for peripheral arterial disease in patients with diabetes mellitus. Ankle-brachial index measured by Doppler remains as the reference method for diagnosing peripheral arterial disease in clinical practice.

5. DECLARATION

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Etical approval: The study comply with the principles laid down in the Declaration of Helsinki "Recommendations guiding physicians in biomedical research involving human subjects", adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964 (and its successive amendments), and the requirements of Spanish law in the field of biomedical research, data protection and bioethics. The study was approved by the institutional review board of our hospital. All patients provided informed consent.

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Differentially expressed genes in adipocytokine signaling pathway of adipose tissue in pregnancy

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ABSTRACT

Objective: To profile the differential gene expression of the KEGG Adipocytokine Signaling pathway in omental compared to subcutaneous tissue in normal pregnancy. Study Design: Subjects included 14 nonobese, normal glucose tolerant, healthy pregnant women. Matched omental and subcutaneous tissue were obtained at elective cesarean delivery. Gene expression was evaluated using microarray and validated by RT-PCR. Differential gene expression was defined as ≥1.5 fold increase at $p < 0.05$. Results: Six genes were significantly downregulated with two upregulated genes in omental tissue. Downregulation of Adiponectin and Insulin Receptor substrate, key genes mediating insulin sensitivity, were observed with borderline upregulation of GLUT-1. There were downregulations of CD36 and acyl-CoA Synthetase Long-chain Family Member 1which are genes involved in fatty acid uptake and activation. There was a novel expression of Carnitine palmitoyltransferase 1C. Conclusion: Differential gene expression of Adipocytokine Signaling Pathway in omental relative to subcutaneous adipose tissue in normal pregnancy suggests a pattern of insulin resistance, hyperlipidemia, and inflammation.

Keywords: Adipose Tissue; Pregnancy; Adipocytokine Pathway; Insulin Resistance

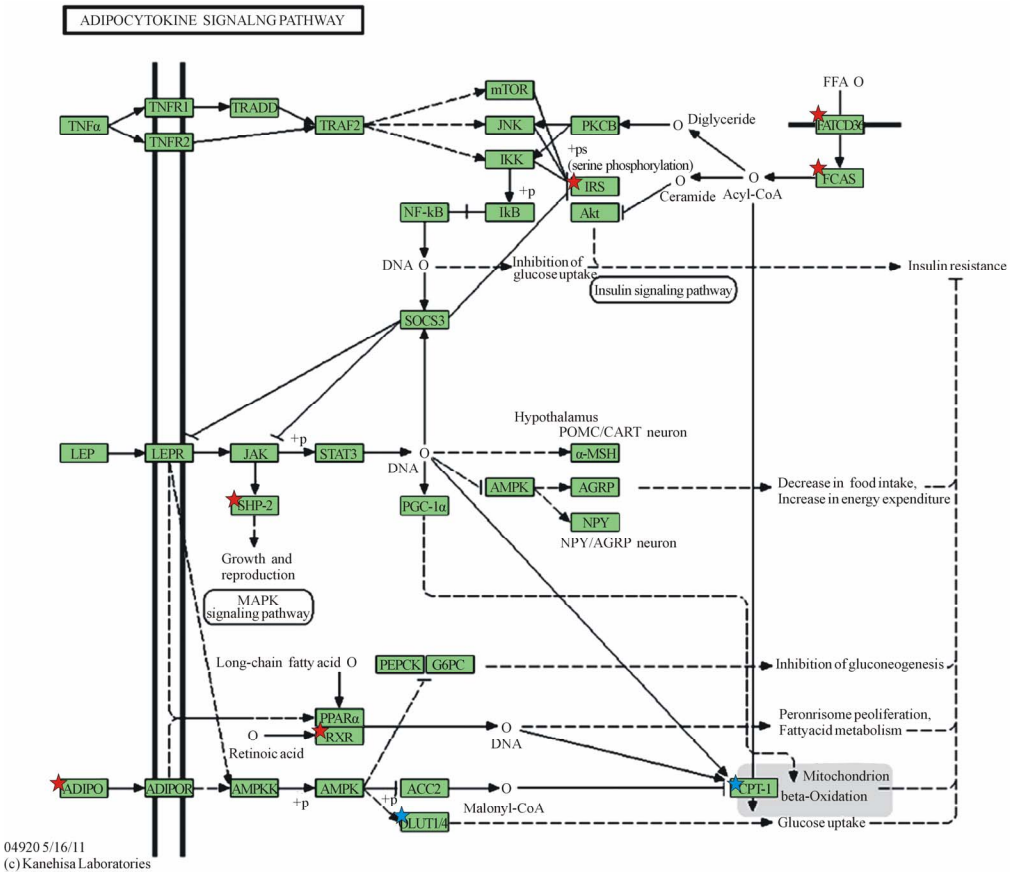
1. INTRODUCTION

Pregnancy is associated with changes in the regulation of glucose metabolism and the development of insulin resistance particularly during the second and third trimesters [1] of all pregnancies.

However, there is no consensus regarding the precise pathophysiology of insulin resistance, which is perceived as causative of gestational diabetes mellitus. It has also been suggested that the observed increase in body fat mass from the 1st trimester throughout pregnancy may be causally associated with decrease in insulin sensitivity [2].

Intra-abdominal (omental or visceral) adipose has been implicated as a major factor in pathophysiology of insulin resistance [3]. It has been observed that visceral fat accumulation increases during pregnancy [4]. Further, Martin et al have noted a significant association between visceral adipose tissue-depth above upper quartile value on ultrasound with positive glucose challenge test in later pregnancy, with no such associations seen for subcutaneous adipose tissue [5].

Adipose tissue is metabolically active, producing adipocytokines that exert effects in energy homeostasis and insulin resistance. The KEGG Adipocytokine Signaling Pathway (**Figure 1**) [6,7] describes signaling cascades arising from the adipocytokines that have been implicated in insulin resistance and sensitivity: TNF-alpha, leptin and adiponectin. Leptin is increased in pregnancies complicated by pre-eclampsia, BMI > 25, gestational diabetes, or hyperinsulinemia [8-11]. Adiponectin is an adipocytokine that has been demonstrated to have antiatherogenic, anti-inflammatory, and antidiabetic roles [12]. Adiponectin has been correlated with insulin sensitivity [13]. Decreased serum adiponectin is consistently seen in women with gestational diabetes independent of body mass index, strongly suggesting a role for adiponectin in modulating insulin resistance during gestation [14]. In late pregnancy, the placenta Produces and secretes adi



Downregulated genes are those marked with red stars: ADIPO (Adiponectin), SHP2 (Protein Tyrosine Phosphatase, Non-Receptor Type 11—PTPN11), RXR (Retinoid X-Receptor, alpha), IRS1 (Insulin Receptor Substrate 1), FATCD36 (CD36 molecule, thrombospondin receptor), FACS (acyl-CoA synthetase long-chain family member 1—ACSL1); Upregulated genes are marked with blue stars as they appear in the KEGG gene listing: SLC20A1 (shown as GLUT-1) and CPT1C (listed under CPT-1). Legend of KEGG gene symbols and full gene names for differentially expressed genes appears below [6,7].

Legend of differentially expressed genes in KEGG adipocytokine signaling pathway (**Figure 1**).

KEGG Gene Symbol	Official Gene Symbol(s)	Gene name(s)
	Downregulated genes	
FATCD36	CD36	CD36 molecule (thrombospondin receptor)
FACS	ACSL1, ACSL3, ACSL4, ACSL5, ACSL6	acyl-CoA synthetase long-chain family member 1, 3, 4, 5, and 6
IRS	IRS1, IRS2, IRS4	insulin receptor substrate 1, 2, and 4
SHP2	LOC344593 or LOC442113 or PTPN11	protein tyrosine phosphatase, non-receptor type 11
ADIPO	ADIPOQ	adiponectin, C1Q and collagen domain containing
RXR	RXRA, RXRB, RXRG	retinoid x receptor alpha, beta, and gamma
	Upregulated genes	
GLUT1/4	SLC2A1, SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 1, solute carrier family 2 (facilitated glucose transporter), member 4
CPT-1	CPT1A, CPT1C, CPT1B or CHKB	carnitine palmitoyltransferase 1A (liver), carnitine palmitoyltransferase 1C, carnitine palmitoyltransferase 1B (muscle) or choline kinase beta

*Note where gene symbols/names are separated by a comma, this denotes different genes, but where they are separated by the word "or" this denotes they are synonyms. **Downregulated/upregulated genes in each gene list are underlined.

Figure 1. KEGG Adipocytokine Signaling Pathway.

ponectin [15]. Adiponectin has also been observed to play a role in adapting energy metabolism at the maternal-fetal interface [15]. Metabolic changes associated with expanding adipose tissue are linked to subclinical inflammation which has been demonstrated to be mediated by cytokines such as TNF-alpha and IL-6 [16]. A signaling cascade arising from TNF-alpha is shown in the KEGG pathway (**Figure 1**), resulting in increased insulin resistance.

There is a paucity of studies examining differential gene expression of the adipocytokine signaling pathway. In the present investigation, we examine differential gene expression in omental and subcutaneous adipose depots in late-term gravid women as relevant to the adipocytokine signaling pathway. We performed this analysis to further elucidate the relative role of the various adipose depots in glucose homeostasis in normal pregnancy in non-obese women. We hypothesize that we will observe changes in differential gene expression in omental adipose consistent with a pattern of relative insulin resistance.

Identifying mechanisms of insulin resistance associated with various adipose tissue depots may help to better elucidate the pathogenesis of insulin resistance, which may have practical implications in the management of diabetes and its complications.

2. MATERIALS AND METHODS

2.1. Adipose Tissue Collection

This is a prospective study on investigating the pathophysiology of insulin resistance and diabetes in pregnancy. We recruited pregnant women between 18 to 45 years during prenatal care. Pregnant women were eligible for this study if they had a BMI < 30; were to be scheduled for an elective cesarean delivery and had a normal glucose challenge test performed during prenatal care. Informed consent was obtained, demographic information was obtained, and patients underwent routine prenatal care. During the cesarean delivery; maternal and cord blood, subcutaneous and omental adipose tissue samples were obtained. The adipose tissues were snap frozen in liquid nitrogen and stored in −80˚C freezer. The patient recruitment and study protocol was approved by Cedars-Sinai Medical Center Institutional Review Board. In this report, we focus on the KEGG adipocytokine pathway. Other KEGG pathways that are relevant to insulin resistance and diabetes in pregnancy that we are studying for other reports are complement and coagulation; Cytokine-cytokine receptor interaction, Arachidonic acid metabolism and insulin signaling pathways.

2.2. RNA Extraction and Labeling

Total RNA was extracted from adipose tissues using RNeasy Lipid Tissue kit from Qiagen following the manufacturer's instructions. Briefly, about 30 mg of frozen adipose tissues were resected and disrupted in 1 ml QIAzollysis reagent with handheld tissue homogenizer (VWR). The homogenates were incubated at room temperature for 5 min followed by mixing with 200 ul chloroform (Sigma Aldrich) and centrifuging at 12,000 g for 15 min at 4˚C. The top aqueous phase of the homogenates containing total RNA were then mixed with 600 ul 70% ethanol (Sigma Aldrich) and loaded onto RNeasy Mini spin column to capture the RNA onto the column's membrane after brief centrifugation at about 10,000 g. On column DNA digestion with RNase-free DNase I set (Qiagen) was carried out to get rid of carry-over genomic DNA. RNA was washed twice and eluted from the membrane with RNase-free water. The concentration and purity of RNA were measured with UV spectrometer (Bio-rad). RNA integrity was checked with Agilent Bioanalyzer with RNA 6000 nano kit.

IlluminaTotalPrep RNA Amplification kit (Ambion) was used to generate biotin-labeled cRNA from total RNA. Briefly, first strand cDNA was made from about 300 ng of total RNA with oligoT7 primer, dNTP, RNase inhibitor, and ArrayScript reverse transcriptase in first strand buffer, incubated at 42˚C for 2 hours. Then second strand cDNA was synthesized at 16˚C for 2 hours with DNA polymerase, dNTP mix, RNase H, and second strand buffer. After purification of cDNA with cDNA-Pure magnetic beads, *in vitro* transcription (IVT) was carried out with biotin-labeling NTPs and IVT enzymes to synthesize biotin-labeled cRNA at 37˚C for 14 hours. Finally, cRNA was purified using RNA binding magnetic beads and eluted with cRNA elution buffer.

2.3. Microarray Hybridization Staining and Scanning

Biotin labeled cRNA (750 ng) was loaded on to Illumina HumanHT-12 V4.0 Expression BeadChip, which comprises probes targeting more than 47,000 transcripts across whole genome, in hybridization buffer with hybridization controls and incubated for 16 hours at 58˚C. After hybridization, BeadChips were washed, blocked with blocking buffer and stained with streptavidin-Cy3 (Amersham Biosciences). Images of stained BeadChips were captured by IlluminaBeadArray Reader.

2.4. Microarray Image Processing and Data Analysis

Images of BeadChip were loaded into IlluminaGenomeStudio for quality determination by evaluation of the present call percentages, signal intensities of hybridization control, biotin controls, and negative controls. Quantile normalization was applied during data processing in GenomeStudio. Signal intensities of each gene/

probe were exported into Partek Genomic Suite 6.5 for high level data analyses. Partek built-in ANOVA model was used to evaluate the impacts of various variables in the study such as different adipose tissues, patient ethnicity, patient-to-patient variability, etc., on the expression data set. Differentially expressed genes (DEGs) of omental fat versus subcutaneous fat were defined as 1.5-fold difference in either direction plus pair-wise *t*-test with Benjamini & Hochberg adjusted $p < 0.05$. Functional classification and pathway analysis were carried out using DAVID Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases, NIAID, NIH) [13,14].

A heat-map is a rectangular tiling of data with cluster trees appended to its margins [15]. Weinstein describes the heat map as follows: in the case of gene expression data, the color assigned to a point in the heat map grid indicates how much of a particular RNA or protein is expressed in a given sample. The gene expression level is generally indicated by red for high expression and either green or blue for low expression. Coherent patterns (patches) of color are generated by hierarchical clustering on both horizontal and vertical axes to bring like together with like. Cluster relationships are indicated by tree-like structures adjacent to the heat map, and the patches of color may indicate functional relationships among genes and samples [16]. In our heat-map (**Figure 2**), tissue samples are displayed on the x-axis while genes are listed on the y-axis. Higher gene expression levels are indicated by red and lower expression levels are indicated by blue or grey. On the vertical and horizontal margins of the tiling there are hierarchical clustertrees arranging the rows and columns so that similar rows or columns of gene expression levels are near each other [15].

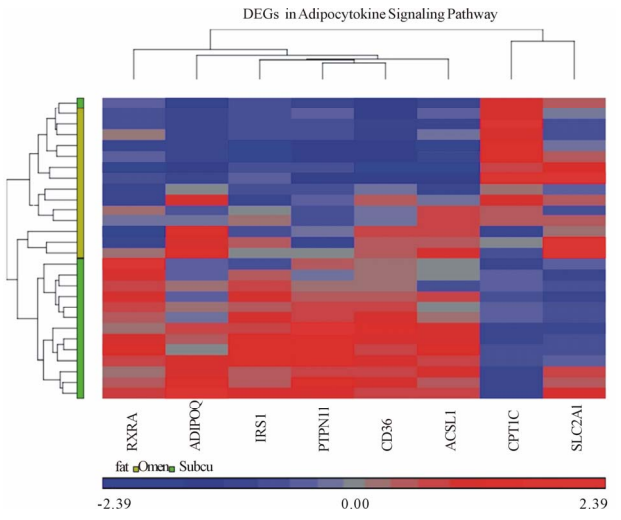


Figure 2. Heat map of differentially expressed genes (DEGs) in Adipocytokine Signaling Pathway. Each column on the horizontal axis represents a tissue sample. (14 subjects) × (2 tissue samples each, omental and subcutaneous) = 28 total tissue samples. Each row on the vertical axis represents one of the 8 DEGs.

2.5. Real-Time PCR Validation of Microarray Results

We used quantitative RT-PCR to verify the differential expression level of several genes based on either their biological functions or chromosomal locations. Confirmation of genes not present in the adipocytokine pathway helped to control for stochastic biological sampling error exacerbated by multiple testing [12]. Procedurally, selected differentially expressed mRNAs were validated with real-time PCR. RNA was first reverse-transcribed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad). About 10 ng cDNA were mixed with gene specific primers (**Table 1**) and Platinum Sybr Green qPCRSuperMix-UDG (Invitrogen). Real-time PCR was carried out in ABI 7000 Sequence Detector (Applied-Biosystems) for 40 cycles. The expression level of human *GAPDH* gene was used as an internal control. Fold changes of omental adipose tissue group versus subcutaneous group were calculated using CT method.

3. RESULTS

The study is based on 14 patients with a body mass index (BMI) <30 who had an elective Cesarean section performed between 2008 and 2010. The mean age was 32.1 years (SD = 6.21), the mean BMI was 23.3 (SD = 2.88), the mean gestational age at delivery was 39 + 0/7 weeks (SD = 4.37 days), and the mean birth weight was 3240 grams (SD = 384.7 grams) (**Table 2**). Regarding the ethnic distribution: 40% were Hispanic, 27% African American, 13% Asian, and 20% were non-Hispanic whites.

Table 1. Primer sequences for PCR validation of microarray results.

GeneSymbol:	REFSEQ_ID:	5' to 3' Forward primer:	Reverse primer:
GAPDH	NM_002046	caggtggtctcct ctgactt	ccaaattcgtt gtcatacca
IL-6	NM_000600.3	gaaagtggctatgggtaagcctac cagtttga	actttccaaga
IL-1B	NM_000576	gggcaagaagta gcagtgtc	gagagcacac cagtccaaa
CLDN1	NM_021101	cctggatttgagt cttggtg	ccgcttacaga tgaagcaat
ADIPOQ	NM_004797	tggctatgctcac agtctca	tctctctgtgc ctctggttc
CFB	NM_001710	cagactatcaggcg ccatttg	gttgctggcaa gtggtagtt
IRS1	NM_005544	acaccgaggata aacactgg	ctacggaagg accacaaaga

Table 2. Patient characteristics for study group.

	Race	Age	Gestational age	Birth weight	Maternal weight at delivery	BMI
Patient 1	Afr Am	33	40 + 0	3213	75.8	22.2
Patient 2	Afr Am	28	39 + 0	3950	77.1	25.5
Patient 3	Afr Am	41	39 + 1	3210	88.9	26.3
Patient 4	Asian	30	39 + 5	2760	59	20.7
Patient 5	Asian	31	38 + 2	3230	78.9	23
Patient 6	Caucasian	20	39 + 0	3400	75.4	20.8
Patient 7	Caucasian	38	39 + 0	3493	89	22.3
Patient 8	Caucasian	36	37 + 2	2850	78.4	24.9
Patient 9	Hispanic	37	39 + 0	2769	56.7	18.7
Patient 10	Hispanic	37	39 + 0	3420	58	21
Patient 11	Hispanic	26	39 + 1	3460	82.6	21.7
Patient 12	Hispanic	38	38 + 5	2630	77.1	23
Patient 13	Hispanic	32	39 + 0	3760	84.8	25.8
Patient 14	Hispanic	23	38 + 5	3210	95.3	29.7
Mean		32.1	39 + 0	3240	77	23.3
Standard deviation		6.21	4.37 days	384.7	12.3	2.88

Afr Am = African American; Age in years; Gestational Age in weeks + days; Birth weight in grams; Maternal weight at delivery in kilograms.

Categorical factors including fat, BMI, and race were analyzed using ANOVA to estimate the contribution of each factor to our data set and allow the identification of significant sources of variation (**Figure 3**). Any factor having a ratio greater than an error of 1 was considered significant. As seen in **Figure 3**, adipose tissue had almost a five-fold effect with an F ratio of 4.77. BMI had a ratio of 2.02 which highlights the impact of BMI and for this reason; we apriori planned to minimize the effect of obesity by excluding from the study any women with a BMI of 30 that would be classified as obesity. Race had a ratio of 1.8. Although the race factor has a value higher than can be contributed to error, when we compared the interaction of race with adipose tissue on differential gene expression using a mixed model ANOVA (column 4) the effect was only 1.25, which is borderline, and suggests minimal to no interference of race on the differential gene expression of adipose tissue.

Six genes of the KEGG Adipocytokine Signaling Pathway orthography were significantly downregulated in omental adipose relative to subcutaneous adipose tissue, (**Table 3**). Two genes were also found to be significantly upregulated (**Table 3)**. The gene most downregulated in omental adipose tissue was CD36, by a factor of 2.05839. The significantly downregulated genes were found in four cascades within the Adipocytokine Signaling Pathway. In the pathway with initial signaling by Adiponectin; both Adiponectin and Retinoid X receptor alpha were significantly downregulated. In the cascade initiated by TNF-alpha; Insulin Receptor Substrate 1 (IRS1), was significantly downregulated with no significant differential expression of TNF-alpha noted. In the pathway with initial signaling by Leptin, Protein Tyrosine Phosphatase Non-Receptor Type 11 (PTPN11 also known as SHP2), was also significantly downregulated without significant differential expression of Leptin. Finally, both CD36 (FATCD36) and acyl-CoA Synthetase Long-chain Family Member 1 (ACSL1, seen in **Figure 2** as FACS) involved in the cascade of free fatty acid uptake and activation were downregulated in omental versus subcutaneous tissue.

Solute Carrier Family 2, Member 1(SCLC2A1) also known as GLUT1 was upregulated with a borderline fold increase of 1.59. GLUT1 is a minor facilitated glucose transporter; however no differential expression of GLUT-4 which is the major glucose transporter was observed. Also upregulated was carnitine palmitoyltransferase 1C (CPT1C); this gene is included in the KEGG pathway as part of the CPT-1 gene complex, which comprises the 3 isoforms of CPT1A; CPT1B and CPT1C. The CPT-1 genes are generally involved in fatty acid oxidation but the specific function of CPT1C isoform is poorly understood.

Our heat-map shows significantly expressed genes and their relative expression in both subcutaneous and omental adipose tissue samples. The heat map analysis was able to differentiate omental and subcutaneous tissues via hierarchical clustering. The tissues clump together as per the color bar beneath the horizontal cluster tree, the grouping supports the hypothesis that the adipocytokine pathway is activated in the omental tissue (**Figure 2**).

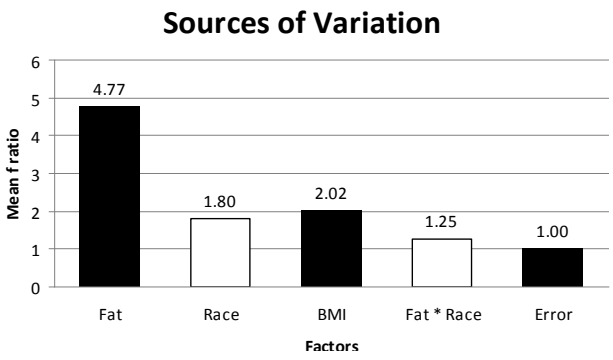


Figure 3. ANOVA model used to evaluate the impacts of the potential confounding variables: fat, race, BMI, fat and race in the differentially expressed genes in the study population. The horizontal column indicates the Mean F Ratio while the various factors analyzed in the multi factor ANOVA are listed horizontally along the x-axis. Any factor that has a Mean F Ratio more than error (1.00) is considered "significant".

Table 3. Differentially expressed genes in omental relative to subcutaneous adipose tissue.

Gene symbol (Symbol in KEGG pathway)	Gene name	Fold-change	P-value	Function
IRS1 (IRS)	Insulin Receptor Substrate-1	−1.771 63	0.000 014 6	Interacts with insulin receptor and enables insulin metabolic actions by signaling PI3K pathway
ADIPOQ (ADIPO)	Adiponectin, C1Q and collagen domain containing	−1.667 34	0.039 814	Signals increased glucose uptake via the GLUT transporters, modulates fatty acid catabolism
RXRA (RXR)	Retinoid X Receptor, alpha	−1.614 26	0.000 015	Activation of PPAR-alpha to promote uptake and utilization of fatty acids
CD36 (FATCD36)	Thrombospondin receptor	−2.058 39	0.000 181	Uptake of free fatty acids (FFA) from plasma
ACSL1 (FACS)	Acyl-CoA synthetase long-chain family member 1	−1.701 68	0.003 205	FFA activation for triacylglycerol synthesis
PTPN11 (SHP2)	Protein tyrosine phosphatase, non-receptor type 11	−1.733 8	0.0 000 256	Has regulatory role in mitogenic activation, metabolic control, transcription regulation, and cell migration
SLC20A1 (GLUT1)	Solute carrier family 2 (facilitated glucose transporter), member 1	1.589 51	0.000 079 4	Plasma membrane glucose transporter, responsible for basal glucose intake In adipose and skeletal muscle
CPT1C (CPT-1)	Carnitine palmitoyltransfer-ase 1C	1.703 3	0.000 060 2	Function not well understood, believed to play a role in regulation of energy homeostasis

Table 4 shows the quantitative RT-PCR results for the 7 positional candidate genes, which confirms the differential expression of all five upregulated genes and the two downregulated genes [19]. For example, for the IRS1 gene, the microarray fold change was −1.77, and the RT-PCR fold change was −1.48. We utilized genes not present in the adipocytokine pathways for external validation.

4. DISCUSSION

The objective of this study was to determine differentially expressed genes in omental when compared to subcutaneous adipose tissue in the KEGG Adipocytokine Signaling Pathway orthography, which may be associated with insulin resistance, inflammation, or adverse pregnancy outcomes.

Figure 1 depicts KEGG Adipocytokine Signaling Pathway and shows the cascades signaled by Adiponectin (ADIPO) and Leptin (LEP), the two major adipocytokines, and TNF-alpha (TNFα), a major inflammatory cytokine. Also depicted is a cascade leading to the uptake and activation of Free Fatty Acids via CD36 (FATCD36) and activation via acyl-CoA synthetase long chain family 1 (FACS).

Table 4. Real-time PCR validation of microarray results.

		Fold Change	
Gene	Ref_seq	Microarray	RT-PCR
IL-6	NM_000600	2.19	1.23
IL-1B	NM_000576	2	1.31
CLDN1	NM_021101	18.61	13.89
ADIPOQ	NM_004797	−1.67	−1.58
CFB	NM_001710	10.62	11.6
IRS1	NM_005544	−1.77	−1.48
PLA2G2A	NM_000300	4.48	3.5

The KEGG pathway cascade arising from TNF-alpha stimulation results in negative regulation of the IRS1 gene. TNF-alpha is an important inflammatory mediator associated with insulin resistance [20], and increased TNF-alpha has been linked to gestational diabetes [21, 22]. A critical function of the IRS1 is to interact with the Insulin Receptor, enabling the metabolic actions of insulin by signaling the PI3K pathway [23]. Thus, IRS1 is a pivotal gene in insulin sensitivity [24-26]. Of note is that IRS1 gene variations have been associated with increased in visceral to subcutaneous fat ratio as determined by computerized tomography of the abdomen [24]. In our study, IRS1 was downregulated in the omental adipose tissue, suggesting increased insulin resistance in that tissue. Another study showed significantly reduced IRS1 mRNA levels in adipocytes from obese compared to lean non-pregnant Pima Indians. Further, a role for IRS1 in the pathogenesis of type 2 diabetes has been suggested because of differential expression of IRS1 variants [23]. We did a PUBMED search using the following phrases "insulin receptor", insulin receptor protein", "adipose tissue", "omentum", "pregnancy", "pregnancy complications", "diabetes in pregnancy" and we could not find any report on downregulation of IRS1 in omental compared to subcutaneous tissue in non-obese healthy, non-diabetic pregnant women; consequently we believe that this finding is a contribution to the literature.

Protein Tyrosine Phosphatase, Non-Receptor Type 11

(PTPN11 also known as SHP2) was also downregulated in the omental adipose depot. The KEGG orthography notes SHP2 (PTPN11) is involved in signaling the MAPK signaling pathway, with consequences to cell growth and development. This is through activation by SHP2 of MAPK/ERK in response to leptin [29]. It has also been demonstrated that interactions between SHP2 and IRS1 promotes binding of IRS1 to the insulin receptor. SHP2, then, acts as an adaptor for insulin receptor and IRS1 forming a multiprotein signaling complex involving all three proteins to enhance glucose uptake. Downregulation of expression of SHP2, in the omental adipose depot [30] may serve to further predispose to insulin resistance.

In another signaling cascade, Adiponectin (ADIPO) was found to be downregulated in the omental depot in our study. As shown in the KEGG pathway, Adiponectin signals increased glucose uptake via the GLUT transporters [29,30]. To establish the involvement of adiponectin in insulin resistance in pregnancy, Cortelazzi *et al.* showed significantly lower adiponectin serum levels in women with GDM than in nondiabetic women at the same gestational ages [16]. Other studies have also shown decreases in serum adiponectin in late normal pregnancy [31,32]. Reports have shown that adiponectin is associated with improved glucose sensitivity, central body fat distribution, and that serum adiponectin concentrations are determined mainly by visceral adipose [33]. Our findings of differential downregulation of adiponectin in omental tissue in healthy non-obese pregnant women adds to this literature and support the association between visceral fat and increased insulin resistance.

Retinoid X Receptor, alpha (RXR) was also downregulated in omental adipose in our study. The KEGG Pathway shows that RXR forms a heterodimer with PPAR-alpha, and that PPAR-alpha is activated via leptin and adiponectin stimulation [34,35]. Activation of PPAR-alpha promotes uptake and utilization of fatty acids by upregulation of genes in fatty acid transport and beta-oxidation [36]. Downregulation of RXR can then lead to diminished fatty acid uptake and utilization in omental adipose tissue. RXR-alpha also forms a heterodimer with Peroxisome Proliferator-Activated Receptor-gamma (PP AR-gamma), not shown on the KEGG pathway, which binds to promoter sites and directly stimulates transcription of adiponectin. Of note, this is the site of action of thiazolidinedione rosiglitazone, which activates the PP AR-gamma-RXR-alpha heterodimers bound to PPAR-gamma response elements in the adiponectin promoter [43]. PPAR-gamma was also found in our study to be downregulated in omental adipose (−1.632 89 fold change, P = 0.006 071 56), further increasing insulin resistance.

CD36 (FATCD36), found to be downregulated in omental adipose, is involved in the uptake of free fatty acids from the plasma [37,38]. ACSL1 (FAT) also downregulated in omental adipose in our study is responsible for activation of free fatty acids for utilization within the cell. Decreased activity of this enzyme decreases the rate of fatty acid storage. Notably, a study showed that the content/activity of fatty acid storage enzymes in omental fat was dramatically lower in those with more visceral fat [39]. Recent evidence supports the role of CD36 in storing fatty acids in adipose tissue. The actions of these proteins prevent the adverse physiologic effects from high circulating free fatty acid (FFA) levels [39,40]. High circulating FFA levels can also lead to pro-inflammatory processes that impair insulin signaling in liver, adipose, and skeletal muscle leading to insulin resistance [41,42]. It has been shown that CD36 membrane levels and turnover are abnormal in diabetes, resulting in dysfunctional fatty acid utilization. Also, variants in the CD36 gene have been shown to increase susceptibility to the metabolic syndrome increasing risk for diabetes and cardiovascular disease [40]. Thus, the downregulation of these two genes in omental adipose tissue may predispose to hyperlipidemia and its sequelae.

GLUT 1 was upregulated in omental adipose in our study. It functions as a basal constitutive glucose transporter, however GLUT-1 is minimally, if at all upre-gulated in the plasma membrane by insulin stimulation in skeletal muscle and adipocytes [44-46]. It has been widely observed that insulin-dependent glucose influx in skeletal muscle and adipocytes relies largely on GLUT-4, not GLUT-1. Moreover, it has been shown that insulin resistance in skeletal muscle and adipose is caused by abnormalities in glucose transport via GLUT-4 [47], which we did not find to be differentially expressed in our study. GLUT-1 was found to be downregulated in skeletal muscle of gestational diabetic women relative to that of normal glucose tolerant pregnant women with no differential expression in subcutaneous adipose tissue. Importantly, GLUT-4 was downregulated in both adipose and skeletal muscle of gestational diabetic women in the same study [48]. Of note, elevation of GLUT-1 expression in adipocytes has been observed in insulin resistant animal models. One study suggests this may be part of a compensatory mechanism for glucose entry in a condition in which normal insulin-stimulated glucose uptake is attenuated [49]. Another report noted that high fat feeding may link GLUT1 upregulation mechanistically via oxidative stress through chronic increased glucose influx to diet-induced insulin resistance [50]. Thus current evidence does not support a role for GLUT1 in insulin sensitivity in adipose tissue.

Carnitine palmitoyl transferase 1C (CPT1C) mRNA was found to be upregulated in omental adipose. The CPT1C isoform is atypical of CPT1 family proteins since

it does not seem to be functionally related to CPT1A and CPT1B but seem to appear in the same category because it has high sequence similarity to them [51]. CPT1C has been shown to be primarily expressed in brain tissue; thus its upregulation noted in our study maybe a novel finding [52,53]. Unlike CPT1A and CPT1B, CPT1C is unable to catalyze acyl transfer from fatty acyl-CoAs to carnitine [53]. Recent studies have shown that CPT1c expression is localized in the ER and not in mitochondrial membrane which suggests that it may be less involved with fatty acid oxidation, and may be closer involved with palmitate transport across ER membrane, or perhaps as a metabolic sensor [51]. These findings underscore the poorly understood involvement of this gene in the regulation of energy homeostasis.

Limitations of this study include the utilization of a heterogeneous ethnic group. It is well accepted that risks of insulin resistance and diabetes may differ by ethnicity [54,55]. However our ANOVA analysis showed the minimal contribution of ethnicity to variation of the differently expressed genes. It is also possible that we could have used a larger sample size. However a review of the literature shows similar sample size in comparable studies [56-58]. We attempted to limit the influence of obesity on our study findings by excluding patients who are classified as obese with a BMI of 30; however we noted that some variation of our study may still be due to BMI since the F ratio was approximately 2 in the ANOVA analysis. A next step would be to perform metabolic testing to confirm function of the expressed genes.

It is acknowledged that adipose tissue is metabolically active and that intra-abdominal fat contributes to the pathophysiology of insulin resistance and inflammation [59]. Elucidation of the mechanisms of metabolic function of adipose tissue is ongoing. Most studies on adipose tissue are in the general population with fewer studies performed on pregnant women. The pathophysiology of adipose tissue function and insulin resistance in pregnancy is still not well understood, and may not necessarily be identical to that in the non-pregnant state. It is well accepted that obesity affects metabolic function, thus in this study we utilized only non-obese pregnant women in order to exclude the effect of obesity and assess the effect of pregnancy. In this study, we have shown the downregulation of several key genes in the KEGG Adipocytokine Pathway in omental when compared to subcutaneous adipose tissue, in which downregulation is linked to insulin resistance or inflammation. We observed borderline upregulation of GLUT-1, and have noted that the literature suggests this not have any significant role in insulin sensitivity in adipose tissue. As discussed, the upregulated gene CPT1C is poorly understood but we saw novel differential expression in omental adipose tissue. We believe that the findings from our study may contribute to literature on the metabolic activity of omental adipose tissue in pregnancy.

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The "metabolic syndrome index": A novel, comprehensive method for evaluating the efficacy of diabetes prevention programs

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ABSTRACT

A myriad of diabetes prevention programs are carried out worldwide to fight against the current type 2 diabetes (T2D) pandemic. The lack of a unified criterion for evaluating the efficacy of T2D prevention programs, however, makes the interpretation of prevention program results difficult, and hinders clear and direct comparisons of different prevention programs. Metabolic syndrome is a strong indicator for diabetes and its complications, holding great promise to become the basis of an intervention evaluation method. The Metabolic Syndrome Index (MSI), proposed here, quantifies the metabolic risk for developing T2D and its complications. The MSI is a novel scale for evaluating the efficacy of diabetes prevention programs because it is a systematic, comprehensive, and stable indicator that reflects the metabolic risk reduction for diabetes and its complications at multiple levels and dimensions.

Keywords: Evaluation; Metabolic Syndrome; Type 2 Diabetes Prevention; T2D

1. INTRODUCTION

In epidemiology, population attributable risk is the common method to describe risk reduction for a disease or an unhealthy condition [1]. However, this method is not applicable for most type 2 diabetes (T2D) prevention programs that involve intensive lifestyle intervention because these programs usually do not include a control group due to the nature of their study designs and ethical concerns. Currently, percent weight loss is a widely used indicator to evaluate the efficacy of T2D prevention programs because of its clear relationship with diet modifycation and exercise—two common constructs in lifestyle intervention [2,3]. Yet, weight change alone may not fully reflect the efficacy of lifestyle intervention in terms of improving prediabetes conditions and reducing the risk of developing T2D and its complications. Recently, Feller and colleagues pointed out that waist circumference provides a better measure of diabetes risk than BMI because development of T2D is especially influenced by visceral fat, which is more metabolically active and produces more hormones and cytokines than other adipose tissues [4]. In addition, many research studies have confirmed that beneficial changes in T2D incidence can be achieved independently of weight loss [5-7].

Metabolic syndrome, which is associated with central obesity and insulin resistance, among several other cardiometabolic components (**Table 1**), has been demonstrated as a reliable predictor of T2D and cardiovascu lardiseases [7-12]. Since cardiovascular diseases such as heart disease and stroke are the major diabetes complications, metabolic syndrome holds great promise to become the basis for measuring risk reduction for diabetes and its complications resulting from lifestyle intervenetions. Many research studies have included the pre-post measurement of metabolic syndrome components in their reports and confirmed that symptoms of metabolic syndrome can be improved by lifestyle interventions [13-18]. However, analysis of each of these components separately may mask or distort the comprehensive change across symptoms because all metabolic syndrome components are mutually linked with strong interactions. Application of a Metabolic Syndrome Index (MSI) overcomes this disadvantage because it incorporatesall metabolic syndrome components into a single, stable, and comprehensive score, reflecting the metabolic risk

Table 1. Diagnostic criteria for metabolic syndrome.

Component	Clinical Cutoff Values
Waist Circumference	≥102 cm in men ≥88 cm in women
Triglycerides	≥150 mg/dL
HDL Cholesterol	<40 mg/dL in men <50 mg/dL in women
Blood Pressure (BP)	≥130 mmHg Systolic BP or ≥85 mmHg Diastolic BP
Fasting Glucose	≥100 mg/dL
Diagnosis	Any 3 of the 5 features above

for diabetes and its complications.

Because metabolic syndrome is a cluster of metabolic risk factors that contribute greatly to the development of T2D, these syndrome components can be thought of as constituting a network of symptoms that reflect the risk for type 2 diabetes and its complications. The MSI is a scaleof that network. The definition and clinical cutoff values of metabolic syndrome was adopted from the criteria proposed by the American Heart Association (AHA) and the National Heart, Lung, and Blood Institute (NHLBI) in 2005 [11].

2. METHODS

The MSI, proposed here, is a five-point scale that assigns one point to each of the five metabolic syndrome components—waist circumference, blood pressure, triglycerides, high-density lipoprotein (HDL) cholesterol, and fasting blood glucose (**Table 1**). The five components are assumed to be equally important for diabetes and its complications because each component is assigned one point only. The validity of index's component equality is assured by the AHA/NHLBI criteria, which is defined as the presence of any three or more of the syndrome's five components. In this way the proposed MSI accounts for the clinical significance of each of the syndrome's risk factor cutoff values, and acknowledges the equal weight placed on each of the syndrome's diagnostic components.

Calculation of the MSI for an individual could not be simpler: a one point score is assigned to any risk factor that exceeds the metabolic syndrome cutoff value, and a score of zero is assigned to any risk factor value that falls below the cutoff value. The MSI value is simply the sum of the scores assigned each of the syndromes five components (**Figure 1**). A range from zero to five quantifies the metabolic risk for diabetes and its complications which can be reduced by lifestyle intervention. A MSI value should be calculated at least twice (before and after intervention) in order to calculate the percentage of the risk reduction potentially resulting from the intervention. Extra measurements during the intervention will provide more information in understanding the responses of metabolic syndrome components to lifestyle interventions and the trend of the MSI.

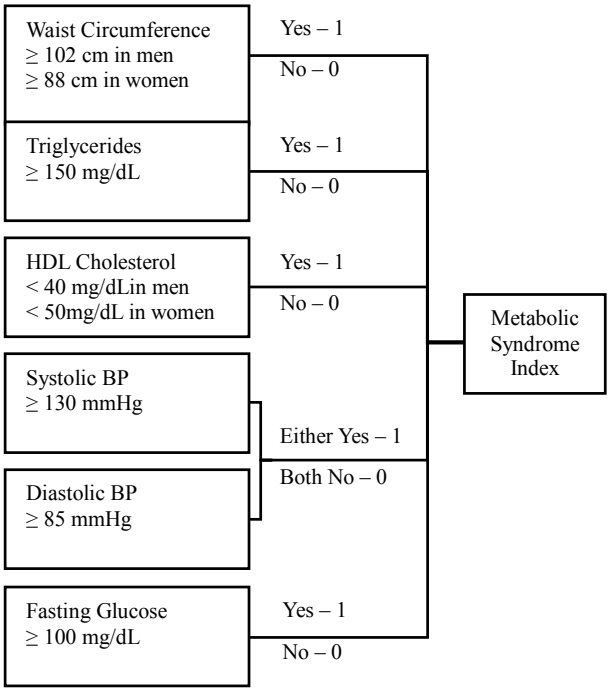

Figure 1. Metabolic syndrome index calculation flow chart.

The percentage of a MSI reduction quantifies the amount of risk for diabetes and its complications that has been avoided. In order to interpret the MSI better, we propose to use a new concept—metabolic risk—in describing the risk reduction caused by lifestyle intervenetion. Metabolic risk for diabetes and its complications is generally attributable to environmental and behavioral factors; in other words, risk that can be modified by lifestyle interventions. This risk reduction can be calculated and expressed as a single MSI value. Additionally, adoption of the MSI value will likely prove convenient for public health professionals because it is a direct reflection of the efficacy of T2D prevention interventions.

3. DISCUSSION

Lifestyle intervention for diabetes prevention programs is a multidimensional process with a series of health promotion strategies at multiple levels. The complexity of diabetes prevention programs necessitates a comprehensive and integrated method to evaluate their efficacy. Unlike weight loss, metabolic syndrome components provide multiple parameters that cover more aspects of risk reduction caused by lifestyle intervention and can provide a more comprehensive evaluation of the strengths and weaknesses of a given diabetes intervenetion. Utilization of a MSI incorporates and integrates di-

verse risk measures into one single parameter that can comprehensivelyquantify the efficacy of that program—both for individual participants, and for the intervention participant group as a whole.

Because the MSI being proposed here is derived from the sum of dichotomous (yes/no) variables reflecting established diagnostic cutoff values for the five risk factor components of metabolic syndrome, it might be argued that the MSI lacks sensitivity to improvements in component risk factors that do not reach these cutoff values. What the MSI may lack in sensitivity, however, it makes up in reliability. The fact that the metabolic syndrome identifies a suite of specific risk factors (present/absent) that have been shown to accurately predict future risk of developing T2D, suggests that any reduction in the MSI constitutes a clear and significant reduction in the future risk for developing T2D. The same cannot be said with respect to improvements in the absolute values of metabolic syndrome risk factor components that do not meet diagnostic cutoff values. As a result, we would suggest that the reliability of the MSI to predict clear and meaningful reductions in the risk of developing T2D sets an appropriate standard for the evaluation of T2D prevention program efficacy.

Application of the MSI provides summarized information in addition to metabolic syndrome components in evaluating diabetes prevention programs. It also serves as a comparison for other evaluation tools used in T2D prevention programs by reporting the amount of metabolic risk reduction for diabetes and its complications that has been achieved. Furthermore, the changes in each of the MSI components provides a more comprehensive evaluation of diabetes prevention program efficacy, showing great potential for helping public health professionals individualize and optimize program curriculums for different target populations. Conventional usage of the MSI in T2D research will establish a unified criterion in evaluating T2D prevention programs and facilitate efficacy comparisons among disparate diabetes interventions.

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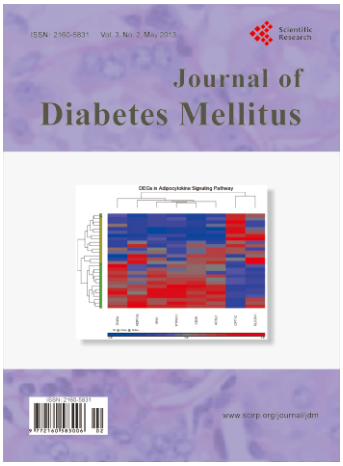

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