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Identification Of Rs266729 Single Nucleotide Polymorphism In Adiponectin Promoter Region Gene Among Diabetic Patients Attending Murtala Muhammad Specialist Hospital Kano State

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Abstract:

The present study involves application of a new concept in molecular biology" Single Nucleotide Polymorphism (SNP "), which describes DNA sequence variation that occurs when a single nucleotide (adenine, thymine, cytosine, or guanine) in the genome sequence is altered (in at least 1% of the population). Specifically, identification of rs266729 SNP in Adiponectin Promoter Region Gene among T2 diabetic patients attending a specialist hospital in Kano State, Nigeria. Today, and in the very near future, DNA sequence information will be much used to establish and confirm diagnoses, to help determine treatment, and to prevent disease through pre-symptomatic identification of genetic risk. This work offered a good trial to identify the association of rs266729SNP (Single Nucleotide Polymorphism) in Type 2 Diabetic patients attending Murtala Muhammad Specialist Hospital Kano State, Nigeria. For achieving this association, the following laboratory techniques have been applied adequately: Blood sample was obtained from twenty-seven (27) participants [(Type 2 diabetic patients (14) and healthy persons (13)] DNA was extracted, followed by Polymerase Chain Reaction (PCR) of AdipQ gene promoter region. The PCR product was sequenced to identify mutation. Results from this study reveal that, the distribution of the minor allele G (OR = 6.88, P = 0.012698) of rs266729 polymorphism was found to be associated with an increased risk of T2D compared to control group. The genetic model form of association showed that the homozygous genotype (GG) and heterozygous genotype (CG) of rs266729 polymorphism were significantly associated with BMI, waist circumference and waist hip ratio. Present study revealed that the minor allele G of rs266729 constitutes a risk factor for the development of type 2 diabetes mellitus (T2DM) in patients attending Murtala Muhammad Specialist Hospital Kano State.

Key words: *Adiponectin, Type 2 diabetes, Single Nucleotide Polymorphism, Single Nucleotide Polymorphism (SNP), Adiponectin Promoter Region Gene, Type 2 Diabetes (T2D), rs266729, Genetic Risk*

1. Introduction

Diabetes mellitus (DM) is defined as a metabolic condition marked by irregularities in the metabolism of glucose, protein, and fats due to insulin deficiency, either absolute or relative, alongside organ system dysfunction[1,2]. Type 2 diabetes mellitus (T2DM) stands as the predominant form of diabetes, arising from a mix of inadequate physical activity, hereditary predispositions, and environmental influences[3,4]. According to the International Diabetes Federation (IDF), by 2015, approximately 415 million individuals worldwide were affected by diabetes, with notably high rates observed in the Middle East and North Africa[2,5]. Within Nigeria, the incidence of diabetes among people aged 20 to 69 is about 1.7%[5]. Prediabetes, characterized by raised fasting glucose levels, often serves as a precursor to the development of diabetes[6]**.** Factors contributing to the risk of developing T2DM include metabolic syndrome characterized by glucose intolerance, high blood pressure, abnormal lipid levels, and central obesity, which are all linked to insulin resistance[7]. Insulin resistance is the condition where the body's peripheral tissues become less responsive to insulin, causing disruptions in glucose metabolism that lead to T2DM [8–10].

Adiponectin, a hormone secreted by fat tissue, is vital for managing fat metabolism, glucose regulation, and inflammation[11]. Lower levels of adiponectin in the plasma are found in individuals with T2DM, metabolic syndrome,

and obesity [11,12]. The APM1 gene, which produces adiponectin, is located on chromosome 3q27 [13]. Genome-wide association studies have identified a significant genetic link to T2DM development, focusing on single nucleotide polymorphisms (SNPs) like rs266729 and rs17300539 in the ADIPOQ gene's promoter region[14,15]. These genetic variations can influence adiponectin levels, thereby affecting insulin sensitivity and playing a role in the onset of T2DM[16,17].

DM represents a major worldwide health challenge, being the fourth primary cause of death globally[18]. The promoter regions of genes, such as the ADIPOQ gene, contain polymorphic sites that might impact gene activity and the risk of developing T2DM[15,19]. Due to its properties in enhancing insulin sensitivity and reducing inflammation, adiponectin is considered a promising target for T2DM therapy[12,20]. Identifying common variants in the ADIPOQ gene could lead to the discovery of new targets for drug development in treating T2DM[15,17,21]. Yet, the relationship between ADIPOQ gene SNPs, especially rs266729, and T2DM risk is still being explored, with studies showing varying results across different ethnic groups[15,22]. Consequently, this research aims to explore the presence of SNPs in the adiponectin gene's promoter region in diabetic patients receiving care at the Murtala Muhammad Specialist Hospital in Kano.

2. MATERIAL AND METHODS

Ethical clearance

The ethical clearance for this research was obtained from Research and Ethics committee of the Ministry of Health Kano state.

Study population

This cross-sectional descriptive study employed convenient sampling techniques, involving a total of twenty-seven (27) participants. Thirteen (13) individuals diagnosed with type 2 diabetes attending the diabetic clinic of Murtala Muhammad Specialist Hospital, and fourteen (14) apparently healthy individuals were included in the study. Venous blood samples, totalling 5ml, were collected from each participant under fasting conditions (8 Hours) using a sterile 21G needle attached to a syringe. Standard venepuncture techniques were utilized, with 3ml of the collected blood transferred into an EDTA specimen bottle, while the remaining 2ml was transferred into a plain blood specimen bottle. The blood in the plain bottle was centrifuged at 3000 rpm for 5 minutes, and the resulting serum was separated for immediate assaying of fasting blood glucose levels. The EDTA blood samples intended for DNA extraction were stored in a refrigerator at -20°C for subsequent genomic analysis.

Anthropometric Parameters

Body mass index (BMI) was determined for each study participant, utilizing their weight in kilograms and divided by their height in meters squared (kg/m^2). Furthermore, measurements of waist and hip circumferences, along with the calculation of the waist-hip ratio, were conducted. Weight was accurately measured to the nearest 0.1 kg using a precisely calibrated weighing scale, and height was assessed to the nearest 0.1 cm with a stadiometer. The waist measurement was taken at the midpoint between the bottom of the last easily felt rib and the highest point of the hip bone, with subjects maintaining an upright position and normal respiration. The circumference of the hips was measured at the broadest part of the buttocks, ensuring the measuring tape was kept horizontal. The waist-hip ratio was then determined by dividing the waist measurement by the hip measurement.

Inclusion Criteria

Participants who were diagnosed of T2DM with the fasting blood glucose ≥100mg/dL were included as cases in the study. Also participants who were non-diabetic with the fasting blood glucose FBS) < 100 mg/dL were included as a control.

Exclusion Criteria

In both groups, exclusion criteria included the following: the use of diabetic medication, pregnancy, chronic kidney disease, liver problems, cancer, acute illness, autoimmune diseases, and infections. Furthermore, individuals who did not provide consent in either group were also excluded from the study.

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Genomic DNA Extraction and Genotyping

Genomic DNA was isolated from peripheral blood samples, using QIAamp DNA Blood Mini Kit Using the protocol provided by QIAGEN, USA, polymerase chain reaction (PCR) was conducted following the extraction of genomic DNA, utilizing specific primers. The primers used were: Forward for rs266729 - ACGTTGGATGATGTGTGGCTTGCAAGAACC, and Reverse - ACGTTGGATGACCTTGGACTTTCTTGGCAC. The PCR protocol included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 45 seconds (extension), with a final extension period at 72°C for 7 minutes. Following PCR, the products were evaluated through gel electrophoresis before being sequenced by the Sanger sequencing technique.

Sequence Analysis of ADIPOQ Gene Promoter Region

The Adiponectin gene's PCR-amplified fragments were sequenced using the Sanger method at Inqaba Biotech Industry (Pty), employing the Nimagen, BrilliantDye Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000, following the instructions provided by the manufacturer. Subsequently, the sequencing reactions were purified using the ZR-96 DNA Sequencing Cleanup Kit. These purified samples were then analyzed on an Applied Biosystems ABI 3500XL Genetic Analyzer equipped with a 50cm array and POP7 polymer. The analysis of the sequence chromatograms was conducted using the FinchTV software.

Identification of rs266729 using Bio-Edit software

The sequencing results were obtained in ABI chromatogram/FASTA file format, and analysis was carried out using Bio-Edit software. The chromatogram peaks were meticulously compared with the specified bases to detect the rs2266729 polymorphism, using a reference marker (CAGATCCTGCCCTTCAAAAAC) acquired from the single nucleotide polymorphism database (dbSNP).

Statistical Analysis

Statistical evaluations were performed using Stata software. The Hardy-Weinberg equilibrium for SNPs was checked using an HWE calculator. The distribution of genotypes and the frequency of alleles among control and Type 2 Diabetes (T2D) groups were analyzed using the chi-square test. The relationship between different genotypes and T2D was investigated by calculating odds ratios and 95% confidence intervals through the use of the natural logarithm. Analysis of the genetic model association was carried out with SNPStats, a web-based tool for SNP analysis (https://www.snpstats.net).

3. RESULTS

Table 1: Anthropometric characteristics of the type 2 diabetic and controls participants

Characteristics	Diabetic (n = 13)	Non Diabetic (n = 14)	P-value
Age (years)	44.67±12.86	42.13±13.19	0.454
Height (meters)	1.59±0.08	1.61±0.10	0.310
Weight (kg)	72.90±16.75	69.20±12.24	0.333
BMI (kg/m2)	28.98±6.55	26.92±5.60	0.194
WC (cm)	110.07±37.15	107.11±56.10	0.810
HC (cm)	117.70±35.54	124.55±58.20	0.585
W:H	0.91±0.05	0.85±0.11	0.014*

FBS	190.27±52.45		87.24±8.75		0.000*
SEX	**N**	**%**	**N**	**%**	
Female	8	61.5	8	57.1	0.8
Male	5	38.5	6	42.9	

Values are expressed as mean ± standard deviation. *p-value obtained by Student's t-test.

p-value obtained from $\chi 2$ test. BMI: body mass index; WC: waist circumference; HC: hip circumference; W: H: waist-to-hip ratio.

Amplification of Fragment of Adiponectin Promoter Region Using Polymerase Chain Reaction (PCR).

Twenty-seven (27) samples of genomic DNA were extracted from both diabetic and non-diabetic participants, out of which thirteen (13) is for diabetic and fourteen (14) for non-diabetic participants.

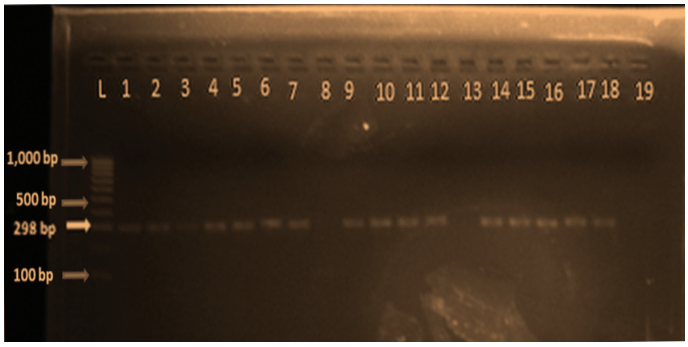

Figure1: Agarose gel electrophoresis picture showing the band of representative sample of amplified Adiponectin promoter region. L is hyperladder 100bp [100-1013bp (bioline, Biosystem)]

Sequencing products analysis of ADIPOQ rs266729 C>G polymorphism

The peaks in the chromatogram revealed the targeted single nucleotide polymorphism. Some of which are shown below.

Rs266729

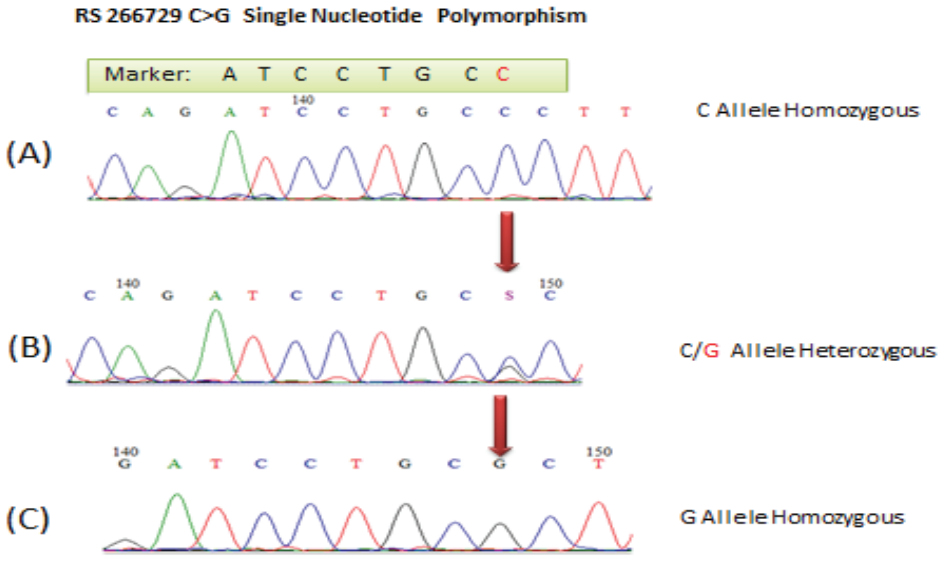

Figure 2: Investigating single nucleotide polymorphisms (SNPs) through Sanger sequencing reveals: (A) The detection of a sole "C" peak, suggesting a homozygous "A" allele. (B) The observation of both "C" and "G" peaks, signifying a heterozygous "C/G" allele. (C) A single "G" peak, indicating a homozygous "G" allele.

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Distributions of rs266729C>G Polymorphism

The distribution of rs266729 C>G polymorphism is shown in Table 1. There was no significant association concerning the genotype distribution of rs266729 between the diabetic and non-diabetic participant ($p<0.05$), suggesting no association of genotype distribution with T2D. Furthermore, the researchers found a significant association of G allele (P=0.012698) with T2D suggesting the association of allelic frequencies with T2D.

Table 2: Distribution of Rs266729 C>G polymorphism in ADIPOQ gene promoter region.

Rs266729 C>G	Diabetic (n = 13)		Non Diabetic (n = 14)		$\chi 2$	P	OR (95 % CI)
GENOTYPE	N	%	N	%			
CC	10	76.92	6	42.86			
GG	0	0	2	14.29			
CG	3	23.08	5	42.85	3.6301	0.056745	6(0.87,41.2)
Allele							
C	23	88.46	17	65.38			
G	3	11.54	9	34.62		0.012698	6.88(1.32,35.77)

Odds ratios (OR) and 95 % confidence intervals (CI) were used for the assessment of risk factors. Significance level: $p<0.05$

Association between Rs266729G>C Polymorphism and Anthropometric Parameters among Diabetic and Non-Diabetic Participants

The study examined the link between the ADIPOQ rs266729G>C polymorphism and various body measurements across all participants. Analysis of genetic models in relation to body mass index (BMI) indicated that individuals with the CG genotype had a higher likelihood of developing diabetes than those with the GG genotype (OR=0.14; 95% CI: 0.02-1.10, P=0.039). When considering the dominant model (CG+GG genotype), there was a significant correlation with BMI compared to the CC genotype (OR=0.12; 95% CI: 0.02-0.82, P=0.019), but no significant association was found for the GG genotype under the recessive model against the CG+CC genotype (OR=0.00; 95% CI: 0.00-NA, P=0.1). The over dominant model with the CG genotype did not show a significant relationship with Waist Hip Ratio against the combined GG and CC (NA) genotypes (OR=0.23; 95% CI: 0.03-1.49, P=0.1) (Table 3).

The investigation also highlighted that the CG genotype was associated with an increased diabetes risk in relation to waist circumference compared to the GG genotype (OR=0.16; 95% CI: 0.02-1.11, P=0.042). A statistically significant association was observed in the dominant model (CG+GG) when compared to the CC genotype (OR=0.12; 95% CI: 0.02-0.83, P=0.018), while the recessive model (GG genotype) did not significantly correlate with waist circumference against the CG+CC genotype (OR=0.00; 95% CI: 0.00-NA, P=0.12). The over dominant CG genotype showed no significant association with diabetes in comparison to the GG and CC (NA) genotypes (OR=0.20; 95% CI: 0.03-1.38, P=0.084) (Table 3).

Regarding waist-to-hip ratio, the CG genotype carriers were found to have an elevated risk of diabetes compared to those with the GG genotype (OR=0.14; 95% CI: 0.02-1.10, P=0.04). The dominant model (CG+GG) showed a significant relationship with waist-to-hip ratio against the CC genotype (OR=0.11; 95% CI: 0.01-0.82, P=0.017), but the recessive model (GG genotype) did not reveal a significant association compared to the CG+CC genotype (OR=0.00; 95% CI: 0.00-NA, P=0.13). The over dominant model with the CG genotype also did not exhibit a significant link with waist-to-

hip ratio against the combined GG and CC (NA) genotypes (OR=0.18; 95% CI: 0.02-1.39, P=0.075) (Table 3).

Furthermore, the analysis considering the combined effect of anthropometric measurements (BMI, waist circumference, and waist-to-hip ratio) indicated that individuals with the CG genotype were more susceptible to diabetes compared to those with the GG genotype (OR=0.13; 95% CI: 0.56-NA, P=0.048). The dominant model (CG+GG) was significantly associated with diabetes compared to the CC genotype (OR=0.11; 95% CI: 0.01-0.83, P=0.018), while the recessive model (GG genotype) did not show a significant correlation with diabetes against the CG+CC genotype (OR=0.00; 95% CI: 0.00-NA, P=0.18). The over dominant CG genotype showed no significant association with the combined anthropometric measurements against the GG and CC (NA) genotypes (OR=31.08; 95% CI: 0.35-NA, P=0.054) (Table 3).

Table 3: Association between Rs266729G>C polymorphism and anthropometric parameter

Response Status	Genetic models	OR (95% CI)	p-value
Body Mass Index	Co-dominant CC vs. (CG)	0.16 (0.02-1.14)	0.039*
	Dominant (CG+GG) vs. CC	0.12 (0.02-0.82)	0.019*
	Recessive GG vs. (CG+GG)	NA	0.1
	Over-dominant CG vs. (CC+GG)	0.23 (0.03-1.49)	0.1
Waist Circumference	Co-dominant CC vs. (CG)	0.16 (0.02-1.11)	0.042*
	Dominant (CG+GG) vs. CC	0.12 (0.02-0.83)	0.019*
	Recessive GG vs. (CG+GG)	0.00 (0.00-NA)	0.12
	Over-dominant CG vs. (CC+GG)	0.20 (0.03-1.38)	0.084
Waist Hip Ratio	Co-dominant CC vs. (CG)	0.14 (0.02-1.10)	0.04*
	Dominant (CG+GG) vs. CC	0.11 (0.01-0.82)	0.017*
	Recessive GG vs. (CG+GG)	0.00 (0.00-NA)	0.13
	Over-dominant CG vs. (CC+GG)	0.18 (0.02-1.39)	0.075
BMI,WHR and WSC	Co-dominant CC vs. (CG)	0.13 (0.02-1.07)	0.048*
	Dominant (CG+GG) vs. CC	0.11 (0.01-0.83)	0.018*
	Recessive GG vs. (CG+GG)	0.00 (0.00-NA)	0.18
	Over-dominant CG vs. (CC+GG)	0.15 (0.02-1.26)	0.054

BMI: Body Mass Index, WHR: Waist Hip Ratio, WSC: Waist Circumference, OR: Odds ratio, CI: Confidence intervals, NA: Not available, p-values: *p<0.05*, **p<0.01 and ***p<0.001 refer as significant

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4. DISCUSSION

The present study identifies rs266729C>G single nucleotide polymorphism in adiponectin promoter region and its associations in relation to BMI, Waist circumference and Waist Hip Ratio between diabetic and non-diabetic participants attending Murtala Muhammad Specialist Hospital Kano State. Adiponectin is essential for enhancing insulin sensitivity, managing energy expenditure, and promoting the oxidation of fatty acids, among various other metabolic functions[23]. The relationship between the prevalence of genetic variation (polymorphism) and the effect of anthropometric parameters on the development of T2D has been widely studied in different geographical location. The visual depiction of the sequenced product shown in Figure 1 illustrates different indicative peak of both the homozygous and heterozygous genotypes and this may be inferred as a two possible nucleotide variation found in a specific location within the genome commonly known as an allele (alternative form of a gene). This two possible nucleotide substitution maybe considered as single nucleotide variation (SNVs) commonly known as single nucleotide polymorphism (SNP) if found present in greater than one percent (1%) of the population. The genetic underpinnings and mechanisms leading to T2D remain largely uncharted, with SNPs identified as key factors potentially elevating the risk of this condition. SNPs serve as a window into understanding variability in disease susceptibility across populations.

This research discovered a significant link (p=0.012698) regarding the prevalence of the minor allele G in the rs266729C>G mutation, as indicated in Table 2, between those with and without diabetes. Notably, the minor allele G of rs266729 was found to be more prevalent in the control group, with a frequency of 34.62%, compared to only 11.54% among the diabetic group. This observation is consistent with findings from [24], who studied the connection between rs266729 and gestational diabetes using a gene chip method. It is theorized that the minor allele G of rs266729 might lead to a reduction in AdipQ expression, potentially making the gene's promoter less susceptible to the inhibitory effects typically seen in diabetes, such as blocking the SP3 suppressor's binding. This theory is supported by numerous studies linking the minor allele G to higher adiponectin levels, suggesting a protective role that could account for its increased occurrence among non-diabetic individuals, even though it is associated with a higher risk of T2D. Consequently, these results corroborate the hypothesis that the rs266729 polymorphism in the adiponectin gene might be a contributing risk factor for T2D among patients seen at the Murtala Muhammad Specialist Hospital in Kano State, Nigeria.

Furthermore, this research examined the association between genetic variations in the ADIPOQ gene and measures such as BMI, waist circumference, and waist-to-hip ratio, revealing significant correlations with the rs266729 C>G polymorphism as detailed in Table 3. The analysis demonstrated that the homozygous (GG) and heterozygous (CG) genotypes of the rs266729 polymorphism were significantly associated with BMI under both codominant (OR= 0.16; 95% CI: 0.02-1.14; p= 0.039) and dominant (OR=0.12; 95% CI: 0.02-0.82; p=0.019) genetic models. Similar significant associations were observed with waist circumference and waist-to-hip ratio under respective genetic models, suggesting this biomarker's involvement in obesity and abdominal fat accumulation, potentially contributing to the pathogenesis of diabetes. This supports the notion that abdominal adiposity may influence the effects of other genetic variations[25]. The findings are consistent with research conducted in various populations, including studies in Asian[26], French Caucasian[27,28], Swedish Caucasian[29,30], and Chinese populations[31,32], while research in Indian[33], Hispanic, African-American[34,35], and other Chinese cohorts[36,37] reported no significant associations with this SNP. The discrepancies in findings across studies might be attributed to the interplay of additional genes, geographical differences, dietary habits, and other factors.

T2DM is recognized as a condition with a polygenic and multifactorial origin, suggesting that anomalies in the ADIPOQ gene's promoter region, such as the rs266729 polymorphism, may either directly contribute to or exacerbate

the risk of developing T2D through interactions with environmental or other genetic factors.

Further research is essential to translate this genetic knowledge with increased sample size into practical benefits for patients, with the most significant gains likely stemming from novel and improved therapeutic approaches based on a deeper understanding of the disease's aetiology. The overarching challenge remains to delineate the roles of numerous genetic and environmental factors, along with their interactions, in the pathogenesis of T2DM, paving the way for comprehensive strategies for prevention, management, and treatment.

Limitation of the Study

One limitation of our study is the relatively small sample size. While the researchers acknowledge that a larger sample size would have provided more statistical power and increased generalizability of their findings, it is important to note that this study was designed as a pilot investigation into the implications of SNPs in their target population.

The decision to start with a smaller sample size was deliberate, as it allowed the researchers to explore initial trends and gather preliminary data on the relationship between SNPs and the outcome variables of interest. This approach aligns with the exploratory nature of the present research and provides a foundation for future studies with larger sample sizes.

Despite this limitation, the study at hand provides valuable insights into the potential associations between SNPs and the outcomes under investigation. Moving forward, the researchers plan to expand their participant pool to further validate and build upon the findings reported in this pilot study.

5. Conclusion

The study showed that the minor allele G of the ADIPQ Rs266729 C>G polymorphism may constitute a valuable risk factor for the development T2D (OR = 6.88, P = 0.012698). Moreover, Waist hip ratio showed a significant association with T2D (P=0.014) and it can serve as an important applicable laboratory predictor of T2D.

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