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# Association of E23K Genotypes to T2DM Complications in subjects in Port Harcourt City, Nigeria.

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***Abstract***
*This study examined the association between the E23K allele variant of the KCNJ11 gene with type 2 diabetes mellitus in a Nigerian population and the possible complications that may arise from the variant. The E23K polymorphism of the KCNJ11 gene results from a substitution of the amino acid lysine to glutamate at codon 23. This alteration causes a critical inhibition of glucose-induced insulin secretion thereby resulting in hyperglycaemia. Hundred consenting Nigerian adults (73 diabetics and 27 non-diabetic subjects) aged at least 40 participated in this study. Genotyping was carried out with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique using BanII restriction digestion enzyme. The restriction fragments were then electrophoresed on DNA grade agarose gel and the bands visualised using a UV transilluminator. The genotypes identified are the EE (150bp band), EK (150bp+178bp bands) and KK (178bp band) genotypes. The KK genotype was preponderant in the diabetic participants (52%) and was followed by the EK genotype (25.9%) while the EE genotype was more in the non-diabetic participants (66.7%). The risks conferred by the different genotypes/allele are as follows: EK (p value = 0.5639; OR = 1.32), EE (p value = 0.000; OR = 0.21), KK (p value = 0.0037; OR = 7.03), K (p value = 0.211; OR = 2.59) and E (p value = 0.0552; OR = O.52). A carrier of the KK genotype is seven times more likely than a non-carrier to develop type 2 diabetes mellitus (p value = 0.0037; 7.03). Only the KK genotype was found to significantly increase the risk of developing type 2 diabetes complications (p value = 0.02; OR = 12.67). The p values of the selected biochemical variables are as follows: leptin = 0.95, fasting blood sugar = 0.15, C-peptide = 0.47, Cystatin C = 0.86, HbA1C = 0.01, insulin = 0.65 and HOMA = 0.65. Of the glycaemic variables analyzed, only HbAlc showed a significant difference between the diabetic and control groups (p value = 0.01) but there was no significant difference in its levels in the different genotypes (p value = 0.64). A significant association between the E23K polymorphism and T2DM as well as complications that could arise from the disease was found in the Nigerian population that was studied. The KK genotype of the E23K polymorphism of the KCNJ11 gene is an independent predictor of Type 2 diabetes mellitus.*

***Keywords: Diabetes, Complications, E23K, Polymorphism, KCNJ11 gene***

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## I. Introduction

The proportion of people with type 2 diabetes is increasing in most countries with 79% of adults with diabetes living in low and middle income countries (International Diabetes Federation Atlas, 2017). The current prevalence rate of T2DM in Nigeria is not known but estimates place it in the region of 8-10% (Ogbera *et al*., 2014). A 2003 study by Nyenwe *et al*., yielded a crude prevalence rate of 6.8% and a standardized prevalence rate of 7.9% of T2DM in Port Harcourt. All regions of the country (even rural settlements) have been affected but the highest prevalence is seen in Southern Nigeria (Uloko *et al*., 2018). Genetic factor has been implicated as a driving force for the current prevalence rate.

Several genetic association studies and genome-wide association scans (GWAS) have been carried out in a bid to identify susceptibility genes and loci associated with this disease (Assman *et al*., 2014).

Some susceptibility genes identified by these genetic studies include TCF7L2 (Nanet *et al*., 2015), PPARG (Engwa *et al*.,2018), SLC30A8, HHEX/IDE (Saxena *et al*., 2007), CDKAL1, CDKN2A/B, IGF2BP2 and FTO (Scott *et al*.,2007), IRSI,ADAM TS9 and GCKR ( Bossegard *et al*.,2009), WFS1 (Sandhu *et al*.,2007), HNF1B (Sparoso *et al*.,2008) and KCNJ11 (Lasram *et al*.,2014).

The KCNJ11 gene is found on chromosome 11 and codes for a 390 amino acid protein that is one of the subunits of the pancreatic $K_{ATP}$ channel ( Rastegari *et al*., 2015).

Several single nucleotide polymorphisms (SNP) have been shown to increase susceptibility to type 2 diabetes mellitus. Thus far KCNJ11 has been found to have 219 SNPs, six of which have been linked to type 2 and they are: rs5219 (E23K polymorphism), rs5215, rs5210, rs5218, rs886288, rs2285676 (Haghvirdiazadeh *et al*., 2015). Studies on the full mutational mechanisms are incomplete and not yet comprehensively categorical

(Hamming *et al.*, 2009) especially in cases where the ABCC8 (rs757110) gene was found in complete linkage disequilibrium with the E23K (rs5219) variant (Hamming *et al*., 2009).

In the E23K polymorphism, there is a substitution of the amino acid lysine to glutamate (AAG –CAG) at codon 23 of the $NH_2$-terminal tail of the Kir6.2 (Haghvirdiazadeh *et al*., 2015; Rastegari *et al*., 2015). Even though lysine is positively charged and glutamate carries no charge at all, there is no remarkable difference in the function and structure of the Kir6.2 proteins (Haghvirdiazadeh *et al*., 2015). It has been reported that the E23K variant may alter the charge of the ATP-binding region, thereby decreasing the sensitivity of the KATP channel to ATP (Haghvirdiazadeh *et al*., 2015).

Similarly, this locus has been associated with increase in fasting and post prandial glucose levels (Shaat *et al*., 2005; Gonet *et al*., 2012) and higher glycated haemoglobin and blood pressure levels (Koo *et al*., 2007; He *et al*., 2008). Pharmacological studies in this polymorphism have revealed varying drug response with some patients having a better therapeutic response to glimepiride and glibenclamide than gliclazide treatment (Javorsky *et al*., 2012). E23K variant carriers have also been found to have a reduced response to sulfonylurea therapy (Holstein *et al*, 2009; El-sisi *et al*., 2011).

The relationship between the E23K phenotype and T2DM risk has been studied in various populations such as the European (Florez *et al*., 2004; Nielsen *et al*., 2003), Arab (Abdelhamid *et al*., 2014; Alsmadi *et al*., 2008), Asian (Sakamoto *et al*., 2007; Koo *et al*., 2007; Zhou *et al.*, 2009) and Tunisian populations (Mtiraoui *et al*., 2012; Lasram *et al.*, 2014). Significant association of E23K with T2DM was found in some European descent populations (Gloyn *et al*., 2003; Nielson *et al*., 2003; Florez *et al*., 2004) and East Asian populations (Sakamoto *et al*.,2007; Koo *et al*., 2007; Zhou *et al*., 2009). In the Arabian populations, the reported results were divergent (Alsmadi *et al*., 2008; Ezzidi *et al*., 2009; Abdelhamid *et al*., 2014) suggesting that these findings cannot be extrapolated to other populations. There is paucity of data available on the E23K allele variant in African and Nigerian populations. There is insufficient knowledge/research in Nigeria of how genetics plays a role in the development of disease and disease progression in the different ethnic groups. Little is known of the prevalence of the E23K allele variant in the Nigerian population. Also lacking are studies on how this variant is linked to the risk of developing T2DM, the possible complications that may arise from it and its usefulness in the areas of risk prediction and determination of disease progression.

## II. Materials And Methods

### 2.1 Research Design and subject characterization

This was a cross sectional study focused to identify E23k allele variants and evaluated A1C, fasting blood sugar, leptin, Cystatin C, C-peptide, insulin and HOMA index in T2DM patients in Port Harcourt City. A total of 100 consenting individuals were enrolled for this study. This number was based on convenient sampling.

This study received ethical clearance from the Rivers State Health Ethics Research Committee. Participants were informed of the nature of the study and consent was obtained. The participants in this study were between 40-77 years of age: 55 males and 45 females. They were drawn from four Nigerian tribes: Ikwerre, Ijaw, Igbo and Ogoni. Seventy- three of them were diabetic and served as the test subjects while twenty - seven of them were not diabetic and served as the controls.

Consenting individuals who are of Nigerian descent aged at least 40 years diagnosed with T2DM for at least one year with glycated haemoglobin level $\geq 6.5$. Individuals less than 40 years of age, who are not of Nigerian descent and pregnant.

### 2.2 Sample Collection and Assays

Ten millilitres of venous blood were collected into fluoride oxalate, EDTA and vacuum tubes: 2mls for fasting blood sugar, 5mls for PCR analysis and 3mls for chemistry analysis.

C-peptide, Insulin, Leptin and Cystatin C were analysed using standard ELISA technique.

Fasting blood sugar was analysed using the glucose oxidase-peroxidase method and HbA1C was analysed using the high performance liquid chromatography technique. Polymerase chain reaction was carried out using the PCR-RFLP method described by Souza *et al*., 2017.

The E23K target was first amplified using a set of primers, the amplicon was Restriction Enzyme (RE) digested and visualized by agarose gel electrophoresis. A 3.0 % agarose gel was prepared by adding 3.0 g of agarose DNA grade to 100 ml of 0.5x TBE (Tris Borate EDTA) buffer, swirling to dissolve and melting in a microwave for 3 minutes. While the mixture was cooling outside, 20 µl of Ethidium Bromide was added. The molten agarose was poured into trays with cassettes and allowed to cool. Ten microlitres of RE digested product was loaded into each well. Electrophoresis was done at 100V for 30 minutes. The gel was visualized in the UV transilluminator and pictures taken using the Genomemini Gel Documentation System. The bands observed after electrophoresis were interpreted thus: 178 bp band only represents the KK genotype, 178 bp + 150 bp bands represents the EK genotype and 150 bp band only represents the EE genotype.

### 2.3 Statistical Analysis

The statistical analysis was carried out using GraphPad Prism Version 8 by GraphPad Software Inc., California.

## III. Results

### 3.1 Association of E23K Genotypes to T2DM Complications

Table 1 shows the association between E23K genotype and risk of developing complications. Of the 10 diabetic subjects that presented with EE genotype only 2 had diabetic complications; the p value was 0.009 and the odds ratio was 0.09 (0.018 – 0.479). Fifteen of the diabetic subjects that were carriers of the EK genotype had complications while the remaining 10 had none; the p value was 0.231 and the odds ratio was 0.566 (0.214 – 1.457). Thirty- four of the thirty eight carriers of the KK genotype had complications and the p value and odds ratio are 0.002 and 12.67 (1.618 -99.150) respectively.

**Table 1:** Association of E23K Genotypes to T2DM Complications

| Genotype | Complications | No complications | P value | Odds ratio (95 % CI) |
|---|---|---|---|---|
| EE | 2 | 8 | 0.009 | 0.09 (0.018 – 0.479) |
| EK | 15 | 10 | 0.231 | 0.56 (0.214 – 1.457) |
| KK | 34 | 4 | 0.002 | 12.67 (1.618 -99.150) |

### 3.2 Logistic Regression of Diabetes Status on Biochemical Variables and E23K Genotypes

Table 2 shows the logistic regression of diabetes status on biochemical variables and E23K genotypes. Alcohol had an odds ratio of 1.48 (0.4 – 5. 13) and a p value of 0.54. The KK genotype had had an odds ratio of 1.12 (0.25 – 5.11) and a p value of 0.88. Smoking, from the logistic regression, was found to be the strongest independent predictor of diabetes type 2 with an odds ratio of 3.70 (1.75 – 7.80) and a p value of 0.00.

**Table 2** Logistic regression of diabetes status on biochemical variables and E23K genotypes

| Predictor | OR | 95 % OR interval | P value |
|---|---|---|---|
| Alcohol | 1.48 | 0.42 – 5.13 | 0.54 |
| KK Genotype | 1.12 | 0.25 – 5.11 | 0.88 |
| Smoking | 3.70 | 1.75 – 7.80 | 0.00 |

### 3.3 Gel bands of RE Digested Products

Figure 1 shows the gel bands of the restricted enzyme digested products. Twenty samples are shown in figure 4.1. L is 100 base pairs (bp) DNA ladder. 178 bp band is KK genotype, 150 bp only is EE genotype while both together is EK genotype. For example, sample 17 is KK genotype, sample 14 is EE genotype and sample 20 is EK genotype.

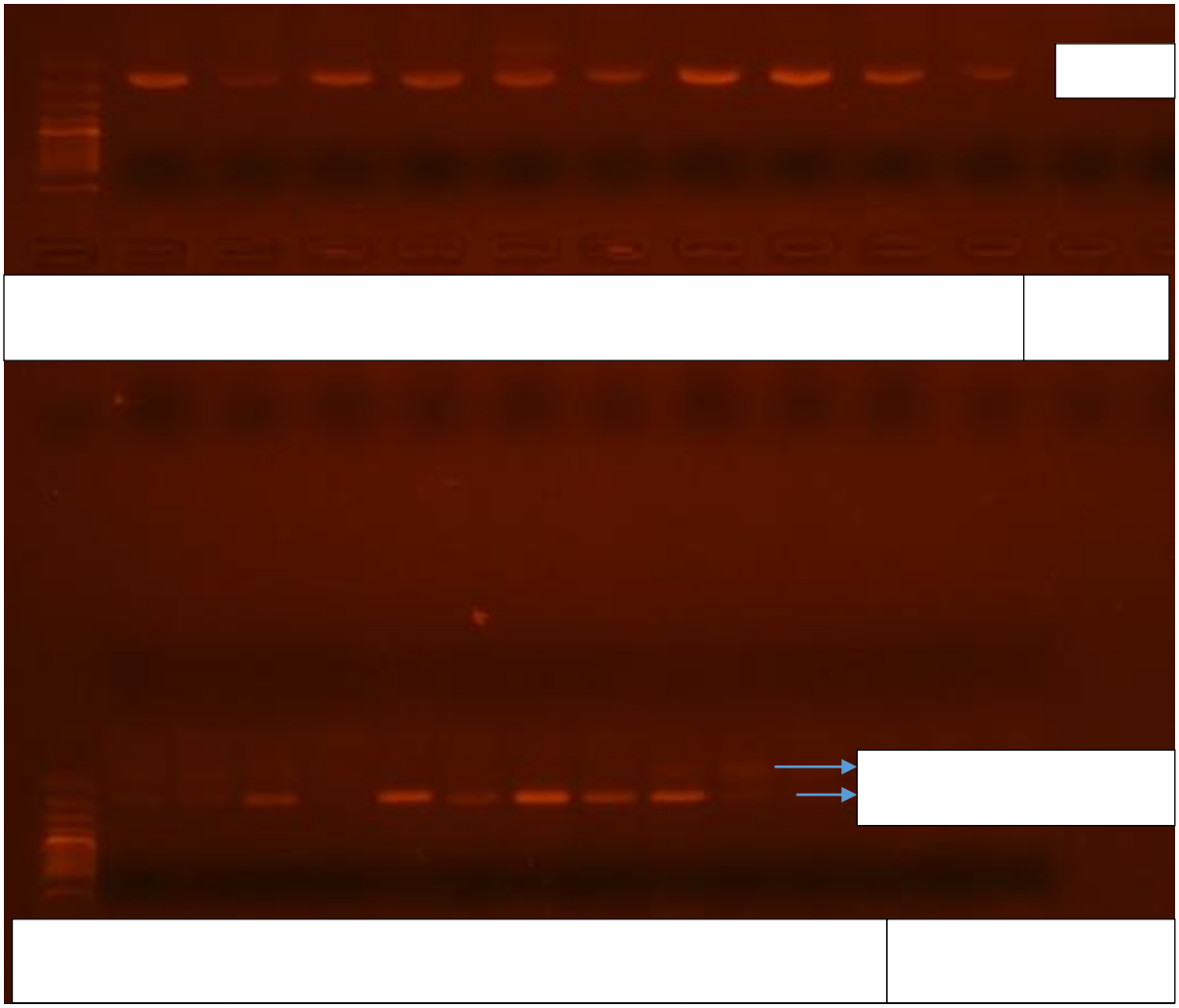

Figure 4.1: Gel bands of RE digested products

**Figure 2Box and Whiskers Plot of HbA1C Levels in Different E23K Genotypes**

Figure 2 is a Box and Whiskers plot showing HbA1C levels in different E23K genotypes. Kruskal Wallis test was performed and there was no significant difference in HbA1C levels in the different genotypes (p value = 0.64).

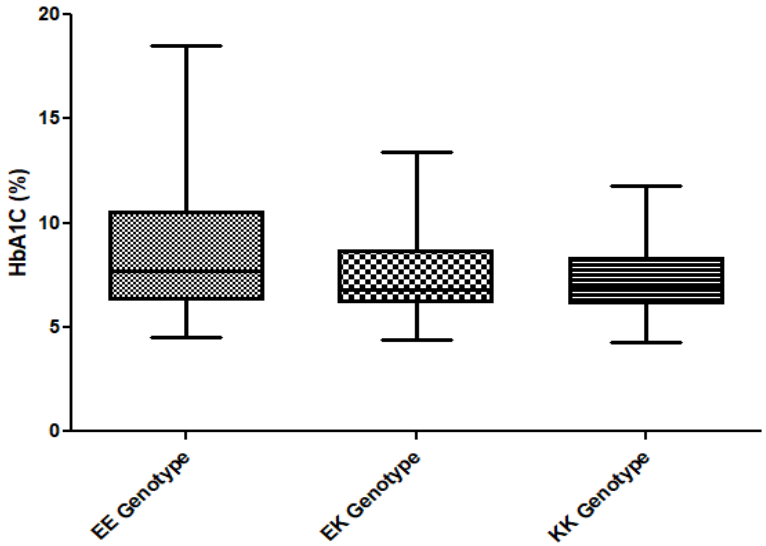


Figure 4.2: Box and Whiskers plot of HbA1C levels in different E23K genotypes

## IV. Discussion

This study examined the association between the E23K polymorphism of the KCNJII gene and type 2 diabetes mellitus complications in a Nigerian population. Mann-Whitney U test was performed on the anthropometric variables of the two groups of subjects (diabetic and non-diabetic) enrolled in this study. Only age (p value = 0.0003) and height (p value = 0.0032) showed a statistically significant difference at $p< 0.05$. Other variables including weight (p value = 0.0589), waist circumference (p value = 0.2547), BMI (p value = 0.9212), systolic pressure (p value= 0.7604) and diastolic pressure (p value = 0.3772) did not show any significant difference. Zadhoush *et al*., 2015 found no significant difference between diabetic patients and non-diabetic control with respect to age, weight, hip circumference, waist circumference, BMI and diastolic blood pressure.

A comparison between the levels of selected biochemical variables present in the diabetic and non-diabetic subjects showed that only HbAlc had a significant difference (p value =0.01). Leptin (p value = 0.95), C-peptide (p value = 0.47), cystatin C (p value = 0.86), insulin (p value = 0.65) and HOMA (p value = 0.65) were not statistically significant. Souza *et al*., (2017) found that HbAlc medians were significantly higher in T2DM patients than the control group (p value = 0.001). The reason for this may be due to the fact that while other variables show the levels present in the participants at the time of sampling, HbAlc measures glycaemic control over the past 3 months. Also, the diabetic participants in the study were mostly people who were managing the disease with drugs and lifestyle interventions. In a study by Zadhoush *et al*., 2015 biochemical changes in the diabetic group without metabolic syndrome (group 1) were found to be statistically insignificant when compared with the control group but were significant when the diabetic group with metabolic syndrome (group 2) was compared with the control group. This suggests that poor disease management is associated with abnormalities in plasma parameters.

The KK genotypes were preponderant in the diabetic participants (52%) compared the non-diabetic participants (7.4%). From our study, having a KK genotype increases risk of T2DM by seven fold (OR =7.03, 95% Cl: 1.59 -31.16). Similarly, Chistiakov *et al*., (2008) and Rastegari *et al*., (2015) found that people with this homozygous genotype had more risk of T2DM with P values of 0.004 and 0.016 respectively. The K allele was more predominant in the diabetic subjects (86.3%) than in the non-diabetic subjects (33.3%). An increased risk of nearly 3 fold (OR=2.59, 95% CI: 1.13 – 5.92) was found in heterozygous carriers of the K allele. In 2013, Asaf *et al*., reported that carriers of the K allele were more predisposed to T2DM provided other factors such as physical and environmental factors were present. This agrees with the findings of Rastegari *et al*., (2015) p value = 0.048 and Christiakov *et al*., (2008) p value = 0.023. Of the three genotypes the KK genotype from this study has the strongest association to risk of developing diabetes complications (p value = 0.002; OR = 12.67; 95% CI: 1.618 -99.150). This may be because substitution of the wild type E (glutamic acid) with an oppositely charged K (lysine) at position 23 in the translated protein would result in a potentially significant restructuring of the core structure and disruption of interactions with other Kir 6.2 subunits; thus providing a basis for altered high-fidelity of the $K_{ATP}$ channel, especially in the homozygous state (Yang *et al.,* 2007). Functional studies have revealed that the KK genotype markedly reduced glucose-induced β-cell insulin release by inducing spontaneous over activity of the pancreatic cells leading to an increase in the ATP concentration for insulin release (Riedel *et al*., 2003).

The frequency of the EK genotype in this study was more in the diabetic participants (34.2%) than in the non-diabetic participants (25.9%) but this is not statistically significant (p value = 0.5639). This is different from a study carried out in an Iranian population where it was observed that the frequency of the EK genotype was more in the non-diabetic subjects (p value = 0.049) (Rastegari *et al*., 2015). However, in both studies the E allele had a higher frequency in the non-diabetic patients: 92.6% for the non-diabetic participants and 47.9% for the diabetic participants (p value = 0.0552) in this study and p value of 0.048 for the Iranian study (Rastegari *et al*., 2015).

The EE genotype was found to be more in non-diabetic subjects (66.7%) than in diabetic subjects (13.7%) (p value = 0.0003). Thus the E allele carriers (OR = 0.52; 95% CI: 0.26 – 1.02) probably have a lower risk of T2DM compared with the carriers of K allele (OR = 2.59; 95% CI: 1.13 – 5.92). This is in agreement with the work of Rastegari *et al*., 2015 who that stated that the higher prevalence of the E allele in non-diabetic subjects suggests that the E allele confers on carriers a lower risk ofT2DM when compared with carriers of the K allele.

This study shows that EE (p value = 0.009; OR = 0.09; 95% CI: 0.018 – 0.479) and EK (p value = 0.0231; OR = 0.36; 95% CI: 0.214 – 1.457) genotypes show no significant association with complications of type 2 diabetes mellitus. On the other hand, the KK genotype increases risk of T2DM complications by twelve fold (p value = 0.002; OR = 12.67; 95% CI: 1.618 -99.150).

HbAlc, which is the only glycaemic variable that was statistically significant, shows no significant differences in its levels in the different genotypes (p value =0.64, Kruskal Wallis Test).

A logistic regression of diabetes status on biochemical markers, lifestyle variables and E23K genotypes showed that smoking of cigarettes is the strongest independent predictor of type 2 diabetes in this study population (p value = 0.00; OR = 3.70; 95% CI: 1.75 – 7.80). This was followed by the KK genotype (p value = 0.88; OR = 1.12; 95% CI: 0.25 – 5.11). Smoking has been demonstrated to have a clear association with increased risk of T2DM (Maddatu *et al*., 2017) and is an independent predictor of elevated HbA1c in persons with type 2 diabetes (Dinardo *et al*., 2019). However, a complete understanding of the mechanism by which underlying pancreatic β-cells pathways are impacted by tobacco abuse is lacking. A study on the effects of smoking on insulin signalling revealed that the skeletal muscle biopsy of smokers had increased Ser636 phosphorylation of IRS-1, which is known to have with negative effects on insulin sensitivity. The same study also showed that smokers exhibited decreased expression of peroxisome proliferator-activated receptor-gamma (PPAR-ɣ) a transcription factor known to promote insulin sensitivity (Bergman *et al*., 2012).

Although the data on the association of this polymorphism to the risk of developing T2DM is inconsistent, (Gloyn *et al.,* 2003; Souza *et al*., 2017; Nielson *et al.,* 2003; Koo *et al.,* 2007; Alsmadi *et al.,* 2008), this study found an association between the E23K polymorphism and the development of T2DM and this is in agreement with other studies (Zhou *et al.,* 2009; Alsmadi *et al.,* 2008; Abdelhamid *et al.,* 2014; Nielson *et al*., 2003). The inconsistency in data may be due to the failure of some studies to detect the modest impact of individual loci, aetiological heterogeneity across populations and small sample sizes (Hirschhorn *et al*., 2002; Souza *et al*., 2017).

The susceptibility of E23K allele has a modest effect (OR 1.15) on T2DM but because it is a high frequency allele it may likely contribute more to population attributable risk (Gloyn *et al.,* 2003; Souza *et al.,* 2009). In addition to the risk conferred on the population by the high frequency allele of E23K is the risk conferred by environmental and physical factors like BMI which have a higher predictive value (Souza *et al.,* 2017). A 2003 study by Nielson *et al.,* reveals an association between E23K and a higher BMI values.

Therefore, higher BMI values/obesity may account for the variations seen in the contribution of E23K to the development of T2DM in different populations.

A complex interaction between multiple genes and environmental factors is believed to be involved in the development of T2DM (Makhzoom *et al*., 2019). Single nucleotide polymorphisms (SNPs) are majorly implicated and the SNPs involved mostly affect the insulin secretion pathway (Basile *et al.,* 2014; Hagvirdizadeh *et al*., 2015; Kwak *et al*., 2016). These SNP include those within the KCNJ11 genes and the interacting genes (Souza *et al*., 2017). It has been reported that rs5219 (E23K) has a strong linkage disequilibrium with rs1799854 located on the ABCC8 gene and lysine carriers at rs5219 almost always carry alanine allele at ABCC8 rs757110 (Hagvirdizadeh *et al*., 2015). It is therefore possible that either or both of these variants produce the effects seen.

## V. **Conclusion**

A significant association between the E23K polymorphism and T2DM was found in the Nigerian population that was studied. This study also found a significant association between E23K and complications that could arise from the disease. Therefore, genotyping E23K in diabetic, prediabetic and non-diabetic individuals may be useful in genetic risk prediction, identifying the possible complications that may arise in patients with these genetic variants and in determining suitable management therapies.

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