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Open-array analysis of genetic variants in Egyptian patients with type 2 diabetes and obesity

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ABSTRACT

Background: Diabetes mellitus is considered a major public health problem worldwide. Susceptibility to diabetes is influenced by both genetic and environmental determinants.
Aims/hypothesis: The aim of the present study was to test for 16 independent single nucleotide polymorphisms (SNPs) in established Type 2 diabetes (T2D) and obesity susceptibility loci by GWAS in a sample of Egyptian patients to find out if there is shared genetic background underlying both disease entities.
Methods: Genotyping was performed using OpenArray® protocol on the QuantStudio™ 12K Flex Real-Time PCR System. In the present case control study a custom array was designed to facilitate cost-effective analysis of selected SNPs related to glycolysis, gluconeogenesis, inflammation, insulin signalling, and immune function.
Results: Seven gene variants showed significant association with the risk of T2D patients including *FCGRA2*, *STAT4*, *CELSR2*, *PPARG*, *EXT2* rs3740878, *GCKR*, *PTGS1*. Factors that significantly affect T2D were obesity ($p < 0.001$) and *GCKR* ($p = 0.001$) and *PTGS1* ($p = 0.001$) gene variants. Gene variants that showed significant or borderline effect on obesity were *MTHFD1*, *EXT2* rs3740878, *GCKR and PTGS1* ($p = 0.03$, 0.017, 0.059, 0.006) respectively.
Conclusions/interpretation: Overlapping genetic aspects should be considered and the presence of risk alleles of different genes together could contribute to the risk of T2D or obesity or both. The *MTHFD1* and *EXT2*rs3740878 gene variants significantly affect obesity and not shared with T2D. Gene variants that showed combined effect on both disease entities were *GCKR* and *PTGS1*. These findings provide a basis for future studies on a larger scale. More stress on the risk gene variants that have a combined impact on both diabetes and obesity is recommended to improve risk prediction and preventive strategies.
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1. Introduction

Diabetes mellitus is considered a major public health problem worldwide. In 2015 the International Diabetes Federation indicated that 415 million adults live with diabetes mellitus in the world. In Egypt over 7.8 million adult cases were reported with 14.9% prevalence [1]. Susceptibility to diabetes is influenced by both genetic and environmental determinants. The major environmental risk factors for Type 2 diabetes (T2D) are obesity and a sedentary lifestyle. Waist-to-hip circumference ratio (WHR), corresponding to fat distribution also has an impact on T2D risk [2,3]. Additionally, chronic inflammation or infections might provoke insulin resistance and thereby contribute to the development of diabetes and its complications [4].

Advances in genotyping technology during the last years have facilitated rapid progress in large-scale genetic studies The large scale genome wide association studies (GWAS) have identified approximately 80 single nucleotide polymorphisms (SNPs) conferring susceptibility to type 2 diabetes (T2D) [5].

The OpenArray®, a new platform technology on the QuantStudio™ 12K Flex Real-Time PCR System accelerates genomic confirmation and screening, enabling unprecedented gene coverage and sample throughput [6]. In the present study a custom array was designed to facilitate cost-effective analysis of independent single nucleotide polymorphisms (SNPs) related to glycolysis, gluconeogenesis, inflammation, insulin signalling, and immune func-

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tion. All of which could provide a rationale for preventive or therapeutic strategies. The common variant associated with T2D, *PPARG*, is a well-established target of thiazolidinedione (TZD) drugs, and used clinically to reduce insulin resistance in T2D patients [7].

Sixteen SNPs at established T2D and obesity susceptibility loci by GWAS were tested using new OpenArray® genotyping protocol among Egyptian patients. Association between genetic variants and the risk of T2D and obesity was analysed to find out if there is shared genetic background underlying both disease entities.

2. Subjects and methods

A case - control study of 74 Egyptian participants; 37 patients with type 2 diabetes mellitus (T2D) (mean age: 48.6 ± 9.7 years; M/F: 16/21) and 37 non-diabetics age and sex matched (mean age: 45.4 ± 10.2 years; M/F: 15/22) were investigated. Sample size has been adjusted to the used Open Array platform. Diabetic patients were recruited from Ein-Shams University and National Institute of Diabetes and Endocrinology in 2014–2015. All laboratory investigations and genetic studies were performed at the Medical Molecular Laboratory Research Unit at National Research Centre, Clinical and Chemical Pathology department. This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans and was approved by the ethical committee of the National Research Centre. All patients and controls provided informed consent for their participation in the study. Patients were subjected to medical history with special emphasis on demographic and related clinical data, age of onset of T2D, family history, history of drug intake and type of treatment for diabetes. Patients with T2D were diagnosed according to American Diabetes Association guidelines [8].

Body weight (WT), height (HT), waist circumference (WC), and hip circumference (HC) were measured using standard methods, and body mass index (BMI) was calculated as weight/height^2 (kg/m^2) for all participants. All participants were then classified according to BMI into two groups; diabetic obese patients (N = 26) with BMI $\geq$ 30 and non-diabetic non-obese control group (N = 36).

2.1. Patient selection

Exclusion criteria:

Patients who receive treatment for inflammatory or chronic infectious disease or malignancy were excluded from the study.

2.2. Blood Chemical analyses

Venous blood samples were obtained from all participants for measuring fasting plasma glucose (FPG), fasting insulin (F Ins), and glycosylated haemoglobin (HbA1c) using standard biochemical analysis. The homeostasis model assessment for beta cell function (HOMA-B) and the homeostasis model assessment for insulin resistance (HOMA-IR) were used to assess insulin resistance. The formulas were as follows:

HOMA-B = Fasting serum insulin $\times$ 20/(FPG $-$ 3.5) (with serum insulin in mU/l and plasma glucose in mmol/l).

HOMA-IR = fasting serum insulin $\times$ FPG/22.5.

2.3. Genetic analyses

All participants were genotyped for: Methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*) (rs2236225); Fc fragment of IgG, low affinity 2A (*FCGR2A*) (rs1801274); signal transducer and activator of transcription 4 (*STAT4*) (rs7574865); complement factor B (*CFB*) (rs547154); Cadherin, EGF LAG Seven-Pass G-Type Receptor 2 (*CELSR2*) (rs599839); fat mass and obesity-associated gene (*FTO*) (rs17817449); rs10811661 upstream of cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*); hemochromatosis gene (*HFE*) (rs1800562); peroxisome proliferator-activated receptor-gamma (*PPARG*) (rs1801282); neuropeptide Y (*NPY*) (rs16147); exostosin-2 (*EXT2*) (rs11037909, rs3740878); glucokinase (hexokinase 4) regulator (*GCKR*) (rs780094); C-reactive protein (*CRP*) (rs2808630); prostaglandin-endoperoxide synthase 1 (*PTGS1*) (rs5788); the intergenic variant of chromosome 11p12 (rs9300039).

Custom array with TaqMan assays were designed by the Bioinformatics Group at Life Technologies to facilitate cost-effective genotyping using QuantStudio™ 12K Flex OpenArray® genotyping protocol (Custom TaqMan® SNP Genotyping kit including plates and accessories). Selected format of OpenArray™ Plates allowed the performance of the required 16 assays for all the samples.

2.3.1. Preparing the DNA samples

Five milliliters of whole blood were collected from each subject into tubes containing ethylene-diamine-tetra-acetic acid (EDTA). Genomic DNA (gDNA) was purified using the QIAcube automated instrument according to the manufacturer's guidelines (Qiagen, Germany). DNA quality and quantity were adjusted at A260/280 ratio between 1.7 and 1.9 by NanoDrop ND-1000 (PeqLab) and normalized to the recommended working concentration at ~50 ng/μL.

The gDNA samples were stored at −80 °C until further use. The TaqMan PCR reaction was performed using TaqMan® OpenArray™ Master Mix (Life Technologies) according to the manufacturer's recommendation.

2.3.2. Setting up the 96 well plates [9]

The concentration of the thawed normalized genomic DNA samples was reviewed. 5.0 microliter (μL) of the DNA samples was put into the appropriate number of wells of a 96-well MicroAmp Optical Reaction Plate according to the selected format of OpenArray™ Plates. Then Tracking the samples from the 96 well plate to the TaqMan® OpenArray™ 384-Well sample plate was performed using sample tracker software provided by the equipment. 2.5 μL of master mix and 2.5 μl of normalized DNA samples were added to the 384-well sample plate. Then the QuantStudio OpenArray AccuFill Software was initialized for loading onto the OpenArray™ plates. Then the plate was immediately sealed and immersion fluid was injected. At this step the sealed OpenArray plate is ready to be loaded into the QuantStudio 12K Flex System and thermal cycling was performed according to the manufacturer's recommendation. Analysis of the genotyping results was performed using provided TaqMan Genotyper software.

2.4. Statistical methods

All test data were converted and manipulated by using SPSS software program version 18.0. Data were analysed, mean and standard deviation were calculated as regards quantitative demographic, anthropometric and biochemical data. The quantitative data were compared and *t* test was applied and p value was established to determine the statistically significant difference between two groups. While numbers and percent were calculated and presented among groups as regards qualitative data. To determine the relationship strength between variables statistical correlation study was performed and coefficient of correlation (r) was measured. Allelic discrimination was analysed using Hardy–Weinberg equilibrium (HWE) analysis tool supplied with the Taqman Genotyper software on the QuantStudio™ 12K Flex Real-time PCR system. Chi-square test was used for testing the association of gene variants with disease entities and for estimating the odds ratios

(ORs) and 95% confidence intervals (CI). Multiple linear regression was applied to determine the linear relationship between a dependent variable and a series of independent variables. Multiple logistic regression was used when studying the effect of one nominal and two or more measurement variables. The difference between the two groups was considered statistically significant when $p < 0.05$, and considered highly statistical significant when $p < 0.01$.

3. Results

The demographics, anthropometric and biochemical findings of the participants are listed in Table 1. Diabetes duration ranged from 0.2 to 22 years. Of diabetic patients 86.5% have positive family history. Of female subjects 44.2% and of male subjects 25.8% were obese with BMI $\geq$ 30. Of diabetic patients 70.3% were on insulin therapy and 29.7% were on hypoglycemic drugs. Anthropometric indices [WT, WC, HC, and waist to hip ratio (WHR)] were significantly increased in diabetic patients when compared to non-diabetic participants with $p < 0.001$. Mean levels of FPG, HbA1c were significantly higher in diabetic patients in comparison to the non-diabetic participants. Highly significant reduction of HOMA-B in the T2D patients was detected compared to non-diabetic participants.

WHR showed highly significant positive correlation with BMI, FPG, HbA1c and HOMA-IR ($r = 0.375$ and $p < 0.001$, $r = 0.269$ and $p = 0.001$; $r = 0.430$ and $p < 0.001$; $r = 0.234$ and $p = 0.004$ respectively). Also, BMI showed highly significant positive correlation with FPG ($r = 0.493$), HbA1c ($r = 0.606$) with $p < 0.001$. Highly significant negative correlation was detected between BMI and HOMA-B ($r = -0.416$ and $p = <0.001$) and borderline significance between WHR and HOMA-B ($r = -0.161$ and $p = 0.051$).

Significantly increased BMI was observed in association with risk variants of *MTHFD1* ($p = 0.016$), *STAT4* ($p = 0.014$), *PPARG* ($p = 0.001$), *EXT2* rs3740878 ($p < 0.001$), *GCKR* ($p = 0.004$), *PTGS1* ($p = 0.004$) and borderline significance with *FTO* variant ($p = 0.07$).

To determine the influence of obesity cofactor in the genetic variation analyses we compared obese diabetic patients (N = 26 and BMI> = 30) with non-obese non-diabetic control individuals (N = 36). Genotype frequencies in all the studied groups are demonstrated in Table 2.

The magnitude of risk (OR) of the different risk alleles for all diabetic and obese-diabetic patients are presented in Table 3. Of all tested genetic variants seven SNPs showed significant association with the risk of T2D including ***FCGR2A***, ***STAT4, CELSR2, PPARG, EXT2*** **rs3740878,** ***GCKR, PTGS1***. Eight SNPs showed significant association with the risk of obesity with T2D including ***MTHFD1***, ***STAT4***, ***CELSR2, FTO, PPARG***, ***EXT2*** **rs3740878**, ***GCKR***, ***PTGS1***.

Significant associations between risk alleles of *PPARG, EXT2* rs3740878, *GCKR, PTGS1* gene variants and increased FPG levels were observed ($p = 0.001$, <0.001, 0.002, 0.001 respectively). Risk alleles of *STAT4, PPARG, EXT2* rs3740878, *GCKR, PTGS1* gene variants showed significant associations with increased HbA1c levels ($p = 0.034$, 0.004, 0.003, <0.001, 0.003 respectively) and decreased HOMA-B ($p < 0.001$, 0.016, 0.001, 0.046, 0.003 respectively). The only significant association with increased HOMA-IR was observed with the risk allele of *STAT4* gene variant ($p = 0.008$). Also *STAT4 and EXT2* rs3740878 risk alleles were significantly associated with reduced F Ins levels ($p = <0.001$ and 0.03 respectively).

With logistic regression analysis we found that factors that significantly affect T2D were obesity and *GCKR* and *PTGS1* gene variants ($p < 0.001$, 0.001, 0.001) respectively (Table 4).

On the other hand gene variants that showed significant or borderline impact on obesity were *MTHFD1, EXT2* rs3740878, *GCKR and PTGS1* ($p = 0.03$, 0.017, 0.059, 0.006) respectively (Table 5).

These findings clarified that the gene variants that significantly affect obesity and not shared with T2D were the ***MTHFD1*** **and** ***EXT2*** **rs3740878** gene variants. ***GCKR*** **and** ***PTGS1*** gene variants showed combined effect on both disease entities.

4. Discussion

In the present study we used a new platform technology on the QuantStudio™ 12K Flex Real-Time PCR System. Custom array was designed to perform genotyping of all selected independent genetic variants in Egyptian patients with T2D with and without obesity. Both T2D and obesity are associated frequently and considered as key components of metabolic syndrome [10]. In this

Table 1
Demographic, biochemical and anthropometric characters of studied subjects.

Variables		Diabetic patients (n = 37) Mean ± SD	Control subjects (n = 37) Mean ± SD	p
Age	Years	48.7 ± 9.8	45.5 ± 10.3	0.176
Sex no (%)	Female	21 (56.8)	22 (59.5)	0.814
	Male	16 (43.2)	15 (40.5)	
Weight	kg	84.9 ± 10.7	72.8 ± 9.0	<0.001[b]
Height	cm	163.1 ± 9.6	168.5 ± 9.3	0.017[a]
BMI	kg/m^2	32.5 ± 4.4	24.9 ± 2.2	<0.001[b]
WC	cm	106.9 ± 11.7	78.7 ± 9.7	<0.001[b]
HC	cm	114.0 ± 10.2	88.8 ± 9.6	<0.001[b]
WHR		0.938 ± .05	0.886 ± .03	<0.001[b]
FPG	mg/dl (mmol/l)	199.1 ± 66.2 (11.06 ± 3.67)	87.5 ± 12.6 (4.86 ± 0.7)	<0.001 [b]
HBA1C	% (mmol/mol)	8.7 ± 2.1 (72.6 ± 22.9)	5.1 ± 0.7 (31.1 ± 6.1)	<0.001[b]
F Ins	mU/l	11.5 ± 14.9	16.5 ± 9.6	0.087
HOMA B	Median (IQR)	16.3 (4.9–59.6)	223.0 (112.3–500.0)	<0.001[b]
HOMA IR		5.3 ± 3.6	3.5 ± 2.1	0.182
Type of treatment	No (%)			
	Insulin	26 (70.3)		
	Oral	11 (29.7)	–	
F history	No (%)			
	Yes	32 (86.5)	–	
	No	5 (13.5)		
DD	Years (range)	8.4 ± 7.1 (0.2–22)	–	

BMI: body mass index, Waist circumference: WC, Hip circumference: HC, WHR: waist-to-hip ratio, FPG: Fasting plasma glucose, F Ins: Fasting insulin, IQR: interquartile range, F history: Family history, DD: Duration of disease.
[a] Statistical significant difference $p < 0.05$.
[b] Statistical highly significant difference $p < 0.01$.

Table 2
Genotype frequencies among all the studied groups.

Gene	Marker SNP	Chromosomal Region	Risk Alleles (Bold)	Genotype	Diabetic patients N = 37 n (%)	ControlN = 37 n (%)	X^2	P value	Diabetic – obese patients[c] N = 26 n (%)	Non-obese non-diabetic control N = 36 n (%)	X^2	P value
MTHFD1	Rs2236225	14:64442127	**G**/A	AA	31 (83.8)	34 (91.9)	1.138	0.286	20 (76.9)	34 (94.4)	4.124	**0.042**[a]
				AG	6 (16.2)	3 (8.1)			6 (23.1)	2 (5.6)		
FCGR2A	Rs1801274	1:161509955	**G**/A	GG	4 (10.8)	0	4.515	0.105	3 (11.5)	0	4.373	0.112
				AG	6 (16.2)	5 (13.5)			3 (11.5)	5 (13.9)		
				AA	27 (73.0)	32 (86.5)			20 (76.9)	31 (86.1)		
STAT4	Rs7574865	2:191099907	**T**/G	TT	26 (70.3)	19 (51.4)	5.446	0.066	20 (76.9)	18 (50.0)	6.666	**0.036**[a]
				GT	5 (13.5)	3 (8.1)			3 (11.5)	3 (8.3)		
				GG	6 (16.2)	15 (40.5)			3 (11.5)	15 (41.7)		
CFB	Rs547154	6:31943161	**T**/G	TT	14 (37.8)	13 (35.1)	1.791	0.408	10 (38.5)	12 (33.3)	1.816	0.403
				GT	14 (37.8)	10 (27.1)			10 (38.5)	10 (27.8)		
				GG	9 (24.3)	14 (37.8)			6 (23.1)	14 (38.9)		
CELSR2	Rs599839	1:109279544	**A**/G	AA	5 (13.5)	0.0	6.815	**0.033**[a]	3 (11.5)	0.0	5.873	**0.05**[a]
				AG	14 (37.8)	11 (29.7)			10 (38.5)	10 (27.8)		
				GG	18 (48.6)	26 (70.3)			13 (50.0)	26 (72.2)		
FTO	Rs17817449	16:53779455	**T**/G	GG	17 (45.9)	23 (62.2)	3.4	0.183	10 (38.5)	23 (63.9)	5.693	**0.058**
				GT	18 (48.6)	14 (37.8)			14 (53.8)	13 (36.1)		
				TT	2 (5.4)	0			2 (7.7)	0		
CDKN2A/B	Rs10811661	9:22134095	**T**/C	TT	7 (18.9)	0	8.913	**0.012**[a]	6 (23.1)	0	10.371	**0.006**[b]
				CT	13 (35.1)	21 (56.8)			8 (30.8)	20 (55.6)		
				CC	17 (45.9)	16 (43.2)			12 (46.2)	16 (44.4)		
HFE	Rs1800562	6:26092913	**A**/G	AA	13 (35.1)	13 (35.1)	6.454	**0.04** [a]	9 (34.6)	12 (33.3)	3.516	0.172
				AG	21 (56.8)	13 (35.1)			14 (53.8)	13 (36.1)		
				GG	3 (8.1)	11 (29.7)			3 (11.5)	11 (30.6)		
PPARG	Rs1801282	3:12351626	**G**/C	GG	14 (37.8)	5 (13.5)	12.111	**0.002**[b]	10 (38.5)	5 (13.9)	9.243	**0.01**[a]
				CG	18 (48.6)	14 (37.8)			12 (46.2)	13 (36.1)		
				CC	5 (13.5)	18 (48.6)			4 (15.4)	18 (50.0)		
NPY	Rs16147	7:24283791	**C**/T	CC	24 (64.9)	25 (67.6)	0.06	0.806	16 (61.5)	24 (66.7)	0.173	0.677
				CT	13 (35.1)	12 (32.4)			10 (38.5)	12 (33.3)		
EXT2	Rs11037909	11:44234064	**T**/C	TT	16 (43.2)	14 (37.8)	1.162	0.559	13 (50.0)	13 (36.1)	1.234	0.54
				CT	18 (48.6)	17 (45.9)			10 (38.5)	17 (47.2)		
				CC	3 (8.1)	6 (16.2)			3 (11.5)	6 (16.7)		
EXT2	Rs3740878	11:44236252	**T**/C	TT	4 (10.8)	0	9.165	**0.01**[a]	4 (15.4)	0	9.755	**0.008**[b]
				CT	12 (32.4)	5 (13.5)			8 (30.8)	5 (13.9)		
				CC	21 (56.8)	32 (86.5)			14 (53.8)	31 (86.1)		
GCKR	Rs780094	2:27518370	**T**/C	TT	11 (29.7)	0	14.457	**0.001**[b]	6 (23.1)	0	11.37	**0.003**[b]
				CT	14 (37.8)	14 (37.8)			12 (46.2)	14 (38.9)		
				CC	12 (32.4)	23 (60.0)			8 (30.8)	22 (61.1)		
CRP	Rs2808630	1:159711078	**T**/C	TT	18 (48.6)	22 (59.5)	2.3	0.317	15 (57.7)	22 (61.1)	1.024	0.599
				CT	15 (40.5)	9 (24.3)			9 (34.6)	9 (25.0)		
				CC	4 (10.8)	6 (16.2)			2 (7.7)	5 (13.9)		
PTGS1	Rs5788	9:122381513	**A**/C	AA	6 (16.2)	0	11.937	**0.003**[b]	3 (11.5)	0	7.854	**0.02**[a]
				AC	17 (45.9)	10 (27.0)			12 (46.2)	10 (27.8)		
				CC	14 (37.8)	27 (73.0)			11 (42.3)	26 (72.2)		
Intergenic	Rs9300039	11:41893816	**C**/T	CC	15 (40.5)	20 (54.1)	1.982	0.371	10 (38.5)	19 (52.8)	1.358	0.507
				CT	19 (51.4)	13 (35.1)			13 (50.0)	13 (36.1)		
				TT	3 (8.1)	4 (10.8)			3 (11.5)	4 (11.1)		

[a] Statistical significant difference $p < 0.05$.
[b] Statistical highly significant difference $p < 0.01$.
[c] BMI ≥ 30.

study we are trying to find out if there is shared genetic background underlying these diseases in a sample of Egyptian patients.

Many previous studies have shown that obesity is associated with increased risk of diabetes [11–13]. In the current work correlation studies revealed significant positive correlation between BMI and central fat accumulation (WHR), and FPG and HbA1c blood levels. Inverse association was detected between BMI and beta cell function (HOMA-B). Also logistic regression analysis demonstrated that obesity is the most significant factor affecting T2D. These findings suggested that management of BMI could significantly improve beta cell function and reduce the risk of T2D.

Of all tested genetic risk variants seven SNPs showed significant association with the risk of T2D including *FCGRA2, STAT4, CELSR2, PPARG, EXT2* rs3740878, *GCKR* and *PTGS1*. On the other hand eight SNPs showed significant association with the risk of obesity with T2D including *MTHFD1, STAT4, CELSR2, FTO, PPARG, EXT2* rs3740878, *GCKR* and *PTGS1*.

4.1. FCGRA2, STAT4 and HFE

To the best of our knowledge, the *FCGR2A* association with T2D has not been previously investigated. *FCGR2A* is a member of a family of immunoglobulin Fc receptor genes found on the surface of many immune-response cells. In the current study *FCGR2A* risk allele showed significant association with risk of T2D. This finding raises the possibility of inflammatory pathogenic mechanism as a risk factor for T2D.

STAT4 is a central inflammatory mediator that is s required for the development of fully functional T helper1 cells. In the present work *STAT4* risk allele showed significant association with the risk of both T2D and obesity. A previous study by Yang et al. suggested that clinical insulin-dependent diabetes mellitus could be prevented in prediabetic individuals at risk through selective inhibition of the *STAT4* activation pathway [14]. A meta-analysis investigated the *STAT4* polymorphisms and diabetes risk found

Table 3
Risk allele frequencies among all the studied groups.

Gene	Marker SNP	Risk Alleles (Bold)	Diabetic patients N = 37 %	Control N = 37 %	Odds ratio (95% CI)	P value	Diabetic – obese patients[c] N = 26 %	Non-obese non-diabetic control N = 36 %	Odds ratio (95% CI)	P value
MTHFD1	Rs2236225	**G**	8.1	4.1	2.1 (0.5–8.9)	0.302	11.5	2.8	4.6 (0.9–23.6)	**0.05**[a]
		A	91.9	95.9			88.5	97.2		
FCGR2A	Rs1801274	**G**	18.9	6.8	3.2 (1.1–9.5)	**0.027**[a]	17.3	6.9	2.8 (0.9–8.9)	0.072
		A	81.1	93.2			82.7	93.1		
STAT4	Rs7574865	**T**	77.0	55.4	2.7 (1.3–5.5)	**0.005**[b]	82.7	54.2	4.0 (1.7–9.5)	**0.001**[b]
		G	23.0	44.6			17.3	45.8		
CFB	Rs547154	**T**	56.8	48.6	1.4 (0.7–2.6)	0.324	57.7	47.2	1.5 (0.7–3.1)	0.25
		G	43.2	51.4			42.3	52.8		
CELSR2	Rs599839	**A**	32.4	14.9	2.7 (1.2–6.1)	**0.012**[a]	30.8	13.9	2.8 (1.1–6.7)	**0.023**[a]
		G	67.6	85.1			69.2	86.1		
FTO	Rs17817449	**T**	29.7	18.9	1.8 (0.8–3.9)	0.125	34.6	18.1	2.4 (1.0–5.5)	**0.036**[a]
		G	70.3	81.1			65.4	81.9		
CDKN2A/B	Rs10811661	**T**	36.5	28.4	1.4 (0.7–2.9)	0.293	38.5	27.8	1.6 (0.8–3.5)	0.209
		C	63.5	71.6			61.5	72.2		
HFE	Rs1800562	**A**	63.5	52.7	1.6 (0.8–3.0)	0.184	61.5	51.4	1.5 (0.7–3.1)	0.262
		G	36.5	47.3			38.5	48.6		
PPARG	Rs1801282	**G**	62.2	32.4	3.4 (1.7–6.7)	**<0.001**[b]	61.5	31.9	3.4 (1.6–7.2)	**0.001**[b]
		C	37.8	67.6			38.5	68.1		
NPY	Rs16147	**C**	82.4	83.8	0.9 (0.4–2.1)	0.826	80.8	83.3	0.8 (0.3–2.1)	0.712
		T	17.6	16.2			19.2	16.7		
EXT2	Rs11037909	**T**	67.6	60.8	1.3 (0.7–2.6)	0.392	69.2	59.7	1.5 (0.7–3.2)	0.277
		C	32.4	39.2			30.8	40.3		
EXT2	Rs3740878	**T**	27.0	6.8	5.1 (1.8–14.5)	**0.002**[b]	30.8	6.9	6.0 (2.0–17.6)	**<0.001**[b]
		C	73.0	93.2			69.2	93.1		
GCKR	Rs780094	**T**	48.6	18.9	4.1 (1.9–8.5)	**<0.001**[b]	46.2	19.4	3.6 (1.6–7.9)	**0.001**[b]
		C	51.4	81.1			53.8	80.6		
CRP	Rs2808630	**T**	68.9	71.6	0.8 (0.4–1.8)	0.719	75.0	73.6	1.1 (0.5–2.4)	0.862
		C	31.1	28.4			25.0	26.4		
PTGS1	Rs5788	**A**	39.2	13.5	4.1 (1.8–9.3)	**<0.001**[b]	34.6	13.9	3.3 (1.4–7.9)	**0.006**[b]
		C	60.8	86.5			65.4	86.1		
Intergenic	Rs9300039	**C**	33.8	28.4	1.3 (0.6–2.6)	0.478	36.5	29.2	1.4 (0.7–3.0)	0.386
		T	66.2	71.6			63.5	70.8		

[a] Statistical significant difference $p < 0.05$.
[b] Statistical highly significant difference $p < 0.01$.
[c] BMI $\geq$ 30.

Table 4
Logistic Regression determining factors affecting diabetes mellitus.

Variable	B	S.E	Wald	df	Sig	Exp(B)
Obesity	5.183	.881	34.613	1	**<0.001**[a]	178.172
FCGR2A	1.600	.859	3.464	1	.063	4.951
CDKN2A/B	-1.071	.640	2.799	1	.094	.343
GCKR	2.007	.607	10.941	1	**0.001**[a]	7.444
PTGS1	2.174	.639	11.582	1	**0.001**[a]	8.795
Constant	-17.346	3.069	31.953	1	.000	.000

Variable(s) entered on step 1: obesity, FCGR2A, STAT4, MTHFD1, CELSR2, FTO, CDKN2A/B, HFE, PPARG, EXT2(rs3740878), GCKR, PTGS1.
[a] Statistical significant difference $p < 0.05$.
[b] Statistical highly significant difference $p < 0.01$.

Table 5
General Linear Model determining genes affecting BMI.[a,b]

Variable	Type III Sum of Squares	Mean Square	F	Sig.
MTHFD1	96.51	96.51	4.834	**0.03**[a]
EXT2 (rs3740878)	116.69	116.69	5.845	**0.017**[a]
GCKR	72.79	72.79	3.646	**0.059**
PTGS1	154.72	154.72	7.749	**0.006**[b]
STAT4 * PTGS1	102.63	102.63	5.36	**0.022**[a]

Variable(s) entered: FCGR2A, STAT4, MTHFD1, CELSR2, FTO, CDKN2A/B, HFE, PPARG, EXT2(rs3740878), GCKR, PTGS1.
[a] Statistical significant difference $p < 0.05$.
[b] Statistical highly significant difference $p < 0.01$.

that rs7574865 polymorphism was significantly associated with diabetes risk, especially diabetes type 1. However, they did not give consistent results for T2D [15]. They postulated the mechanisms by which chronic inflammation or infections could provoke insulin resistance and thereby contribute to the development of diabetes and its complications. Macrophages release cytokines that cause cells of liver and muscle to be insulin resistant. Another mechanism that is more consistent with the current study is to affect

insulin secretion through decreasing beta cell mass. This is evidenced by the highly significant decrease in the fasting insulin levels and HOMA-B in association with *STAT4* gene variant. Also *STAT4* gene was the only variant that showed significant association with insulin resistance (HOMA-IR) in the studied patients. Additionally *STAT4* effect on T2D might be mediated by its impact on obesity. Several mechanisms were proposed to understand the links between obesity and inflammation. Obesity increases the severity of pancreatitis through upregulation of the inflammatory response. Obesity reduces the number of T-regulatory cells and increases the number of $CD8^+$ T cells, which promotes macrophage recruitment and increases levels of the proinflammatory hormone leptin and decreases its anti-inflammatory counterpart, adiponectin [16].

The hemochromatosis gene (*HFE*) codes for a protein that functions to regulate circulating iron uptake by regulating the interaction of the transferrin receptor with transferrin. This gene has been suggested to be involved in the etiology of type 2 diabetes mellitus in previous studies [17,18].

In the present work the *AA* and *AG* genotype frequencies were significantly higher in diabetic patients compared to non-diabetic participants with p = 0.04. A previous study speculated that the relatively strong association of increased serum ferritin levels with abnormal glucose tolerance raises the possibility that progressive iron accumulation in the pancreas contributes to beta cell dysfunction and abnormal glucose tolerance [18]. In addition the HFE protein functions to regulate the expression of hepcidin, a secreted hepatocyte peptide that is the principal regulator of iron homeostasis. Hepcidin production increases with iron loading and inflammation [19]. These findings added evidence for the link between inflammation and T2D pathogenesis.

4.2. PPARG

PPARG gene is a target for the hypoglycemic drugs known as thiazolidinediones. *PPARG* is one of the few genes that have been confirmed to be associated with insulin resistance [20]. The *PPARG* rs1801282 *C* > *G* polymorphism also known as Pro12Ala, a SNP in exon 2 of *PPARG*, encodes a proline → alanine substitution at amino acid residue 12. The more common (*C*) allele encodes the 'Pro' amino acid at this SNP position [20,21].

In our results rs1801282 was investigated and *PPARG* Ala-allele showed highly significant association with T2D risk (p < 0.001). The *GG* genotype frequency was highly significantly increased in diabetic patients (p = 0.002). The presence of Ala-allele was significantly related to increased blood levels of FPG and HbA1c and decreased HOMA-B supporting its impact on glucose metabolism and beta cell function.

These results are in line with the previous Finnish Diabetes Prevention Study that investigated 522 subjects with impaired glucose tolerance and subjected them to either placebo or a lifestyle intervention. They reported a twofold increase in risk of developing T2D among alanine carriers in the placebo arm when compared to proline homozygotes P/P [22]. On the other hand, the much larger Botnia Prospective Study (N = 2293) documented a hazard ratio for developing diabetes of 1.7 among P/P homozygotes, a result which was statistically significant [23].

In our results the Ala-allele bearers had a significantly higher risk of obesity (p = 0.001) and the presence of the gene variant was significantly associated with increased BMI.

4.3. EXT2

Exostosin (EXT) proteins may affect beta-cell mass and insulin secretion capacity in humans, and render subjects at a higher risk of developing type 2 diabetes when exposed to environmental risk factors [24,25].

In the current work we studied two SNPs of (*EXT2*) gene (rs3740878 and rs11037909). We found an association between rs3740878 and the risk of T2D which is consistent with previous studies [26]. The risk allele frequency was highly significantly increased in diabetic patients compared with non-diabetic individuals. The same was observed when comparing obese diabetic patients with non-obese non-diabetic individuals. We found a significant association between the risk allele of rs3740878 and increased FPG, HbA1c, F Ins blood levels and BMI and with reduced HOMA-B. These findings support the impact of this gene variant on glucose metabolism, beta cell function and obesity. Logistic regression analysis showed that *EXT2* rs3740878 gene variant significantly affects obesity only and did not reach significance with T2D indicating that its role in T2D might be mediated by its association with obesity.

Marginal association for the rs3740878 (OR = 1.07, 95% CI = 0.99–1.16, p = 0.09) was previously reported in a replication and meta-analysis [27]. Three single nucleotide polymorphism (SNP), rs3740878, rs11037909 and rs1113132, in the *EXT2* gene were genotyped in a Chinese case control study and suggested that the *EXT2* gene might not have a major role in the development of type 2 diabetes in the Chinese population. The same was found in a Lebanese case control study [28,29]. Gene–environment and gene-gene interactions should be considered for these discrepancies in literature.

4.4. GCKR

The *GCKR* gene product is a regulatory protein that inhibits glucokinase in liver and pancreatic islet cells by binding non-covalently to form an inactive complex with the enzyme. In the current work the *GCKR* rs780094 risk allele was significantly associated with risk of both type 2 diabetes and obesity. The *TT* and *CT* genotypes were significantly more frequent in both disease entities. The risk variant showed a significant association with increased FPG and HbA1c blood levels and BMI and reduced HOMA-B. These are consistent with observations in white Europeans and Han Chinese individuals [30,31]. Conversely Tam et al. observed significant associations of the minor T-allele of *GCKR* rs780094 with decreased FPG (P = 0.013) [32]. Regression analysis in the present work demonstrated statistical significance with both T2D and obesity indicating its impact on both disease entities.

4.5. CELSR2

The protein encoded by this gene (Cadherin EGF LAG seven-pass G-type receptor 2) is a member of the flamingo subfamily, part of the cadherin superfamily. In the current study, the *CELSR2* risk allele frequency was significantly increased in diabetic patients when compared with non-diabetic individuals. The same was found when comparing obese diabetic patients with non-obese non-diabetic individuals. The risk allele showed significant association with risk of T2D and obesity. In the present study no impact of the gene variant could be revealed on both T2D and obesity by logistic regression analysis and no association could be detected between *CELSR2* risk allele and BMI or glycaemic level. Ahluwalia et al. identified three loci (*CELSR2*, *HNF1A*, and *GCKR*) significantly associated with T2D. They stated that the association with the *CELSR2* locus has not been shown previously [33]. Other investigators previously studied the lipid-metabolizing genetic polymorphisms and the BMI. They confirmed their association with dyslipidemia, however, they did not find association with higher BMI [34,35]. From these findings the *CELSR2* might be associated

significantly with both T2D and obesity with no recognizable impact on both diseases.

4.6. PTGS1

PTGS1/*COX*-1 gene was selected for genotyping in the present study based on its role in regulation of angiogenesis in endothelial cells and inflammatory response. It has been suggested that the hypercoagulable state in T2D could be as a result of disturbances of platelet activation, aggregation and lifespan, in addition to the pro-inflammatory state and endothelial dysfunction [36,37]. Endogenous prostanoids have long been known as inhibitors of insulin secretion [38]. There is evidence that genetic variants in the *PTGS1* gene alter the metabolism of arachidonic acid to prostaglandin H2 and prostaglandin F2, the precursors of prostacyclin, thromboxane, and several other prostaglandin metabolites [39].

In the present study the *PTGS1* risk allele frequency was highly significantly increased in diabetic patients when compared with non-diabetic individuals. The same observation was obtained when comparing obese diabetic patients with non-obese non-diabetic individuals. The risk allele was significantly associated with increased FPG, HbA1c blood levels, BMI and decreased HOMA-B. Regression analysis indicated the effect of this gene variant on both T2D and obesity that could be related to its impact on glucose metabolism and obesity resulting in reduced beta cell function.

4.7. FTO

Within the *FTO* gene, many SNPs appear to be co-inherited. We selected one of these SNPs (rs17817449) for genotyping. The risk allele frequency showed increased significance in obese diabetic patients when compared with non-diabetic non-obese individuals. There is significant association with the risk of obesity. The presence of *FTO* risk allele showed borderline significance with increased BMI. No significant association could be demonstrated with T2D. Thus This gene variant might affect T2D through its relation to obesity and BMI.

A study of Quebec individuals reported that rs17817449 was significantly associated with BMI, weight and waist circumference under an additive model, and in addition, influenced fasting insulin and HOMA-IR [40]. Another study in Mexican women demonstrated that the *FTO* rs17817449 TT genotype predisposes its carriers to fat deposition in the thoracic and breast region. Individuals carrying this genotype showed a gynoid phenotype in Mexican women [41]. Consistent with our findings Frayling et al. indicated that the diabetes risk associated with the *FTO* locus is mediated by obesity [42].

4.8. MTHDF1

MTHFD1 is a key gene associated with three sequential enzymatic reactions in folic acid metabolism. Huang et al. stated that subjects with the genotype *AA* of the *MTHFD1* variant (rs2236225) had a significantly lower susceptibility to T2D compared with subjects with the *GG* or *GG* + *GA* genotypes [43]. In the present work the *MTHFD1* risk allele was significantly associated with obesity risk but not with T2D. Also the frequency of the *GA* genotype was significantly increased in obese diabetic patients when compared with non-obese non-diabetic individuals. The risk allele was significantly associated with increased BMI. These findings demonstrated the impact of *MTHFD1* gene variant on obesity risk with possible secondary impact on T2D.

4.9. CDKN2A/B

In the current study significant association was found between the risky TT genotype and T2D patients and highly significant association in obese-diabetic patients. A meta-analysis by Li et al. indicated significant association between rs10811661 polymorphism upstream of *CDKN2A*/B and T2D [44]. Consistent observations have been detected in Japanese and Vietnamese population studies [45,46]. Conversely in Czech Slavonic population study the association between rs10811661 SNP and susceptibility to T2D was not confirmed [47].

5. Conclusion

The inflammatory pathogenic mechanism and obesity are major factors underlying the risk of T2D. Overlapping genetic aspects should be considered and the presence of risk alleles of different genes together could contribute to the risk of T2D or obesity or both. The *MTHFD1* and *EXT2*rs3740878 gene variants significantly affect obesity and not shared with T2D. Gene variants that showed combined effect on both disease entities were *GCKR* and *PTGS1*. These preliminary findings provide a basis for future studies on a larger scale. More stress on the risk gene variants that have a combined impact on both diabetes and obesity is recommended to improve risk prediction and preventive strategies.

Contribution statement

The clinical and chemical pathology team (Hanaa R.M.Attia, Solaf A. Kamel, Mona H. Ibrahim, Heba A. Farouk, Amany H.A. Rahman) participated in planning the work and preparing design of the research and final approval of the version to be published. They were responsible for conducting the laboratory work, analysis and interpretation of data and making modifications as appropriate as the work progresses. Nevine I. Musa and Ghada H. Sayed were responsible for proper selection and sample processing of the diabetic cases and controls. Corresponding author was also responsible for drafting the work and revising it critically for important intellectual content and preparing the paper for submission.

Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript

Compliance with ethical standards

The study was an outcome of scientific event held in the National Research Centre (NRC) in April 2015 at the Medical Molecular Research Unit of the Medical Division. The Clinical and Chemical Pathology team organized the workshop including theoretical as well as practical sessions on OpenArray new technology supported by both STDF project ID.17364 and NRC. All the ethical rules of the NRC medical research committee were followed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. Informed consent was obtained from all individual participants.

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