

Study of DNA Methylation in offsprings of Individuals with Type 2 Diabetes mellitus

Abstract:

Introduction: Epigenetic is study of changes in gene expression that occur by changing the DNA methylation and remodeling chromatin. It may also be affected by the environment. It is an important pathogenic mechanisms in complex multifactorial diseases such as type two diabetes. Recent genome-wide association studies have identified a number of genetic variants that explain some of the inter individual variation in diabetes susceptibility. Thus keeping in view, the role of epigenetic mechanisms particularly DNA methylation, the present study was conducted to find out DNA methylation (Whole DNA) in offsprings of individuals with type 2 Diabetes Mellitus.

Methods: For the present study, the families of one hundred Diabetic individuals were enrolled. All the off springs of diabetics above the age of 18 years were recruited for the present study. All the samples collected were analyzed for Fasting plasma glucose, Glycosylated Hemoglobin, Lipid profile complete and DNA methylation (5mcytosine)

Results: The study included 100 diabetic individuals and their off springs in the age range of 18-35 years. DNA methylation measured as 5mc% was significantly ($p < 0.001$) more in IFG and Diabetic offsprings as compared to normal individuals both males and females.

Conclusions: Altered DNA Methylation in individuals of the age group 18-24 years indicates towards deranged metabolism in these individuals, leading to impaired fasting glucose and making them more prone to type 2 Diabetes Mellitus and its complications at a later age.

Key Words: Diabetes Mellitus, Offsprings, Epigenetics, DNA Methylation

Introduction:

“Diabetes mellitus is one of the most common non-communicable metabolic disorders which is manifested by hyperglycemia, resulting due to defects in insulin secretion, insulin action or both. The incidence of diabetes mellitus is increasing along with increased proportion of individuals with impaired fasting glucose or prediabetes. The disease formerly considered to be affecting middle aged is incident now in young and adolescent as indicated in previous studies”¹. “It has been well documented that environmental factors play an important role in pathogenesis of Diabetes Mellitus leading to epigenetic modifications which include Methylation of DNA and histone modification”². These are classified as Epigenetics or epigenetic mechanisms.

“Although there is no uniform definition of Epigenetics, it is defined as a heritable change in gene function that occurs without a change in nucleotide sequence. Epigenetic is presently described as the study of changes in gene expression that occur not by changing the DNA sequence, but by modifying DNA methylation and remodeling chromatin”³. “In recent years, major advances in the understanding of epigenetic mechanisms have established them as key players in several cellular processes including cell differentiation, aging, DNA replication, and repair”⁴⁻⁷.

“These modifications, including acetylation, methylation, phosphorylation, and ubiquitination, altering the interaction between the histones, DNA and nuclear proteins, therefore affecting gene transcription and regulating gene silencing or expression”⁶. Epigenetic effects may also be affected by the environment, making them potentially important pathogenic mechanisms in complex multifactorial diseases such as type 2 diabetes. DNA methylation is commonly associated with gene silencing and contributes to X chromosomal inactivation, genomic imprinting and transcriptional regulation of tissue- specific genes during cellular differentiation.

“Although obesity, reduced physical activity, and aging increase susceptibility to type 2 diabetes, many people exposed to these risk factors do not develop the disease. Recent genome-wide association studies have identified a number of genetic variants that explain some of the inter individual variation in diabetes susceptibility”⁸⁻¹².

Thus, keeping in view, the role of epigenetic mechanisms particularly DNA methylation the present study was conducted to find out DNA methylation (Whole DNA) and its association with Type 2 Diabetes mellitus.

Material and Method: The present study was conducted in Multidisciplinary Research unit GMC Amritsar in Association with Department of Biochemistry and Medicine at Government Medical College and Guru Nanak Dev Hospital Amritsar. The study was conducted after approval from the Ethical committee (Code No GMCIEC00095). For the present study, the families of one hundred Diabetic individuals were enrolled after obtaining informed consent. All the off springs of diabetics individuals in the age group of 18 to 35 years were recruited for the present study.

Inclusion criteria

All the offsprings of known Diabetics belonging to the age group of 18-35 years were included in the present study.

Exclusion criteria

The subjects with liver disease, renal disease, thyroid disease, tuberculosis, hypertension, pancreatitis, individuals on drugs like gluco-corticoids, Nicotinic acid, Thyroid hormones, β -adrenergic antagonists and thiazide diuretics, drug addicts patients with endocrinopathies such as acromegaly, patients with down syndrome were excluded from the present study.

Sample collection was done by calling the family members in outpatient department. All the members were advised to observe at least 8 hours fast. To comply with the instructions the sample

collection was started at 7.30 am in the morning. Samples collected were labeled using a number code for a family. All the samples were transferred to multi-disciplinary research unit for serum separation, processing, and storage for further analysis. All the samples collected were analyzed for

1. Fasting plasma glucose¹³
2. Glycosylated Hemoglobin¹⁴
3. Lipid profile complete (Total cholesterol¹⁵, HDL¹⁶, LDL¹⁷, VLDL and Triglycerides¹⁸)

The samples were stored at -70°C to estimate DNA methylation (5mcytosine¹⁹) DNA was isolated by using Standard Phenol Chloroform method described by Adeli and Oqbonna 1990²⁰ and quantification of DNA was done using Agarose Gel Electrophoresis method as described by (Sambrook et al 1989)²¹. After quantification the methylation of DNA was estimated using commercially available ELISA kits.

The data thus generated was analyzed statistically using student "t" test for comparison of mean of two groups. ANOVA was used to compare means of more than one group. Pearson's coefficient of correlation was used to calculate correlation between various numerical parameters. $p < 0.05$ was considered to be statistically significant.

Results: The study included 100 diabetic individuals and their off springs in the age range of 18-35 years. All the participants in the study were evaluated for fasting plasma glucose, glycated Hemoglobin, lipid profile and DNA methylation. The offsprings were categorized depending on age as Group I, II and III and as Normal, IFG and Diabetic depending on the levels of Fasting Plasma Glucose and HbA1c as per ADA criteria

The three groups depending on their age were Group I $\geq 18-24$ years, Group II $>24-30$ years and Group III $>30-35$ years. The maximum number of individuals belonged to age group II i.e. $>24-$

30 years of age. All the offsprings were segregated according to gender i.e. males and females. Some of the individuals gave the history of being diabetic. The observations thus made are reported as under.

| Age Group | Gender | Glucose mg% (Mean \pm S.D) | | | Glycated Hemoglobin % (Mean \pm S.D) | | |
|--------------------|--------|------------------------------|---------------------|--------------------------------|--|-------------------|------------------------------|
| | | Normal | IFG | diabetic | Normal | IFG | diabetic |
| \geq 18-24 years | Male | 84.7 \pm 1.2 | 111.7 \pm 7.4** | 160.4 \pm 2.9** \ddagger | 4.47 \pm 0.55 | 5.8 \pm 0.35** | 6.47 \pm 1.46** \ddagger |
| | Female | 82.37 \pm 1.3 | 109.7 \pm 5.6** | \ddagger 201.85 \pm 7.9** | 4.35 \pm 0.48 | 5.87 \pm 0.40** | 6.71 \pm 1.61** \ddagger |
| >24-30 years | Male | 84.0 \pm 1.3 | 112.85 \pm 6.7** | 170.18 \pm 3.2** | 4.41 \pm 0.6 | 5.85 \pm 0.26** | 6.4 \pm 1.09** \ddagger |
| | Female | 81.38 \pm 1.32 | 110.72 \pm 7.6** | \ddagger 195.05 \pm 8.17** | 4.35 \pm 0.52 | 5.75 \pm 0.64** | 6.39 \pm 1.28** \ddagger |
| >30-35 years | Male | 86.11 \pm 1.22 | 111.4 \pm 7.9** | 180.46 \pm 3.36** \ddagger | 4.38 \pm 0.52 | 5.8 \pm 0.51** | 5.8 \pm 1.22 |
| | Female | 79.31 \pm 1.33 | 109.79 \pm 6.56** | 198.19 \pm 8.8** \ddagger | 4.45 \pm 0.50 | 5.69 \pm 0.56** | 6.59 \pm 0.92** \ddagger |

Table 1 Comparison of Fasting Plasma Glucose and Glycated Hemoglobin in Normal IFG and Diabetic Individuals belonging to different Age groups

\ddagger p < 0.05 when males were compared with females

** p < 0.001 when IFG and Diabetics were compared with normal individuals

\ddagger p < 0.05 when IFG and diabetics were compared amongst each other

In the Age group \geq 18-24 years It was observed that the levels of fasting plasma glucose were more in diabetics as compared to IFG and normal individuals (both in males and females). When males and females were compared amongst each other it was observed that in diabetic females the values were more as compared to males, whereas in IFG and normal individuals the value of fasting glucose amongst males and females did not show any significant variation. Levels of glycosylated hemoglobin were more in diabetic individuals as compared to normal and IFG both in males and females when males and females were compared amongst each other it was

observed that although in diabetic females the values of glycosylated hemoglobin were more as compared to diabetic males, yet the difference was not significant.

In Age group >24-30 years It was observed that the levels of fasting plasma glucose were more in diabetics when compared to normal and IFG both in males and females when males and females were compared amongst each other it was observed that levels of fasting plasma glucose were more in diabetic females as compared to diabetic males the variation was statistically significant ($p < 0.05$). In other two groups i.e. normal and IFG the levels of glucose in males and females did not differ much. Levels of glycosylated hemoglobin were more in diabetic individuals as compared to normal and IFG both in males and females however when the levels were compared amongst males and females the levels did not vary significantly.

In age group >30-35 years the levels of fasting plasma glucose were more in diabetic individuals as compared to normal and IFG both in males and females. The levels of fasting plasma glucose were more ($p < 0.05$) in diabetic females as compared to males. Levels of glycosylated hemoglobin were more in diabetics ($p < 0.001$) as compared to normal whereas there was no variation when diabetics were compared with IFG male individuals. As far as females were concerned the levels of glycosylated hemoglobin were more in diabetic individuals as compared to normal and IFG. When males and females were compared amongst each other it was observed that levels of glycosylated Hb were more in diabetic females as compared to males, the variation was significant ($p < 0.05$).

Table 2 Comparison of Total Cholesterol and S. Triglycerides in Normal IFG and Diabetic Individuals belonging to different Age groups

| Age Group | Gender | Total cholesterol mg% (Mean \pm S.D) | | | Triglycerides mg % (Mean \pm S.D) | | |
|--------------------|--------|--|------------------|--------------------|-------------------------------------|--------------------|--------------------|
| | | Normal | IFG | diabetic | Normal | IFG | diabetic |
| \geq 18-24 years | Male | 183 \pm 3.54 | 173 \pm 4.0 | 201 \pm 3.7" ‡ | 116 \pm 5.43 | 116 \pm 4.13 | 191 \pm 4.97" ‡ |
| | Female | 185 \pm 3.77 | †198 \pm 3.9* | †219 \pm 5.7" ‡ | 106 \pm 4.48 | ††159 \pm 8.85** | 187 \pm 9.28" ‡ |
| >24-30 years | Male | 186 \pm 3.81 | 191 \pm 4.26 | ‡215 \pm 4.41** | 113 \pm 5.91 | 143.7 \pm 8.98** | 149 \pm 9.70 |
| | Female | 189 \pm 3.13 | 201.8 \pm 3.15 | 210 \pm 3.57 | 116 \pm 4.03 | 155.6 \pm 1.07** | ‡†173 \pm 1.38** |
| >30-35 years | Male | 187.2 \pm 3.7 | 189.3 \pm 4.54 | 200.1 \pm 5.88 ‡ | 153.4 \pm 5.2 | 157 \pm 8.1 | 189.9 \pm 9** ‡ |
| | Female | 191.9 \pm 4.38 | †203.9 \pm 5.1 | †214.9 \pm 4.1 ‡ | 147 \pm 6.2 | †177.5 \pm 8.3** | 182.3 \pm 1** ‡ |

† p < 0.05 when males and females were compared amongst each other

** p < 0.001 when IFG and diabetics were compared with normal individuals

* p < 0.05 when normal and IFG were compared amongst each other

†† p < 0.001 when males and females were compared amongst each other

‡ p < 0.05 when IFG and diabetics were compared with each other

In age group of >18-24 years The levels of total cholesterol were more (p<0.05) in diabetic individuals as compared to normal and IFG both in males and females when males and females were compared amongst each other it was observed that in IFG and diabetic females the value of total cholesterol was more as compared to IFG and diabetic males, this difference was statistically significant (p<0.05) .No variation was observed when males and females were compared amongst each other . Levels of serum triglycerides were more in diabetic individuals when compared to normal and IFG both in males and females when males and females were compared amongst each other it was observed that the values of triglycerides were more in IFG females and diabetic males as compared to IFG males and diabetic females. The change in levels in IFG females as compared to IFG males was statistically significant (p<0.001)

In the age group of >24-30 years Levels of total cholesterol were more in IFG, and diabetic individuals as compared to normal male individuals. When males and females were compared amongst each other it was observed that in diabetic males the levels were more as compared to diabetic females but this difference was statistically insignificant . The levels of triglycerides were more in diabetic individuals as compared to normal and IFG both in males and females, the variation amongst normal and IFG males was insignificant. In females the levels of triglycerides were more in IFG individuals as compared to normal; this increase was statistically significant ($p<0.001$) whereas when IFG and diabetic individuals were compared amongst each other the variation was insignificant . When males and females were compared amongst each other it was observed that levels of triglycerides were more in IFG and Diabetic females as compared to IFG and Diabetic males this variation was significant ($p<0.05$).

In the age group of >30-35 years Levels of total cholesterol were more in IFG, and diabetic individuals as compared to normal in male and female individuals. When males and females were compared amongst each other it was observed that in IFG and diabetic females the levels were more as compared to males and this difference was statistically significant ($p<0.05$) . The levels of triglycerides were more in diabetic individuals as compared to normal and IFG both in males and females, the variation amongst normal and IFG males was insignificant. In females levels of triglycerides increased as the status of females changed from normal to Diabetic through IFG. This change was statistically significant ($p<0.001$). When males and females were compared with each other, it was observed that in IFG females and diabetic males the increase in levels of triglycerides was statistically significant ($p<0.001$).

Table 3 Comparison of Lipoproteins in Normal IFG and Diabetic Individuals belonging to different Age groups

| Age Group | Gender | LDL mg% (Mean ±S.D) | | | VLDL mg % (Mean ±S.D) | | | HDL mg% (Mean ±S.D) | | |
|--------------|--------|---------------------|------------|---------------|-----------------------|------------|--------------|---------------------|-----------|------------|
| | | Normal | IFG | diabetic | Normal | IFG | diabetic | Normal | IFG | diabetic |
| 18-24 years | Male | 111 ± 3.3 | 120±3.6 | 135.64±3.6**‡ | 18 ±9.94 | 21±8.5 | 23 ± 1.08 | 50.8±8.07 | 50.0±5.8 | 47.18±7. |
| | Female | 111 ± 3.4 | 115±4.3 | 135.26±4.9**‡ | 21± 8.94 | 31±1.79* | ‡37±1.85** | 51.49±6.2 | 47.2±7.58 | 47.0±9.9 |
| >24-30 years | Male | 112 ± 4.2 | 115.6±3.29 | 133 ±4.61**‡ | 23 ± 1.21 | 28.75±1.79 | 29.94±1.94 | 50.45±6.6 | 50.78±7.9 | 50.90±6.76 |
| | Female | 112± 3.6 | 119.55±3.3 | 127±4.75**‡ | 23.55± 8.26 | 31.12±2.14 | ‡34.77±2.76* | 49.78±7.6 | 48.40±6.2 | 49.94±4.11 |
| >30-35 years | Male | 109.5±4.2 | 100.7±4.6 | ‡115.7 ±5.7 | 30.4 ±1.6 | 31.6±1.0 | 37.9±1.8* | 51±6.9 | 47.4±7.2 | 45.7±6.2 |
| | Female | 115.4 ± 4.4 | ‡114.7±3.9 | ‡133.6±4.0**‡ | 29.6 ± 1.1 | 35.5±1.6 | 36.4±2.1 | 50 ± 7.8 | 46.6±5.2 | 46.5±4.5 |

†p< 0.05 when males were compared with females,

**p< 0.001 when IFG and diabetics were compared with normal individuals,

*p<0.05 when IFG, Diabetics and normal were compared with each other,

‡p<0.05 when IFG and diabetics were compared amongst each other

In the age group of >18-24 years Levels of LDL-C were more in diabetic individuals when compared to normal and IFG individuals. When males and females were compared amongst each other it was observed that levels of LDL-C was more in IFG males as compared to females, but the difference was not significant statistically. Similar trend was observed for VLDL. The variation in the levels of HDL in normal, IFG and Diabetics both males and females was statistically insignificant.

In the age group of >24-30 years levels of LDL increased significantly ($p<0.05$) when IFG, Diabetic and normal males and females were compared amongst each other. Although levels of LDL increased in Diabetic males than females the change was statistically not significant.

In the age group of >30-35 year when LDL-C levels were compared in normal, IFG and diabetic individuals it was observed that levels of LDL-C were more in diabetics as compared to normal and IFG both in male and female individuals but this change was not significant statistically in males whereas in females this change was statistically significant ($p<0.001$). In other two groups the variation was insignificant. When males and females were compared amongst each other it was observed that levels of LDL-C were more in IFG, and diabetic females as compared to males. The increase in levels of LDL-C in diabetic females as compared to diabetic males was statistically significant ($p<0.05$). Levels of VLDL-C did not vary significantly when diabetics were compared with IFG and normal individuals both in males and females. When males and females were compared amongst each other it was observed that the variation in all the groups was insignificant. Levels of HDL-C decreased in diabetic male individuals as compared to normal and IFG, this variation was statistically insignificant. In females although the levels of HDL-C decreased the levels were same in IFG and diabetic individuals and the change as compared to normal was insignificant.

Table 4 Comparison of DNA methylation in Normal IFG and Diabetic Individuals belonging to different Age groups

| Age Group | Gender | 5mc% (Mean \pm S.D.) | | |
|--------------|--------|------------------------|-------------------|--------------------|
| | | Normal | IFG | diabetic |
| 18-24 years | Male | 2.06 \pm 0.36 | 3.56 \pm 0.63* | 19.93 \pm 8.45* |
| | Female | 2.84 \pm 0.38† | 3.63 \pm 2.05* | 5.09 \pm 0.83*†† |
| >24-30 years | Male | 3.25 \pm 0.29 | 4.07 \pm 0.51* | 6.11 \pm 2.1* |
| | Female | 2.71 \pm 0.58† | 4.23 \pm 1.3*† | 5.24 \pm 1.5*† |
| >30-35 years | Male | 1.97 \pm 1.3 | 4.76 \pm 0.70* | 4.78 \pm 3.01* |
| | Female | 2.94 \pm 0.69† | 3.87 \pm 1.3*†† | 4.56 \pm 2.97*† |

*p<0.001 when normal and IFG individuals were compared amongst each other

†p<0.05 when males and females were compared with each other

††p<0.001 when males and females were compared with each other

In the age group of 18-24 years significant increase in DNA methylation (p<0.001) was observed when Normal, IFG and Diabetic males were compared with each other. When Diabetic males and females belonging to the age group of >24-30 years were compared, a significant increase (p<0.05) in 5mc% was seen in males as compared to females belonging to the same age group. Similar trend was seen in individuals belonging to the age group of >30-35 years where the variation amongst males was significant (p<0.05).

Discussion: Diabetes mellitus is a metabolic disorder due to lack of insulin secretion or action or both, resulting in persistent hyperglycemia. Earlier type 2 Diabetes mellitus was considered a disease of middle aged but with prevalent lifestyle changes i.e. increasing obesity and sedentary lifestyle in young individuals the prevalence has increased in adolescents and individuals as young as 18 years of age. As reported in previous studies¹ that the age of onset of type 2 Diabetes mellitus is decreasing and there is increased prevalence of insulin resistance in siblings of patients of type 2 diabetes mellitus, the present study focused on family members of Diabetics i.e. off springs of diabetics in the age group of 18-35 years. As depicted in Table 1 females belonging to age group of $\geq 18-24$ years had higher plasma glucose and glycated hemoglobin. The females belonging to this age are in their reproductive years and it has been stated that estrogen and sex hormone binding globulin are also associated with insulin resistance in women, thus increased levels of glucose²². More the level of glucose more is the glycosylated Hemoglobin.

Dyslipidemia at a young age of >18-24, >24-30 and >30-35 years is indicative of insulin resistance. Levels of triglycerides are more due to increased levels of glucose in circulation although levels of insulin may also be increased, but the response generated was not significant in lowering the levels of glucose to normal limits in individuals belonging to this age group.

As observed in the present study levels of lipids were more in IFG females belonging to various age groups. Similar observations have been made by OMP Ganda (1985)²³. Although indiabetics poor metabolic control is the reason for this change in individuals with IFG this may be partly explained by the fact that these individuals are still not aware of their status of lipid profile and hence are at an increased risk of developing complications.

DNA methylation is one of the markers of epigenetic variation which has been linked to type 2 diabetes mellitus and its related risk factors including insulin resistance. The genetic basis for developing type 2 diabetes has been recognized for a long time. The concordance of type 2 diabetes mellitus in monozygotic twins is approximately 70% compared with 20-30% in dizygotic twins and a sibling of an affected individual has about 3-time higher risk for developing type 2 diabetes mellitus than the general population²⁴.

The incidence of type 2 diabetes mellitus has increased dramatically over the past decade which is too short a period for consideration of genetic alterations in the genome. Therefore, it is likely that environmental factors including diet and sedentary lifestyle play a significant role in development of type 2 diabetes mellitus.

Epigenetics in the current study was studied by estimating DNA methylation. The levels of 5mc% were increased significantly in individuals with IFG and diabetes (both males and females) especially in the age group of 18-24 years (males). 5mc% indicates the excess of methylation of the genome which is an alarming situation. In individuals with IFG similar changes were observed which a matter of concern is again because persistent hyperglycemia leads to various complications associated with Diabetes mellitus.

Persistent hyperglycemia can lead to increased DNA methylation of β cells, thus leading to decreased pancreatic function. As age is an important risk factor for development of Type 2 Diabetes Mellitus increased expression of 5mc% at an early age predisposes the individual to an increased risk of development of Diabetic complications at an early life²⁵. As DNA methylation also plays a role in controlling energy metabolism by storing and releasing lipids, so excess lipids will accumulate leading to dyslipidemia as evident from the results²⁶.

DNA methylation is also affected by environmental factors which includes exposure to insecticides, pesticides and food adulterants, the process of changes in DNA will not stop, so the individuals with IFG at a tender age of >18-24 years need to be advised dietary and life style modifications so as to slow down the rate of development of Type 2 Diabetes mellitus and Associated complications.

Epigenetic changes can lead to various changes in genome which along with various environmental factors can affect muscle, liver and adipose tissue energy metabolism leading to β cell death and may also lead to metabolic memory which is expressed as predisposition to type 2 Diabetes mellitus, can alter the response to treatment²⁷, lead to risk for development of complications of Type 2 Diabetes mellitus and if these changes happen in females it may lead to transmission to the next generation.

Conclusions:The present study concludes that hyperglycemia, altered lifestyle and obesity are risk factors for development of type 2 diabetes mellitus, but these changes are occurring at an early age i.e. 18-24 years (young adolescents) which is a matter of concern. Increased DNA Methylation in these individuals indicates towards deranged metabolism and development of inflammation in these individuals. These changes can be reversed by changing the dietary and lifestyle pattern as Epigenetics is affected by lifestyle.

Ethical Approval and Consent

The study was conducted after approval from the Ethical committee (Code No GMCIEC00095).

For the present study, the families of one hundred Diabetic individuals were enrolled after obtaining written consent.

References

1. Sukhraj Kaur and Mridula Mahajan. Declining age of onset of type 2 diabetes Mellitus in Northwest Punjabi population. *Journal of Clinical and Diagnostic Research*, 2011 June, vol-5(3):425-429
2. Bird A. Perceptions of Epigenetics. *Nature* 2007;447: 396-398
3. Mohn, F., Schubeler, D. Genetics and epigenetics: Stability and plasticity during cellular differentiation. *Trends Genet.* 2009; 25: 129- 136.
4. Calvanese, V., Lara, E., Kahn, A., Fraga, MF. The role of epigenetics in aging and age-related diseases. *Ageing Res Rev.* 2009; 8: 268-276.
5. Hiratani, I., Gilbert, DM. Replication timing as an epigenetic mark. *Epigenetics* 2009; 4: 93- 97.
6. Huertas, D., Sendra, R., Munoz, P. Chromatin dynamics coupled to DNA repair. *Epigenetics* 2009; 4: 31-42.
7. Waki, H., Yamauchi, T., Kadowaki, T. The epigenome and its role in diabetes. *CurrDiab Rep.* 2012; 12 (6): 673-85.
8. Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 2007;316:1331–1336
9. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL et al A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science* 2007;316:1341–1345
10. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D et al A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 2007;445:881–885

11. Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, et al Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science* 2007;316:1336–1341
12. Zeggini E, Scott LJ, Saxena R, Voight BF, Marchini JL, Hu T et al Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet* 2008;40:638–645.
13. P. Trinder, 1969, 'Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *J. clin. Path*, vol 22, 158-161
14. Klenk, DC, Hermanson, GT, Krohn, RI et al 1982, 'Determination of glycosylated hemoglobin by affinity chromatography: comparison with colorimetric and ion-exchange methods, and effects of common interferences, *Clinical Chemistry*, vol. 28, no. 10 pp 2088-2094.
15. Charles, C, A'Lucy S, P, Cicely S, et al 1974, 'Enzymatic Determination of Total Serum Cholesterol, *Clinical Chemistry*, vol. 20, no. 4, pp 470-475.
16. Brustein, M, Scholnick, HR, Morfin, R, 1970, 'Rapid method for the isolation of lipoprotein from human serum by precipitation with polyanions, *J Lipid Res*. Vol 11, pp 583–595.
17. William, T, Friedwald, Robert, I, Levy and Donald, S, Fredrickson 1972, 'Estimation of concentration of low density lipoprotein cholesterol in plasma, without the use of preparative ultracentrifuge,' *Clinical Chemistry*, vol 18, no 6, pp 499-502.
18. Michael, W, Gowan, Mc, Joseph, D, et al 1983, 'A peroxidase coupled method for the colorimetric determination of Serum triglycerides, *ClinChem*, vol 29/3, pp 538-542.
19. Tyson A Clark, Xingyu Lu, Khai Luong, Qing Dai, Matthew Boitano, Stephen W Turner, Chuan He et al Enhanced 5-methylcytosine detection in single-molecule, real-time sequencing via Tet1 oxidation *BMC Biology* 2013 volume 11, Article number: 4
20. Adeli K and Ogbonna G. Rapid purification of human DNA from whole blood for potential application in clinical chemistry laboratories. *Clinical Chemistry* 36(2): 261-264.
21. Sambrook J, Fritsch E.F. and Maniatis T 1989. *Molecular Cloning: A laboratory manual*. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Press; 1989.

22. Sumika Matsui, Toshiyuki Yasui and Minoru Irahara. Association of Estrogen and Testosterone with Insulin Resistance in pre and post menopausal women with and without hormone therapy. *Int J EndocrinolMetab* 2013 (11)2: 65-70
23. Ganda, OP, Stuart, SJ, and Gleason, RE , ‘Alterations in Plasma Lipids in the Presence of Mild Glucose Intolerance in the Offspring of Two Type II Diabetic Parents’, *Diabetes Care*, 1985 vol. 8, no. 3, pp 254-260.
24. Hana Alzamil Elevated serum TNF α is related to obesity in Type 2 Diabetes Mellitus and is associated with glycemic control and Insulin Resistance. *Hindawi Journal of Obesity* 2020: vol 2020 Article ID 5076858.
25. Cajsadavegardh, Sonia Garcia-Calzon, Karl Bacos, Charlotte Ling. DNA methylation in the pathogenesis of type 2 diabetes in humans. *Molecular Metabolism* 2018;14; 12-25.
26. Gillberg L, Jacobsen SC, Ribel-Madsen R, et al. Does DNAmethylation of PPARGC1A influence insulin action in firstdegree relatives of patients with type 2 diabetes? *PLoS One*.2013;8:e58384. DOI: 10.1371/journal.pone.0058384.
27. VelmuruganMannar, Hiya Baro, Deepika Patel, SaurabhAgsta, MazharDalvi and VikashBundela. Epigenetics of the pathogenesis and complications of type 2 Diabetes Mellitus. *Review in Endocrinology*. 2023: 19 (1); 46-53.