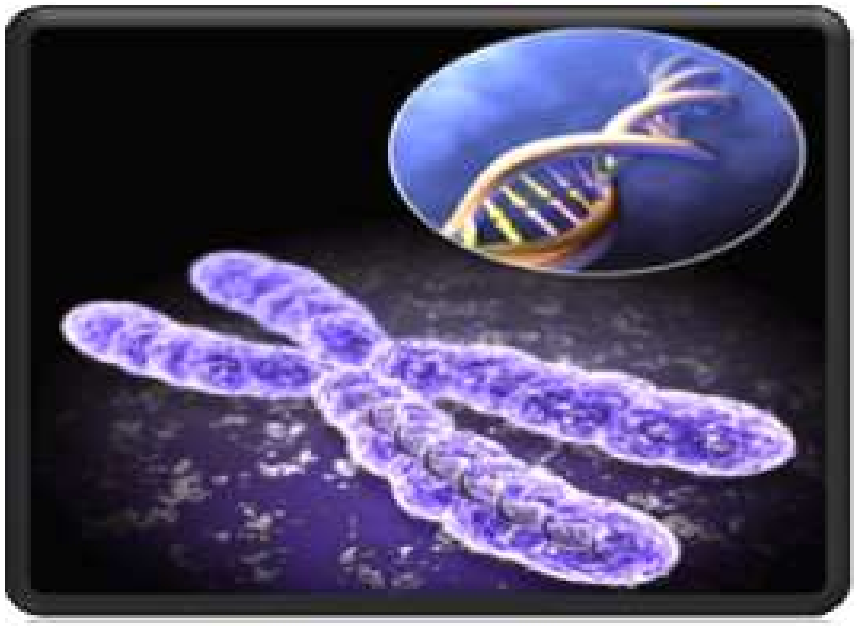
DNA Markers Susceptible to T2DM



Identifying DNA Markers Susceptible to Type 2 Diabetes Mellitus (T2DM) in Unborn Babies

Thesis Paper

ASIAN UNIVERSITY FOR WOMEN

Topic: Identifying DNA Markers Susceptible to Type 2 Diabetes Mellitus (T2DM) in Unborn Babies

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ABBREVIATIONS

A	Adenine	**MTHFR**	Methylene Tetra Hydro Folate Reductase
ACE	Angiotensin-1 Converting Enzyme	**PCR**	Polymerase Chain Reaction
APOE	Apolipoprotein E	**PPARγ2**	Peroxisome Proliferator-Activated Receptor
BMI	Body Mass Index	**P-SH**	Protein Thiol
C	Cytosine	**RFLP**	Restriction Fragment Length Polymorphism
CAD	Coronary Artery Disease	**SNPs**	Single Nucleotide Polymorphisms
FBS	Fasting Blood Sugar	**SOD**	Superoxide Dismutase
FBMI	Fat Body Mass Index	**T**	Thiamine
FPG	Fasting Plasma Glucose	**T1DM**	Type 1 Diabetes Mellitus
GDM	Gestational Diabetes Mellitus	**T2DM**	Type 2 Diabetes Mellitus
HGP	Hepatic Glucose Production	**TBARS**	Thio-Barbituric Acid Reactive Substances
IR	Insulin Resistance	**THF**	Tetrahydrofolate
LBMI	Lean Body Mass Index	**VEGF**	Vascular Endothelial Growth Factor
MDA	Malondialdehyde		

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May 7, 2013

Abstract:

The aim of this study was to identify DNA markers that can be used to diagnose the susceptibility and potential risk of developing Type 2 Diabetes Mellitus (T2DM) in unborn babies. Data was collected from articles obtained from online databases such as Google Scholar, Pub Med and medical journals. Various articles reported that MTHFR, PPARγ2, APOE, and ACE gene polymorphisms are associated with the development and progression of T2DM. However, the studies' results largely varied across different populations since T2DM is a complex multi-factorial polygenic disease. Further studies are therefore required to better identify and understand the significance of DNA makers and their direct correlation to predisposition to T2DM.

Key words:

Type 2 Diabetes; DNA markers; Polygenic disease; MTHFR; PPARγ2; APOE; ACE; Polymorphisms.

Identifying DNA Markers Susceptible to Type 2 Diabetes Mellitus (T2DM) in Unborn Babies

CHAPTER: 1

1.0 Introduction:

Diabetes mellitus is a chronic lifelong condition which refers to a group of metabolic disorders affecting the ability to use the energy found in consumed food (Nordqvist, 2013). An estimated 366 million people are suffering from diabetes worldwide, and according to the International Diabetes Federation, the global diabetes epidemic is getting worse, causing a death every seven seconds worldwide. It is estimated that diabetes causes 4.6 million deaths every year and that health systems spend $465 billion annually fighting the disease (Cheng, 2011). According to World Health Organization (WHO) 2013, more than 80% of diabetes deaths are recorded in low and middle income countries, and the disease will be the 7th leading cause of death by 2030 (WHO, 2013). There are three major types of diabetes mellitus: i) Type 1 Diabetes Mellitus (T1DM)- is a condition when pancreas fails to produce enough amount of insulin. T1DM is characterized by low or absent levels of endogenously produced insulin and dependence on exogenous insulin due to pancreatic β-cell damage. Since patients with T1DM require either insulin injection or insulin pumps, this form of diabetes was also known as "insulin-dependent diabetes mellitus" (IDDM) or “juvenile diabetes” (Alemzadeh and Ali, 2011). This is the most common endocrine metabolic disorder found in children and adolescence, and,

therefore, it is also known as “childhood onset-diabetes”. Common Symptoms of T1DM include excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger, weight loss, vision changes and fatigue. These symptoms may occur suddenly (WHO, 2013). ii) Type 2 Diabetes Mellitus (T2DM)- a state of insulin resistance when cells fail to use insulin properly. T2DM is basically a consequence of insulin resistance occurring at the level of skeletal muscle, liver, and adipose tissue, with various degrees of β-cell impairment. This form of diabetes was previously known as non insulin-dependent diabetes mellitus (NIDDM) or "adult-onset diabetes" (Alemzadeh and Ali, 2011). Details about T2DM are given in the sections below. iii) Gestational Diabetes Mellitus (GDM)- a form of diabetes developed in pregnant women with no previous record of diabetes. GDM takes place due to sudden elevation of fasting blood sugar level in non-diabetic pregnant women which often lead to the development of T2DM and sometimes, T1DM. According to the World Health Organization 2011, about 4% of all pregnant women are affected by GDM (Willacy, 2012). This paper focuses inclusively on T2DM and the DNA markers associated with the development and progression of T2DM.

1.1 Overview of Type 2 Diabetes Mellitus (T2DM):

T2DM is the most common form of diabetes, affecting 85-90% of all people with the diabetes worldwide (WHO, 2013). As mentioned above, T2DM was previously known as “adult onset diabetes” since it was thought that only older people can get this disease. Although from the current data, it has been found that people with T2DM are usually >30 years old, it is now increasingly diagnosed in children and adolescents (Willacy, 2012). It develops when the body cannot use enough insulin to break down glucose, inflating blood sugar levels. Type 2 diabetes is

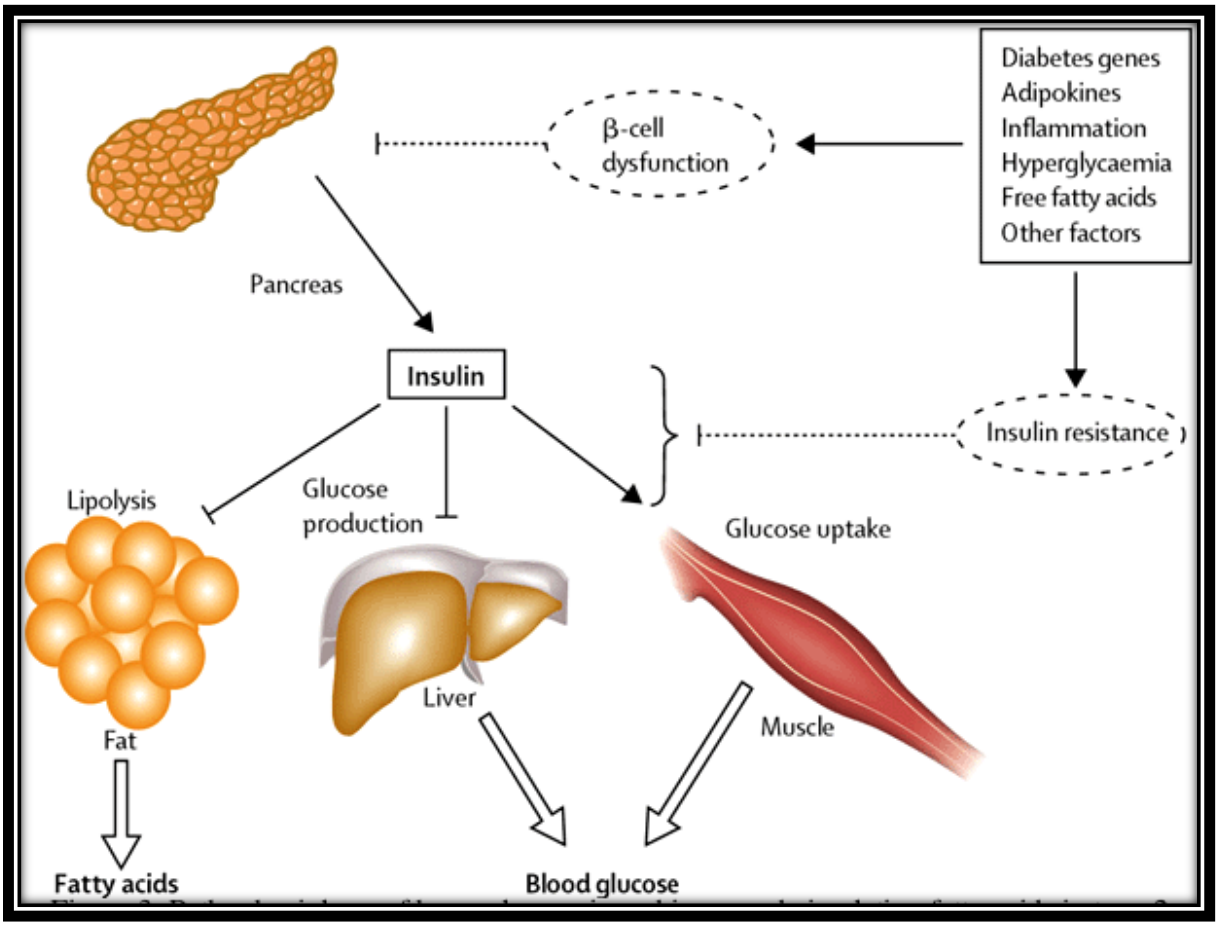
often tied to obesity and also to age (Cheng, 2011). Experts are still working on finding out the significant correlation between weight gain, age and progression of T2DM. One explanation is that people usually gain weight when they get older and also, older people are usually less physically active (Nordqvist, 2013). All racial groups are affected by this disease, but increased prevalence is found in people of South Asian, African, African-Caribbean, Polynesian, Middle-Eastern and American-Indian ancestry (Willacy, 2012). Researchers from the University of Edinburgh, Scotland, found that men with low testosterone level have higher risk of developing type 2 diabetes. According to their findings, testosterone acts on fat cells through molecules known as androgen receptors, which enable the testosterone to activate genes linked to obesity and diabetes. Many scientists also believe that a protein called RBP4 plays a crucial role in regulating insulin resistance when testosterone is impaired (University of Edinburgh, 2012).

1.2 Pathophysiology of T2DM:

The pathophysiology of T2DM is complicated since multiple factors are involved in it. T2DM involves at least two primary pathogenic mechanisms. These are: i) a progressive decline in pancreatic islet cell function resulting in reduced insulin secretion and inadequate suppression of glucagon secretion, and ii) peripheral insulin resistance resulting in a decreased metabolic responses towards insulin. These mechanisms result in inadequate uptake, storage, and disposal of ingested glucose accompanied by elevated hepatic production of glucose and profound hyperglycemia. Thus, the pathogenic mechanisms in T2DM involve not only insulin, but also glucagon, and it is the interplay between these two processes that is a key component in T2DM development and progression (Spellman, 2010). As the physiology of glucose homeostasis

requires the close cooperation of a number of organ systems, hormonal secretions, and neural signaling complexes, the disruption of any of these processes may lead to the development of T2DM. Besides other predisposing risk factors such as overweight and obesity, poor diet (e.g. rich in cholesterol), and lack of exercise, environmental contaminations (e.g. endocrine disrupting chemicals in plastics) (Bergman *et al.*, 2012) and some genetic factors, insulin resistance (IR) has long been recognized as a primary cause of T2DM (Campbell, 2009). Insulin resistance is said to be present when the biological effects of insulin are less than expected for both glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in the liver. In the fasting state, however, muscle accounts for only a small proportion of glucose disposal (less than 20%) whereas endogenous glucose production is responsible for all the glucose entering the plasma. Endogenous glucose production is accelerated in patients with type 2 diabetes or impaired fasting glucose. The pathophysiology of hyperglycemia is shown in figure 1 (Stumvoll *et al.*, 2005).

Figure 1: Pathophysiology of hyperglycaemia and increased circulating fatty acids in type 2 diabetes mellitus (T2DM).



Adapted from: Stumvoll *et al.*, 2005.

Recent research in disease pathogenesis however suggests that IR is neither a necessary nor sufficient condition for development and progression of T2DM. The reason is that despite having significant correlation between IR and T2DM, many people with IR do not develop the disease and similarly, many people with T2DM do not have IR (Campbell, 2009). Studies have

shown that the people with T2DM have markedly reduced (by about 30%) peripheral glucose uptake in response to increasing insulin levels with compared to normal people. Also, the basal hepatic glucose production (HGP) and the fasting plasma glucose (FPG) are significantly greater in T2DM than non-diabetic people. Figure 2 shows that after consuming carbohydrate meals, people with T2DM have higher levels of glucose and glucagon, and lower level of insulin compared to the healthy individuals. As the insulin-stimulated glucose uptake and the insulin-stimulated glycogen synthesis are reduced in people with T2DM, their glucose level stays elevated in the periphery (Spellman, 2010). After food consumption, diabetic people have an elevated level of blood glucose compared to the normal people. As normal people have lower glucose level compared to T2DM patients, the insulin level gets accelerated in the normal people which is indicated by blue lines in figure 2. On the contrary, as glucagon and insulin work inversely, the glucagon level is higher in T2DM patients compared to normal people (Figure 2).

CHAPTER: 2

2.0 Goals of the Study:

In addition to the above mentioned prevalence, and complications of T2DM, the initial interest in studying T2DM was embedded during assisting a Diabetes Project under Prof. Georgia Guldan of AUW and the visits and interviews at the Chittagong Diabetes Hospital in 2010. Subsequently, "Genes and Genomics," an academic course of Fall Semester 2012, provoked the interest and enthusiasm in doing a study to identify DNA markers directly and indirectly associated with the potential risk of developing Type 2 Diabetes Mellitus (T2DM) in unborn babies (e.g. fetus and embryonic cells). In the long run, the genetic information and understandings obtained from this study will hopefully help early diagnosis of T2DM susceptibility and thus reduce the current prevalence of T2DM especially in children by altering the disease onset.

CHAPTER: 3

3.0 Materials and Methods:

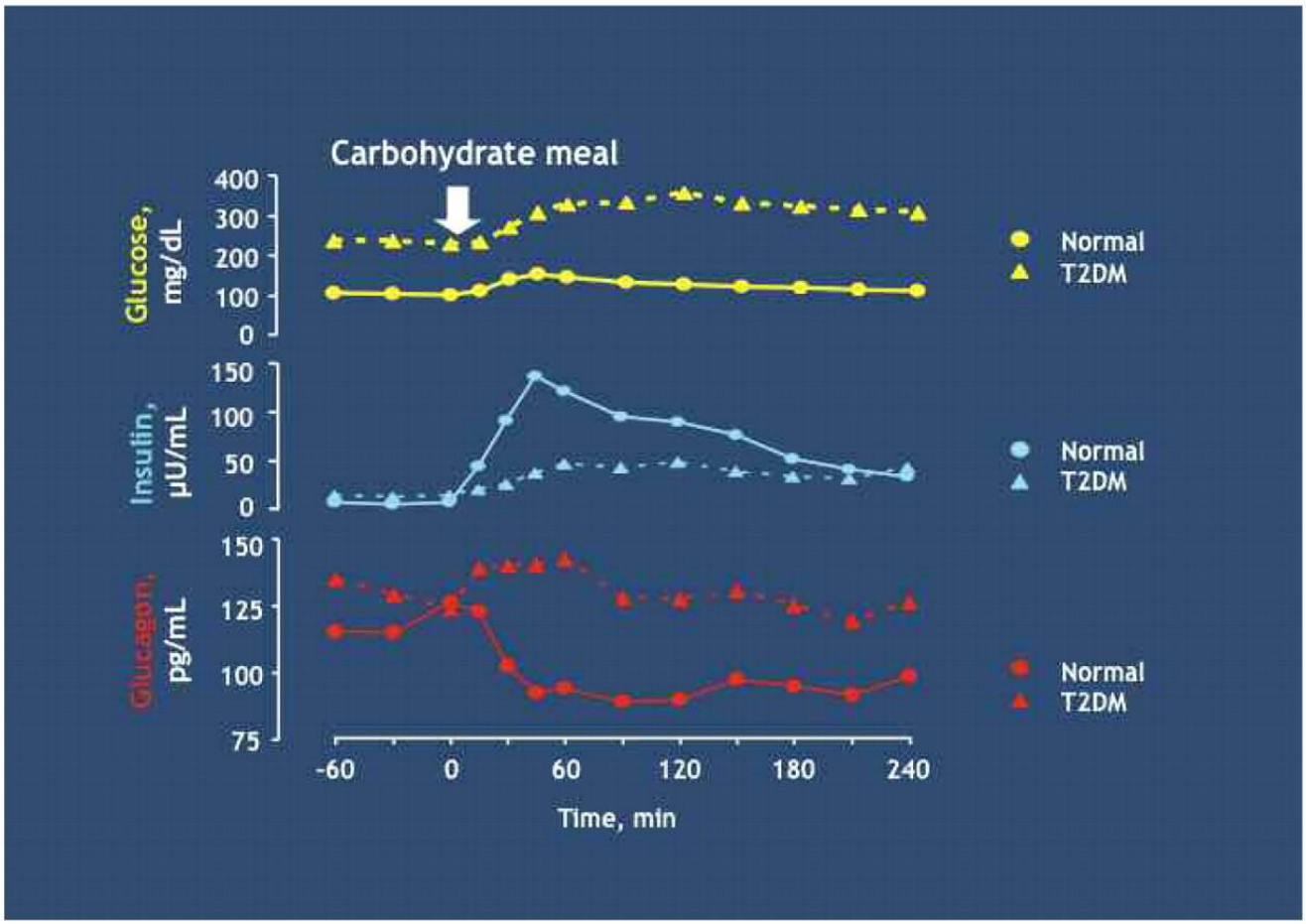
As there was no wet lab performed in the study, the method of the study is therefore literature review, obtaining data from different studies performed in different populations during different time periods. The materials of this study are online based information. Data and relevant figures were obtained from articles available on online databases such as Google Scholar, Pub Med, HINARI and Medical Journals. The collected information was succinctly presented throughout the paper. Almost 20 papers were studied, and the majority of those papers were published online between 2000 and 2013. Some frequently used keywords in the literature review search were: "History of T2DM"; "Prevalence of T2DM"; "Physiology of T2DM"; "Pathophysiology of T2DM"; "DNA markers of T2DM"; "Chromosomal changes due to T2DM"; "DNA damage due to T2DM"; "Association of MTHFR with T2DM"; and "Genetic polymorphisms causing T2DM". The articles were chosen based on titles that are most relevant to the study topic of research and also derived from international organizations or publishers. The wet lab experiments mentioned in those articles and studies commonly used Spectrophotometric methods, Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), and Chelex based methods. The majority analysis was performed using some software such as EXCEL, SPSS, and STATA SE 9.0. Also, to assess the association between variables and to draw conclusions from the experimental data, test statistics such as Analysis of variance (ANOVA), Chi square test, Spearman's rank correlation coefficient, linear regression analysis, multiple regression analysis were frequently used. The results obtained from different sources and experiments were compared and analyzed in the paper.

CHAPTER: 4

4.0 Results:

This section contains graphs and tables about oxidative DNA damage, genetic variants regulating vascular endothelial growth factor and some gene polymorphisms obtained from wet lab experiments and studies run in different populations and geographical locations.

Figure 2: Plasma glucose, insulin, and glucagon profiles in response to ingestion of a carbohydrate meal in normal subjects and patients with type 2 diabetes mellitus (T2DM).

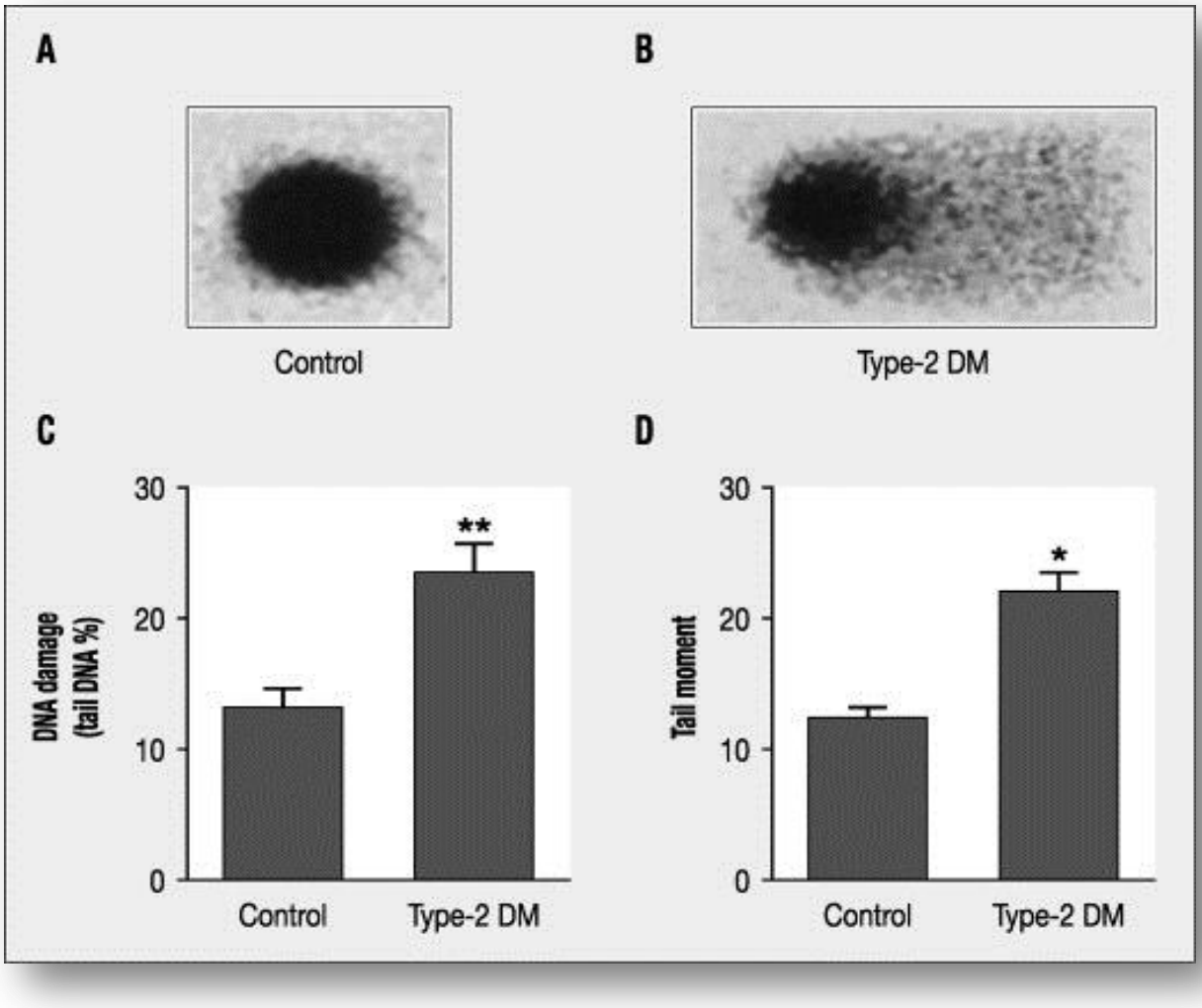


Adapted from: Spellman, 2010.

The correlations between glucagon, insulin and glucose over time in diabetic and non-diabetic people after consuming carbohydrate diets are depicted in Figure 2. The yellow lines show that fasting blood glucose (FBG) levels are 100 mg/dl and 250 mg/dl in normal and T2DM people respectively (Time: -60 to 0 min). After food consumption, diabetic people have an elevated level of blood glucose compared to the normal people. Food consumption leads the insulin level to get accelerated in the normal people, whereas the insulin level is much lower in people with T2DM which are indicated by blue lines in figure 2. On the contrary, the glucagon level is higher in T2DM patients compared to normal people as shown by the red lines.

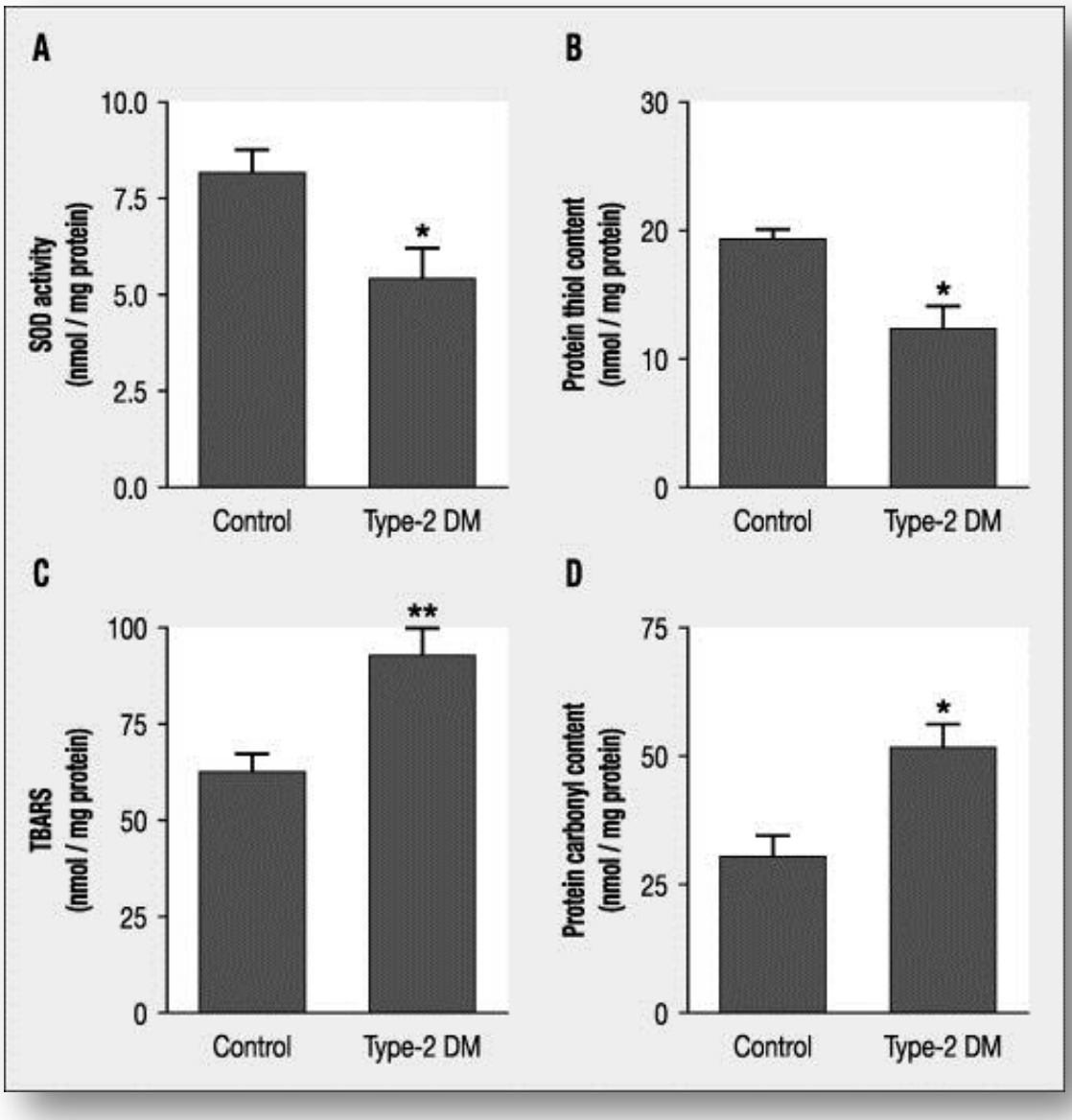
The association of oxidative DNA damage with the progression of T2DM is represented in Figure 3, 4 and 5. Figure 3 shows that people with T2DM have almost 25% of tail DNA damage, whereas the control people have only 12% (C). Also, tail moment, which is another indicator of DNA damage, is higher in T2DM patients compared to the normal populations (D). Figure 4 exhibits that people with T2DM have lower levels of serum activity of superoxide dismutase (SOD) and protein thiol (P-SH) content and higher level of thio-barbituric acid reactive substances (TBARS) and protein carbonyl content compared to these levels found in the control group. The result from linear regression analysis which demonstrates that oxidative DNA damage- tail moment and tail DNA %, and fasting blood glucose level are positively related is shown in Figure 5.

Figure 3: Comet images of blood cells from diabetic patients: the extent of DNA damage was assessed by comet assay, coupled with silver staining, using lymphocytes from the controls (A) and type 2 DM patients (B). Quantitative analysis of comet images shows tail DNA (%) (C) and tail moment (D). Data are expressed as means ± SEM. **P* < 0.05; ***P* < 0.01 (Bonferroni test).



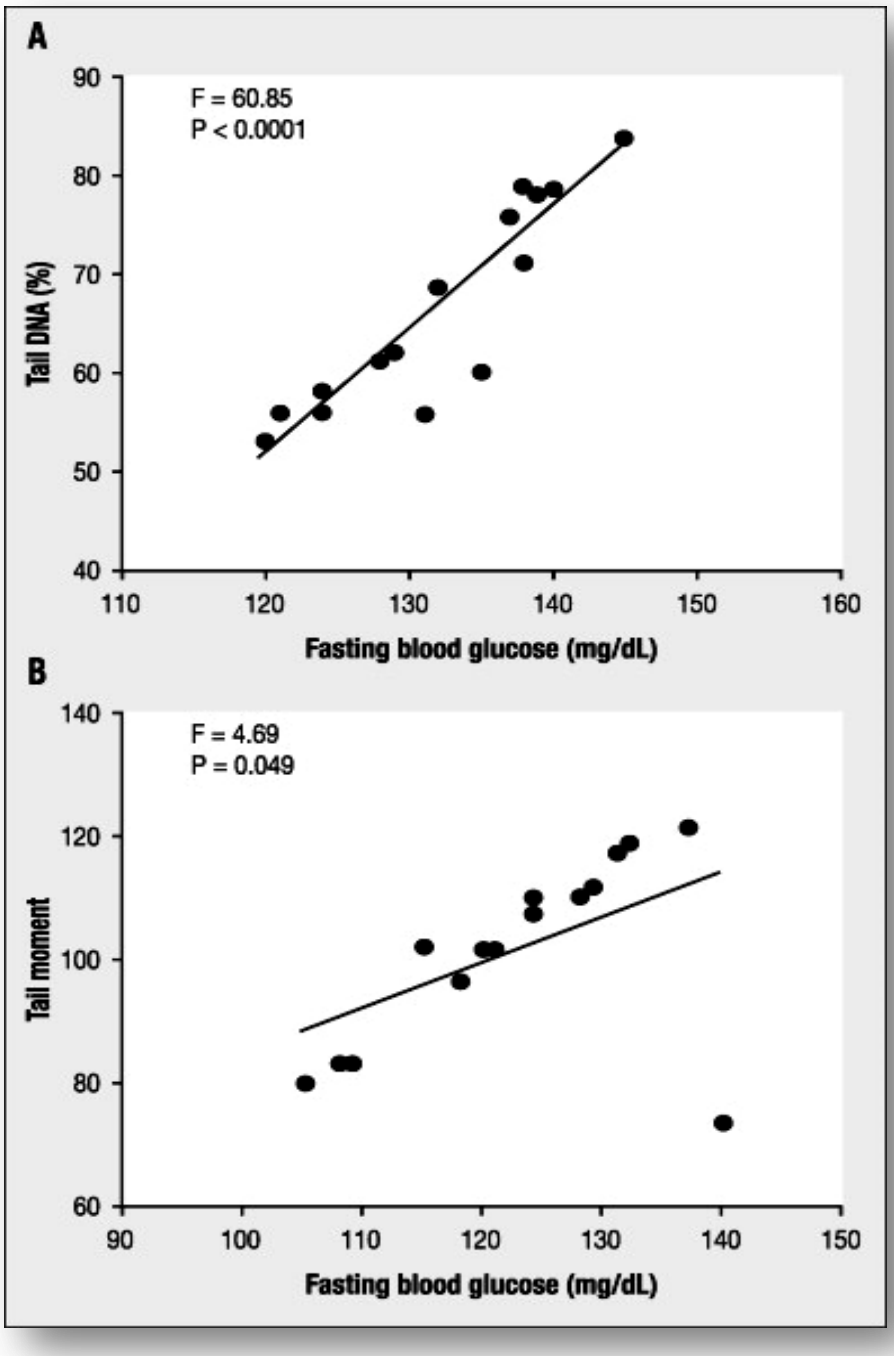
Adapted from: Arif *et al.*, 2009.

Figure 4: Antioxidant and prooxidant status in the controls and type 2 diabetes patients: levels of SOD (A) and protein thiol (B) were measured in the non-diabetic controls and T2DM patients. Changes levels of TBARS (C) and protein carbonyl content (D) were calculated in both study groups. Data are expressed as means +/- SEM *P<0.05; **P<0.01.



Adapted from: Arif *et al.*, 2009.

Figure 5: The stepwise progression analysis shows that the tail DNA percentage (A) and the tail DNA moment (B) are positively related with the fasting glucose level in people with T2DM.



Adapted from: Arif *et al.*, 2009.

Table 1: Association of SNPs rs6921438 and SNPs rs10738760 with T2DM susceptibility in two European case-control studies.

SNPs	Studies	X frequency (%)	*N*	YY (%)	YX (%)	XX (%)	OR (95% CI)*	*P*
rs6921438 (G-allele = X A-allele = Y)	**French controls**	52.5	3,745	861 (23.0)	1,834 (49.0)	1,050 (28.0)	-	-
	French cases	55.6	6,908	1,367 (19.8)	3,402 (49.2)	2,139 (31.0)	**1.15 (1.07;1.22)**	**3.7×10^{-5}**
	Danish controls	50.8	2,600	590 (22.7)	1,324 (50.9)	686 (26.4)	-	-
	Danish cases	51.8	3,524	857 (24.3)	1,753 (49.7)	914 (25.9)	1.02 (0.94;1.10)	0.66
	Combined analysis	-	-	-	-	-	-	0.28
rs10738760 (A-allele = X G-allele = Y)	**French controls**	50.4	3,756	916 (24.4)	1,896 (50.5)	944 (25.1)	-	-
	French cases	50.6	6,914	1,733 (25.1)	3,362 (48.6)	1,819 (26.3)	0.98 (0.91;1.06)	0.63
	Danish controls	51.2	2,592	611 (23.6)	1,308 (50.5)	673 (26.0)	-	-
	Danish Cases	50.9	3,512	827 (23.5)	1,796 (51.1)	889 (25.3)	1.04 (0.96;1.12)	0.40
	Combined analysis	-	-	-	-	-	-	0.93

*OR from additive logistic regression models adjusted for age and gender.
T2D, type 2 diabetes; *OR*, odds ratio; *CI*, confidence interval; *P*, P-value.
doi:10.1371/journal.pone.0055921.t001

Adapted from: Bonnefond *et al.*, 2013.

A study conducted in two different European populations, French and Danish, to find out the correlation between SNPs rs6921438 and SNPs rs10738760 with the progression of T2DM is given in Table 1. The Table shows that in French populations, SNPs rs6921438 is directly related to the people with T2DM; whereas none of the SNPs seem to be correlated with T2DM in the Danish populations.

Table 2: Genotype and allele frequencies of MTHFR CC, MTHFR CT and MTHFR TT polymorphisms in North Indian population

Genotype and allele frequencies of *MTHFR* and *PPARγ2* in healthy controls and type 2 diabetes cases.

MTHFR						
Group	Alleles & genotype	C	T	CC	CT	TT
Control	N (%)	124(70)	52(30)	49(56)	26(29)	13(15)
Diabetic cases	N (%)	107(61)	67(39)	35(40)	37(43)	15(17)
	OR/95%CI	0.67/(0.42-1.05)	1.49/(0.95-2.33)	0.54/(0.29-0.98)	1.76/(0.94-3.30)	1.20/(0.53-2.70)
	P-value/chi sq	0.08/3.13	0.08/3.13	0.041/4.18	0.07/3.20	0.66/0.198
	Power	0.963(0.200)	0.963(0.200)	0.982(0.313)	0.966(0.273)	0.763(0.067)

Adapted from: Raza *et al.*, 2012.

Table 3: MTHFR gene polymorphisms across different ethnicities in different geographical locations.

MTHFR gene polymorphism in different ethnic groups.

Ethnicity	MTHFR		Control	T2DM
Indian (Present study)	Genotype	CC	49(56)	35(40)
		CT	26(29)	37(43)
		TT	13(15)	15(17)
	Allele	C	124(70)	107(61)
		T	52(30)	67(39)
Turkish Yilmaz et al. (2004)	Genotype	CC	101(47)	121(49)
		CT	20(9)	30(12)
		TT	93(44)	98(39)
	Allele	C	295(69)	340(68)
		T	133(31)	158(32)
Egyptian Amal et al. (2011)	Genotype	CC	32(80)	24(60)
		CT	6(15)	10(25)
		TT	2(5)	6(15)
	Allele	C	70(88)	58(73)
		T	10(12)	22(27)
Tunisian Mtiraoui et al. (2006)	Genotype	CC	270(68)	163(45)
		CT	94(23)	135(38)
		TT	36(9)	62(17)
	Allele	C	634(79)	461(64)
		T	166(21)	259(36)
Brazilian Errera et al. (2006)	Genotype	CC	36(34)	44(46)
		CT	57(53)	41(43)
		TT	14(13)	10(11)
	Allele	C	129(60)	129(68)
		T	85(40)	61(32)
Chinese Sun et al. (2009)	Genotype	CC	78(55)	94(44)
		CT	38(27)	73(34)
		TT	26(18)	48(22)
	Allele	C	194(68)	261(61)
		T	90(32)	169(39)
Iranian Barraz et al. (2009)	Genotype	CC	113(54)	148(53)
		CT	80(39)	102(36)
		TT	14(7)	31(11)
	Allele	C	306(76)	398(71)
		T	108(24)	164(29)

Frequency in percentages is in parentheses.

Adapted from: Raza *et al.*, 2012.

The polymorphisms of MTHFR gene among the normal and diabetic people in North India are shown in Table 2, whereas Table 3 represents polymorphisms among normal and diabetic people cross different ethnicity and geographical locations. In Table 2, the genotypic frequencies of MTHFR CC are 56 and 40 in control and T2DM cases respectively. However, in terms of MTHFR CT, the frequency is higher (43) in diabetic patients compared to the frequency (29) in non-diabetic population in North India. Considering different geographical locations, Table 3 represents that the MTHFR CC polymorphism has similar frequency ranging between 40 and 46 in most populations, including Indian, Tunisian, Brazilian and Chinese with T2DM. However, in Iranian and Egyptian populations, the frequencies are quite high- 53 and 60 respectively.

Table 4: Allele frequencies of PPAR γ2 gene polymorphisms in Japanese populations.

subjects	number	Pro12Pro	Pro12Ala	Ala12Ala	Ala frequency
control	116	110	6	0	0.05
type 2 DM	402	363	39	0	0.10[a]

[a] χ^2=2.33, p=0.13 versus control

Adapted from: Kawasaki *et al.*, 2002.

Table 5: Clinical characteristics of 116 healthy and 402 diabetic subjects classified in accordance with their genotype of the Pro12Ala polymorphism of the PPAR γ2 gene

	control				type 2 diabetes		
	n	Pro12Ala	Pro12Pro	P-value	n	Pro12Ala	Pro12Pro	P-value
Age (years)	116	52.2 ± 2.3	53.5 ± 0.9	0.570	402	56.2 ± 1.9	55.3 ± 0.7	0.673
Treatment								
Non-treatment						4	41	0.86[a]
Diet therapy						2	33	0.44[a]
Oral agent						22	163	0.41[a]
Insulin therapy						9	61	1.00[a]
BMI (kg/m²)	116	23.6 ± 0.9	22.6 ± 0.3	0.330	340	22.0 ± 0.8	23.5 ± 0.2	0.020
LBMI (kg/m²)[b]	116	16.5 ± 0.2	16.0 ± 0.1	0.148	340	16.4 ± 0.3	16.8 ± 0.1	0.186
FBMI (kg/m²)[b]	116	7.1 ± 0.9	6.7 ± 0.2	0.654	340	5.6 ± 0.5	6.8 ± 0.2	0.016
Fasting plasma glucose (mmol/l)	116	5.05 ± 0.11	5.18 ± 0.06	0.420	364	8.66 ± 0.54	8.55 ± 0.17	0.980
HbA1c (%)	116	4.8 ± 0.1	4.8 ± 0.1	0.730	360	8.7 ± 0.30	8.9 ± 0.12	0.774
Fasting insulin (pmol/l)	116	30.9 ± 6.46	28.7 ± 1.44	0.680	367	46.8 ± 6.9	62.6 ± 5.52	0.184
Total cholesterol (mmol/l)	116	5.72 ± 0.52	5.53 ± 0.08	0.940	377	5.30 ± 0.15	5.33 ± 0.07	0.503
Triglyceride (mmol/l)	116	1.10 ± 0.26	1.28 ± 0.08	0.610	377	1.33 ± 0.09	1.51 ± 0.05	0.516
HDL cholesterol (mmol/l)	116	1.94 ± 0.18	1.76 ± 0.05	0.270	373	1.37 ± 0.07	1.24 ± 0.02	0.009
LDL cholesterol (mmol/l)	116	3.26 ± 0.41	3.21 ± 0.08	0.970	373	3.36 ± 0.13	3.39 ± 0.08	0.582
HOMA IR	116	1.0 ± 0.3	0.9 ± 0.5	0.960	364	2.3 ± 0.3	3.1 ± 0.3	0.115
Clamp IR		ND[c]	ND		86	4.5 ± 2.3	5.1 ± 3.5	0.960

Data are means ± SE, unless otherwise indicated. Statistical analyses were assessed by Mann-Whitney's **U**-test.

[a]Chi-square test was performed.

[b]Lean body mass index (LBMI) and fat body mass index (FBMI) were calculated by dividing lean body mass (kg) and fat body mass (kg) by square of height (m²), respectively.

[c]ND, not done

The study results shown in Table 4 and Table 5 are conducted in Japanese populations to see the association of PPAR γ2 gene polymorphisms with T2DM. The allele frequency of the Ala variant did not differ significantly between diabetic subjects and normoglycemic control subjects CX2=2.33, p=O.13), as shown in Table 4. From the data presented in Table 5, it can be seen that among the diabetes subjects, the Pro12Pro homozygotes showed significantly higher body mass index (BMI) than those with the Pro12Ala variant (p=0.020), while there was no association between genotype and BMI in the controls. Furthermore, diabetic subjects with Pro12Pro showed significantly higher fat body mass index (FBMI) than those with Pro12Ala (p=0.016), while no association between genotype and lean body mass index (LBMI) was observed.

Different allelic and genotypic frequencies of APOE gene and their predisposition to T2DM are represented in Table 6. APOE and ACE gene polymorphisms and their associations with the progression of T2DM are shown in Table 6 and Table 7. APOE 3–3 genotype was most common in both groups: 87% in diabetics and 76% in controls as shown in Table 6. The 2–3 genotype was present in both groups; however the frequency was higher in the control group-control 7.2% and diabetic 4.4%. The diabetic sample showed higher frequency of *E3 allele than controls. Although some ORs were above 1 (e.g. 3–3 genotype = 1.41 and *E3 allele = 1.70), none of the odds ratio achieved statistical significance. In Table 7, ACE-DD genotype patients were significantly younger, heavier and had higher BMIs compared to individuals having ACE-II genotype.

Table 6: Associations of APOE gene polymorphisms with the risk of T2DM and CAD compared to healthy controls represented as unadjusted OR.

Genotype	Controls (n = 149)	T2DM (n = 155)	Unadjusted OR (95% CI)	p-value	T2DM + CAD (n = 147)	Unadjusted OR (95% CI)	p-value
E2/E2	2	1	0.48 (0.02 - 6.78)	0.616	1	0.50 (0.02 - 7.16)	1.000
E3/E3	113	117	0.98 (0.56 - 1.71)	0.942	88	0.48 (0.28 - 0.81)	0.003
E4/E4	1	4	3.92 (0.41 - 93.19)	0.371	1	1.01(0.06 - 16.36)	1.000
E2/E3	12	2	0.15 (0.02 - 0.72)	0.005	11	0.92(0.36 - 2.33)	0.854
E2/E4	0	1	-	1.000	0	-	-
E3/E4	21	30	1.46 (0.76 - 2.81)	0.219	46	2.78(1.50 - 5.16)	**0.0004**
Allele ε2	16	5	0.29 (0.09 - 0.85)	0.011	13	0.81(0.36 - 1.81)	0.580
Allele ε3	259	266	0.91 (0.56 - 1.48)	0.691	234	0.57(0.36 - 0.90)	0.107
Allele ε4	23	39	1.72 (0.97 - 3.06)	0.047	49	2.37(1.36 - 4.15)	**0.0009**

Adapted from: Chaudhary *et al.*, 2012.

Table 7: Frequencies of APOE and ACE gene polymorphisms.

Genotype numbers, %, allele frequencies and odds ratios of APOE and ACE gene polymorphisms in North West Indian diabetics and controls

Locus and genotype	Diabetics		Controls		Disease association analyses			
	Observed number	%	Observed number	%	Odds ratio	95% CI	χ^2	*P* value
APOE								
Type								
E2E2	1	1.11	1	1.03	1.08	0.01–85.54	0.003	1.000
E2E3	4	4.44	7	7.22	0.60	0.12–2.46	0.648	0.421
E3E3	78	86.67	74	76.29	1.41	0.58-3.49	0.381	0.537
E2E4	2	2.22	0	0.00	ND			
E3E4	5	5.55	13	13.40	0.38	0.10–1.20	2.46	0.117
E4E4	0	0.00	2	2.06	ND			
Total genotypes	90		97					
Alleles	No	AF ± S.E.	No	AF ± S.E.				
*E2	8	0.044 ± 0.015	9	0.046 ± 0.015	0.96	0.31–2.86	0.000	1.000
*E3	165	0.917 ± 0.021	168	0.866 ± 0.025	1.70	0.83–3.59	1.97	0.117
*E4	7	0.039 ± 0.014	17	0.088 ± 0.020	0.42	0.14–1.10	2.93	0.087
Total alleles	180		194					
HWE χ^2P	0.337		0.109					
ACE (I/D)								
Type								
II	20	22.22	30	31.91	0.61	0.30–1.24	2.18	0.140
ID	38	42.22	43	45.75	0.87	0.46–1.56	0.23	0.630
DD	32	35.56	21	22.34	1.92	1.00–3.70	3.92	0.048*
Total genotypes	90		94					
Alleles	No	AF ± S.E.	No	AF ± S.E.				
*I	78	0.433 ± 0.039	103	0.547 ± 0.038	0.63	0.41–0.97	4.38	0.036*
*D	102	0.567 ± 0.039	85	0.453 ± 0.038	1.58	1.03–2.44	4.38	0.036*
Total alleles	180		188					
HWE χ^2P	0.183		0.457					

AF: allele frequency, S.E.: standard error, ND: not done.
*Significant at 5% level.

Adapted from: Singh *et al.*, 2006.

Table 8: Descriptive information of people with T2DM with reference to APOE and ACE gene polymorphisms.

Descriptive information (mean and S.D.) of T2DM patients with reference to genotypes

Number	Number	Age	Weight (kg)	Height (cm)	BMI	FBS (mM/L)
Patients						
Total	90	50.64 ± 12.05	62.63 ± 13.60	165.72 ± 9.04	22.85 ± 4.87	9.15 ± 2.93
Female	32	50.03 ± 11.93	61.66 ± 12.98	162.00 ± 10.24	23.55 ± 4.80	8.14 ± 1.95
Male	58	51.72 ± 12.48	62.47 ± 13.77	170.26 ± 6.99	21.51 ± 4.38	10.21 ± 3.65
APOE						
E22/E23/E24						
Total	7	54.43 ± 11.96	64.71 ± 12.91	167.28 ± 11.02	23.08 ± 3.86	9.38 ± 2.45
Female	4	57.25 ± 13.40	67.75 ± 12.84	165.74 ± 12.85	24.64 ± 3.51	9.61 ± 2.77
Male	3	50.67 ± 11.06	60.67 ± 14.47	169.33 ± 10.27	21.00 ± 3.86	9.07 ± 2.49
E33						
Total	78	49.67 ± 12.02	62.36 ± 13.81	165.30 ± 9.04	22.88 ± 5.03	8.94 ± 2.71
Female	27	48.41 ± 11.39	61.37 ± 12.86	161.34 ± 10.14	23.65 ± 4.89	7.86 ± 1.77
Male	51	50.33 ± 12.40	62.88 ± 14.38	167.39 ± 7.71	22.48 ± 5.10	9.52 ± 2.95
E34						
Total	5	60.60 ± 8.26	64.00 ± 13.66	170.18 ± 5.39	22.02 ± 4.26	12.04 ± 5.37
Female	1	65.00	45.00	165.10	16.51	9.78
Male	4	59.50 ± 9.11	68.75 ± 9.91	171.45 ± 5.29	23.40 ± 3.40	12.61 ± 6.03
ACE						
ACE-II						
Total	20	51.50 ± 11.23	62.65 ± 12.61	166.88 ± 7.38	22.52 ± 4.47	10.51 ± 3.91
Female	6	48.50 ± 11.15	61.17 ± 14.32	161.71 ± 5.25	23.52 ± 6.18	7.89 ± 2.34
Male	14	52.79 ± 11.43	63.29 ± 12.33	169.09 ± 7.16	22.09 ± 3.73	11.64 ± 3.96
ACE-ID						
Total	38	52.66 ± 11.44	61.24 ± 15.06	165.10 ± 10.55	22.55 ± 5.49	8.07 ± 1.99
Female	14	53.57 ± 9.67	59.71 ± 15.12	161.65 ± 13.43	22.80 ± 4.65	7.87 ± 1.85
Male	24	52.13 ± 12.52	62.13 ± 15.28	167.11 ± 8.10	22.40 ± 6.01	8.19 ± 2.10
ACE-DD						
Total	32	47.72 ± 13.00	64.28 ± 12.57	165.74 ± 8.19	23.41 ± 4.41	9.58 ± 2.77
Female	12	46.67 ± 14.30	64.17 ± 9.98	162.56 ± 8.32	24.44 ± 4.51	8.57 ± 1.97
Male	20	48.35 ± 12.50	64.35 ± 14.14	167.64 ± 7.69	22.80 ± 4.35	10.18 ± 3.04

Adapted from: Singh *et al.*, 2006.

CHAPTER: 5

5.0 Analysis and Discussions of Key Genetic Factors for T2DM:

As mentioned earlier that T2DM is a complex multi-factorial polygenic disease, it is difficult to identify the genetic factors which have significant direct correlation with T2DM. Various studies reported that Oxidative DNA damage, Genetic Variants Regulating VEGF, MTHFR, PPARy2, APOE, and ACE gene polymorphisms are associated with the development and progression of T2DM. These associated factors are thoroughly discussed and analyzed in the following sections.

5.1 Oxidative DNA Damage:

Several studies suggest that the increased oxidative stress as well as alternation of antioxidant capacity is related to type 2 diabetes complications. A study was done in 2009 in Bangladeshi population to measure the serum antioxidant status in T2D patients and to assess its correlation with oxidative DNA damage (Arif *et al.*, 2009). 32 people with T2D and 25 non-diabetic people were included in the study where the level of DNA damage in lymphocytes was quantified by comet assay, also known as Single Cell Gel Electrophoresis (SCGE). Using spectrophotometric methods, serum level of malondialdehyde (MDA) and protein carbonyl, as well as activity of superoxide dismutase (SOD) and protein thiol (P-SH) were measured in both case and control groups. The results show that the people with high level of DNA damage (mean comet tail DNA) (Figure 3), have significantly higher level of malondialdehyde (MDA), lipid peroxidation, protein oxidation, Glycosylated Hemoglobin (Hb A1c), Fasting Glucose Level (FGL) (Figure 5), and significantly decreased level of SOD and P-SH compared with the control

group (Figure 4). The study suggests that the status of oxidant-antioxidant imbalance is one of the mechanisms leading to the DNA damage detected in the lymphocytes of T2D patients.

5.2 Genetic Variants Regulating VEGF:

Some studies claim that the vascular endothelial growth factor (VEGF) is directly involved in tissue growth and organ repair processes. Elevated circulating VEGF levels play a role in T2DM and micro vascular complications. A genome-wide association study has found two common single nucleotide polymorphisms (SNPs)- rs6921438 and rs10738760 which are directly associated with the circulation of VEGF. Several studies were done in French and Danish T2DM populations and the results showed that the French population has an association with SNPs rs6921438 which increases both the circulating VEGF levels and the risk of T2DM. However, the findings were not confirmed in the Danish population (Table 1). Multiple studies could not find any association of SNPs rs10738760 at all with T2DM. The study concluded with the assumption that the link between genetic variants regulating VEGF levels and the risk of developing T2DM might be indirect and more complex than expected (Bonnefond *et al.*, 2013).

5.3 MTHFR Polymorphisms:

MTHFR is the gene that provides the complete instruction for the biosynthesis of the metabolically important enzyme called methylenetetrahydrofolate (CH3-THF) reductase (Sace, 2011). The MTHFR enzyme, made by the MTHFR gene, converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5,10-methylenetetrahydrofolate reductase is known as NADPH. The MTHFR gene is found on the short arms of Chromosome 1 and the gene is made up of 20,373 base pairs. Although MTHFR enzyme production is the major

function of MTHFR gene, it produces incorrect enzyme if the gene is mutated (Ben, 2011). The MTHFR enzyme catalyzes the conversion of folic acid into its biologically active form which is tetrahydrofolate (THF). An MTHFR gene mutation impairs the efficiency of the enzyme to produce enough tetrahydrofolate that the body needs. When the body is deficient in tetrahydrofolate as a result of an MTHFR mutation, serious diseases can develop (Sace, 2011). At least 40 mutations in the MTHFR gene have been identified in people with homocystinuria, a metabolic disorder when homocysteine builds up in the bloodstream, and the amount of methionine is reduced (MTHFR, 2011). The most common variants of MTHFR are: at chromosomal position 677 where "C" gets replaced by "T" and at 1298 where "A" gets replaced by "C" (Ben, 2011).

It is shown in Table 2 that people with T2DM have higher MTHFR CT frequency which is 43 compared to the normal population who has a frequency of 29 for the same genotype. On the other hand, the frequency of MTHFR CC is lower in T2DM patients (44) compared to the frequency found in the normal people (56) (Table 2). Since MTHFR CC polymorphism has similar frequency in the most populations, such as Indian, Tunisian, Brazilian and Chinese, with T2DM as shown in Table 3 (Raza *et al.*, 2012), several studies claim that the MTHFR CC genotype is a good DNA marker for T2DM.

5.4 PPARγ2 Gene Polymorphisms:

The peroxisome proliferator-activated receptor (PPAR) γ is a nuclear receptor, which is an important regulator of adipocyte differentiation and a modulator of intracellular insulin signaling events. The PPAR γ gene generates two isoforms: PPAR-γl and PPAR-γ*2*. These are

transcript from different promoters and formed through alternative splicing. The human PPAR-γ2 gene is most abundant only in adipose tissue (Kawasaki *et. al.*, 2002).

The peroxisome proliferator activated receptor (PPARγ2) gene and type 2 diabetes are significantly associated with each other, suggested by several genetic studies (SACN, 2011). In people with low birth weight, the Pro12Pro polymorphism of the PPARγ2 gene has been shown to be associated with increased insulin resistance and elevated plasma insulin concentrations (Lindi, 2005). Regarding insulin resistance, however, there was no difference in clamp index between Pro12Ala and Pro12Pro variants. The allele frequencies of PPAR γ2 gene polymorphisms in Japanese populations presented in Table 4 has a p-value of 0.13 which indicates that the data are not statistically significant. In Table 5, in diabetic subjects, body mass index (BMI) has statistically significant association with the Pro12Ala variant at p-value: 0.020 and fat body mass index (FBMI) has statistically significant association with Pro12Pro variant at p-value: 0.016. However, looking at the variants in non-diabetic people in Table 5, it can be concluded that none of these variants are significantly associated with BMI and FBMI, since the test statistical p-values are not equal to or below 0.050.

The data presented in Table 4 and Table 5 suggest that the Pro12Ala polymorphism of PPAR γ2 does not influence insulin resistance, rather affects the body composition in Japanese diabetic subjects (Kawasaki *et al.*, 2002). Also, people with normal birth weight do not show this phenomenon and therefore some studies suggest that there are environmental interactions which affect the polymorphisms of PPARγ2-Pro12Ala to modify the expression of PPARγ2 and T2DM (SACN, 2011).

5.5 APOE Gene Polymorphisms:

Several studies claim that the apolipoprotein E (APOE) gene is a risk factor for T2DM, though the results are inconsistent. APOE gene polymorphism directly influences plasma lipid concentration, but its association with the increased risk of T2DM is still unclear. The APOE gene polymorphism was tested by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in Bangkok province (Singh *et al.,* 2006). The case-control study was carried out on a total of 451 samples including 149 normal control subjects, 155 subjects with T2DM, and 147 subjects with T2DM complicated with CAD (Coronary Artery Disease). Univariable and multivariable logistic regression analyses were used to identify the possible risks of T2DM and CAD. These results indicate that ε4 allele has influence on lipid profiles and is associated with the development of both T2DM with and without CAD. Furthermore, it increased the risk among the subjects with obesity and/or smoking, the conditions associated with high oxidative stress. From Table 6, it can be concluded that the ε4 allele containing genotypes is the major predictor of development of both T2DM and CAD as it has a p-value of 0.0009. The study also demonstrates strong association of E3/E4 genotype with development of CAD in T2DM patients at p-value: 0.0004 (Chaudhary *et al.,* 2012). However, the study in Punjab mentioned in the following section came up with a conclusion that though APOE*4 allele frequency is low in diabetics, there is no significant and direct association of APOE with T2DM (Table 7). These data strongly suggest that underlying genetic factors are playing significant role in addition to nutritional and environmental factors in terms of APOE gene expression, correlating with T2DM.

5.6 ACE gene polymorphisms:

A study performed in Punjab, India suggested that the angiotensin-1 converting enzyme (ACE) I/D polymorphisms are associated with T2DM (Singh *et al.*, 2006). Polymerase chain reactions (PCRs) were used to analyze the available genetic data from 90 patients and 97 random healthy controls. The results showed that DD genotype and *D allele of ACE are associated with T2DM (OR = 1.90, $p < 0.05$, and OR = 1.58, $p < 0.05$, respectively) (Table 7). Recessive and multiplicative mode of inheritance for *D allele provided the strongest support for the association. DD-33 and ID-23 combinations (ACE–APOE) showed higher odds of 2.01 and 2.14, respectively. ACE polymorphism is positively associated with T2DM in Indian population; however, the synergistic effects of DD-33 and ID-23 are also evident (Table 8).

ACE*D allele is associated with increased bradykinin degradation which in turn may influence blood flow and glucose uptake at skeletal tissue level which is the primary site of insulin resistance in T2DM. The genetic association between ACE I/D and certain complications of diabetes may be stronger compared to the association between general T2DM and ACE I/D polymorphism. The ACE polymorphism may also be informative when examined in combination with life style variables such as BMI, diet, and physical activity. In this study only BMI and FBS were examined. These parameters showed interesting trends (Table 8), which can be examined further in a comprehensive large scale study (Singh *et al.*, 2006).

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6.0 Limitations:

Some of limitations of this review paper are accessibility to online based data, inadequate genetic information regarding T2DM from unborn babies, and time restriction. Limitations commonly found in the experiments mentioned in the journal articles are small sample size, no single direct and strongly associated marker predispose to T2DM, and lack of technology and funding shortage in the developing countries like Bangladesh.

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7.0 Recommendations:

More information and practical data are required to analyze genetic factors in large populations to have better understanding about the directly related DNA markers predisposed to the T2DM progression. To achieve that goal there should be broader collaboration between clinics, laboratories, local and international researchers and scientists in terms of sharing information, knowledge, findings and understandings. Microarray technology can be used to detect the differences in normal people vs. T2DM patients and the obtained genetic level differences can be uploaded in a database system. This genomic database will help future research and studies. After doing all these literature reviews and research, it can be recommended that epigenetic factors should also be considered in identifying markers directly

associated to T2DM. For example, the study done by Arif *et al.* suggests that the presence of ascorbic acid in diets reduces the amount of oxidative DNA damage and downgrades hyperglycemia. As previous genetic studies such as fat, yellow Agouti mice experiment showed that changing in diet during pregnancy highly influences gene expression in offspring by turning on or off the genetic switches. From these information, it can be recommended that increasing the amount of ascorbic acid in pregnant woman's diet can potentially help reducing the oxidative DNA damage in the unborn and thereby altering the onset of T2DM in children. Future studies should be done by changing diets such as low cholesterol and high ascorbic acid in the diets of pregnant women to see its effects on the T2DM onset in unborn.

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8.0 Conclusion:

Since T2DM is a complex multi-factorial polygenic disease, various genetic and epigenetic factors are involved in the process of disease development and progression. More studies and research are required in this field to identify a narrow range of markers which are strongly correlated to the development of T2DM. Primary detection in unborn will help to reduce the incident rate as well as the disease complicacy and onset which would be beneficial for worldwide public health.

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