

Impact of Peroxisome Proliferator-Activated Receptor Gamma SNP C161T gene on Lipid Parameters Linked to Diabetes Mellitus in Cotonou (Benin)

Nicodème W. Chabi^{1,2,*}, Martin N. CAKPO AKLAN³, Adé M. Dominique SANNI², Christian T. R. KONFO⁴,
Jonas SOSSA-HIFFO², Daniel K. AMOUSSOU-GUENOU⁵, DAHOUENON-AHOUSSE Edwige⁴,
BABA-MOUSSA Lamine⁶

¹Laboratory of Teaching and Research in Food Microbiology, Department of Food Technology Engineering, Polytechnic School of Abomey, University of Abomey, 01P.BP 2009 Cotonou, Benin

²Laboratory of Biochemistry and Molecular Biology, Department of Biochemistry and Cell Biology, Faculty of Science and Technology (FAST), University of Abomey-Calavi, Cotonou, Benin

³Pharmacy Training and Research Unit, Faculty of Health Sciences, University of Abomey-Calavi, Cotonou, Benin

⁴Laboratory of Study and Research in Applied Chemistry, Polytechnic School of Abomey, University of Abomey-calavi, 01 BP 2009 Cotonou, Benin

⁵Department of Endocrinology-Metabolism, National Hospitals University Center, Faculty of Health Sciences, University of Abomey-Calavi, Cotonou, Benin

⁶Laboratory of Biology and Molecular Typing in Microbiology, Faculty of Science and Technology (FAST), University of Abomey-Calavi, 05 BP 1604, Cotonou, Benin

*Corresponding author: nicodeme.chabi@gmail.com

Abstract Diabetes is one of the most prevalent metabolic diseases worldwide. Prevent or delay the onset of diabetes may be the better management of this condition. It is in this context that we have chosen to investigate the genes involved in the homeostasis of blood sugar and lipids. The purpose of this study was to examine the effects of the C161T polymorphism Peroxisome proliferator-activated receptor gamma (PPAR γ) on lipid parameters in Benin subjects (n=41) diagnosed with type 2 diabetes and controls (n=8). Anthropometric data in consideration were: body mass index (BMI), height, weight, waist circumference, hip turn. The biochemical parameters in consideration were: fasting glucose, total cholesterol (TC), HDL-cholesterol and LDL-cholesterol, triglycerides (TG) and total protein. Genomic DNA was obtained from white blood cells present in the buffy coats of type 2 diabetic subjects and controls. The mutations analysis in the coding region of PPAR gene was carried out by PCR assay while the C161T polymorphism was analysed by PCR-RFLP. It is clear that obesity was a prognostic factor for type 2 diabetes ($p=0.05$). Using the correlation test, on one hand we observed a correlation between BMI, sex, height and weight, respectively, $p=0.051$; $p<0.001$; $p<0.001$; and secondly a correlation between BMI and lipid parameters, $p=0.012$. The frequency of the wild allele " C " of the C161 polymorphism was 63.27% and that of the allele transferred " T " (T161) was 36.73%. No significant difference was observed between the SNP C161T and lipid variables TC, TG, HDL-C, LDL-C ($p>0.05$). However, we observed a low level of TG in carriers " TT " than for " CT " and "CT +TT" (0.17g / L TT vs 0.73g / L CT vs 0.69g / CT + TT). The results show that the C161T polymorphism in the PPAR γ gene is not associated with blood lipid variables in type 2 diabetes of the study population. However, a reduction of triglycerides in serum was observed in individuals carrying the variant " TT " C161T polymorphism of the PPAR γ gene.

Keywords: PPAR γ , PCR-RFLP, SNP C161T, diabetes mellitus, blood lipid parameters, BMI

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1. Introduction

Non-communicable diseases are chronic conditions slow growing. They include four main types of diseases: cardiovascular diseases (heart attacks and strokes), cancer,

chronic respiratory diseases (chronic obstructive pulmonary disease and asthma) and diabetes mellitus. They are the leading cause of death worldwide: 63% of the world's annual deaths are due to these diseases [1].

Diabetes mellitus results from a chronic metabolic disorder of carbohydrates, lipids and proteins due to a relative or absolute deficit in insulin secretion and / or

action [2,3]. This is a constellation of metabolic and pathological abnormalities with a variety of environmental and hereditary causes [3]. Diabetes is one of the most common non-communicable spread throughout the world; its current prevalence in the world is around 347 million people [1].

In sub-Saharan Africa, epidemiological data for individual countries often vary due to socio-cultural differences and variations between urban and rural settings and variations in diagnostic methods used in the various surveys. Like all over the world, type 2 diabetes is the predominant form in sub-Saharan Africa with more than 90% of diabetes cases [4].

There are two main types of diabetes mellitus: type 1 diabetes and type 2 diabetes. Type 1 diabetes is characterized by insufficient insulin secretion due to the destruction of β cells of the pancreas islets of Langerhans; its signs may already appear from childhood. Type 2 diabetes, on the other hand, is characterized by chronic hyperglycaemia due to poor use of glucose in peripheral tissues using glucose [1]. Diabetes mellitus has become a public health problem today. Indeed, its prevalence is growing rapidly in the world: for all age groups it was estimated at 2.8% in 2000 (171 million people) with a projection in 2030 of 4.4% (366 million people). In Benin, this prevalence increased from 1.1% in 2001 [5] to 2.6% in 2008 [6]. Particular in Cotonou, its prevalence increased from 3.3% in 2002 to 4.6% in 2009 [7]. Type 2 diabetes alone accounts for 90% of all diagnosed diabetes cases. It has an insidious character, its cardio-vascular, renal and ocular complications are serious and its taken care of is for life and very expensive [8]. Preventing or delaying the onset of type 2 diabetes will then constitute the best management of this condition. It is in this context that we have chosen to investigate genes involved in the homeostasis of blood glucose and lipids. Insulin resistance is the first essential defect characteristic of type 2 diabetes. Its installation is correlated with several factors including the increase of the free fatty acids circulating in the blood and the accumulation of lipids in non-adipose tissues (liver and muscle). The stimulation of PPAR γ by its ligands improves this situation by reorienting the storage of fatty acids in adipose deposits and the regulation of hormones secreted by adipocytes which have an impact on glucose homeostasis [9]. This study focused on assessing the impact of the C161T polymorphism of the Peroxisome Proliferator-Activated Receptor Gamma gene on the risk of type 2 diabetes and lipid variables associated with type 2 diabetes in Cotonou (Benin). The objective of this study was to evaluate the impact of the C161T polymorphism of the Peroxisome Proliferator - Activated Receptor gene on the lipid variables associated with type 2 diabetes in Cotonou.

2. Materials and Methods

2.1. Study Population

The study included Type 2 diabetics, male and female, with a fasting blood glucose level of 1.26 g / L or over, aged 25 or more. Non-diabetics, male and female, with a fasting glucose level greater than or equal to 0.7 g / L and less than or equal to 1.1 g / L, an age greater than or equal to 25 years.

We did not include in our study pregnant women, Subjects under 25 years of age, Type 1 diabetics.

All participants in this study have signed an informed consent form and the ethics committee has validated the research protocol under the number N° 5049/MS/DC/SGM/DRF/SA.

In total, our sample consisted of 49 participants: 41 type 2 diabetics and 8 non-diabetics.

2.2. Processing of Biological Material

The procedure of samples analysis was divided into three main parts: biochemical parameter determination, DNA extraction and techniques applied to DNA amplification (PCR).

After collection, the samples were immediately centrifuged. The plasma was collected in two Eppendorf tubes and used for the determination of biochemical parameters (blood glucose, total cholesterol, HDL-cholesterol, triglycerides, LDL-cholesterol and total proteins). The leukocyte layer was recovered and washed twice with 1X PBS. It was then distributed in two Eppendorf tubes and will be used for DNA extraction. After DNA extraction, the PCR was made to amplify the PPAR γ gene. The amplicons obtained was then digested with the restriction enzyme EcoR 72I and the digested products were visualized on UV table.

All biochemical parameters were determined using ELITECH Clinical Systems SAS.

2.3. Biochemical Parameters

2.3.1. Blood Glucose Assay with Glucose Oxidase

Blood glucose was performed by colorimetric assay. From the stock glucose solution at 5.5 mmol / L, diluted solutions were prepared at 1/2, 1/4, 1/8, 1/16 and 1/32. Then, 30 mL of each solution of glucose were added. Distilled water was used as control. Tubes were kept in a water bath for 10 min at 37°C before measuring the absorbance with spectrophotometer at 500 nm. The standard curve $A = f([\text{glucose}])$ was plotted. The same procedure was performed with the serum to obtain blood glucose. Concentration was calculated with the formula:

$$C = (\text{OD sample}) / (\text{OD standard}) \times C \text{ standard.}$$

2.3.2. Triglycerides (TG) Assay

Triglycerides were assayed according to the method reported by [10]. The glycerol released after hydrolysis of triglycerides by lipoprotein lipase was converted to glycerol-3-phosphate glycerokinase. Glycerol 3-phosphate was subjected to the action of the glycerol phosphate oxidase to form dehydroxyacetone phosphate and hydrogen peroxide. The latter in presence of peroxidase oxidizes a chromogen group 4- aminoantipyrine / phenol to form a coloured compound in red. Absorbance was read at 500 nm.

2.3.3. Triglycerides Assay

Cholesterol esters were hydrolysed by a cholesterol ester hydrolase into fatty acid and cholesterol. Both were then oxidized by cholesterol oxidase to A4-cholestenone

and hydrogen peroxide. The latter, in the presence of peroxidase, oxidizes the chromogen 4-amino-antipyrine in a red coloured compound of maximum absorbance at 505 nm.

2.3.4. HDL-Cholesterol Assay

LDL-cholesterol was precipitated by a haemolysis tube in which 50 µl of reagent A (ELITECH Kit) and 500µL of serum were introduced. The sample was mixed by vortex and let for 10 minutes and then centrifuged at 4000 rpm/min for 15 min. After centrifugation, the cholesterol associated with high density lipoprotein (HDL)-cholesterol was assayed in 50 µl of supernatant which was added to 1000µL of reagent A (ELITECH Kit). The optical density of the mixture was read with spectrophotometer at 500 nm after 5min of incubation at 37°C. LDL-cholesterol was estimated by the following formula: LDL-cholesterol (g / L) = total cholesterol (g / L) -HDL-cholesterol (g / L) - triglycerides (g / L) / 5 if triglycerides are less than 3.4 g/L.

2.3.5. Protein Assay

Protein assay was performed by the Biuret method as reported by [11]. In alkaline solution, the proteins form with the cupric ions a coloured complex with absorbance measured at 540 nm. The determination of different concentrations was done using a calibration curve of human serum albumin.

2.4. DNA Extraction

DNA extraction was performed by the conventional phenol-chloroform protocol which involves five (05) steps: leukocyte recovery, cell treatment, DNA extraction by Protein Separation, Nucleic Acid Precipitation and DNA Retention [12].

2.5. Amplification of the PPAR Gamma Gene by PCR

The human PPAR gamma gene was amplified by the pair of primers [13]:

Primer forward: 5'-CAA GAC AAC CTG CTA CAA GC-3'

Primer reverse: 5'-TCC TTG TAG ATC TCC TGC AG-3'

The reaction medium was composed of reagents as: 10X PCR buffer, 50 mM MgCl₂, DNTP 200 µM, Primer forward 10 µM, Primer reverse, 10 µM, Taq 5U / µL and distilled water in total volume of 20 µL.

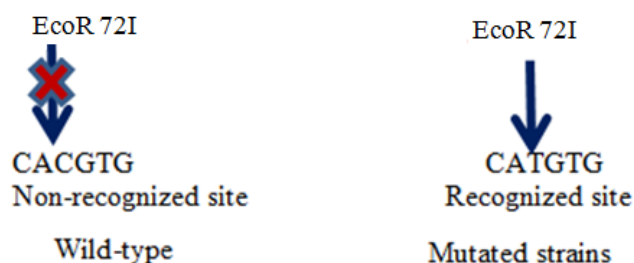
The samples were subjected to initial denaturation at 94°C for 5 minutes followed by 35 cycles, each cycle consisting of 30 seconds denaturation at 94°C., 30 seconds hybridization of Primers at 56 ° C and 1 minute extension at 72°C. The final extension was performed at 72°C. for 5 minutes [13,14].

To verify the amplification of PPAR γ gene, electrophoresis (1% agarose gel) followed by revelation (on a UV table) of the DNA amplicons which was carried out. The amplicons digestion (at 37°C/2hours) of the exon 6 of the PPAR γ gene was then carried out by the restriction enzyme EcoR 72I followed by electrophoresis (in 2.5% agarose gel) and the restriction fragments were visualized on the UV table.

The primers used to amplify a portion of exon 6 of the gene PPAR γ gave fragments of 200 pb. The expected results after digestion of the amplicons by EcoR 72I were as follows:

- In wide homozygotes CC we will have two fragments of different size: 120 and 80 pb;
- In the heterozygous CT mutants there will be three fragments of 200, 120 and 80 pb;
- In homozygous TT mutants there will be a single fragment of 200 pb [13].

The length of exon 6 of the PPAR γ gene is 249 pb. The nucleotide sequence of exon 6 from 159th to 164th is CACGTG. This sequence is not recognized by the enzyme EcoR 72I. But, the mutation (SNP C161T) of this sequence reveals the EcoR 72I recognition site. After digestion, the genotypic results will give three groups: wild-type (non-mutated) homozygous CC strains (CACGTG), mutated strains (CATGTG) homozygotes TT and CT heterozygotes [13]. Representation below had showed the recognition site of EcoR 72I on the exon 6 of the PPAR γ gene.



2.6. Statistical Analysis

The values of biochemical parameters were presented through histograms. The Chi2 test was used to assess the distribution of qualitative variables in the diabetic or non-diabetic groups. The Mann-Whitney test was used to make ANOVA analysis of each biochemical parameter. The figures were made using GraphPad Prism 5.0. All statistical analysis were conducted using Stata software version 12.0. The significance threshold was 5%.

3. Results

3.1. Socio-demographic, Clinical and Biological Characteristics of the Subjects

3.1.1. Distribution of Subjects by Sex

49 participants including 41 diabetics and 8 non-diabetics were enrolled in the study. 58.54% were female and 41.46% were male (Table 1).

Table 1. Distribution of subjects by sex

Sex	Non-diabetics (8)	Diabetics (41)
Female (%)	62.5	58.54
Male (%)	37.5	41.46
Total (%)	100	100

3.1.2. Distribution of Subjects by Body Mass Index (BMI)

For diabetics, 39.2% had a normal weight, 34.15% were overweight and 19.2% were obese. On the other hand, in non-diabetics, 50% had a normal weight, 25% were overweight and 12.5% were obese (Figure 1).

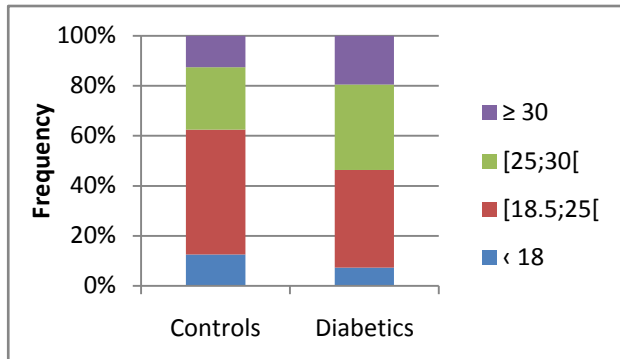


Figure 1. Distribution of subjects by BMI (BMI in kg / m²: <18.5 = lean; [18.5; 24[Normal weight; [25; 30 [= overweight; ≥ 30 = obesity)

3.1.3. Clinical and Biological Characteristics of the Study Population

Table 2 shows that only the waist circumference, hip circumference and BMI have a statistically significant difference respectively: $p = 0.0466$; $P = 0.0437$ and $p = 0.05$ between controls and patients. Regarding the biological characteristics only the glycaemia showed a statistically significant difference between controls and patients: $p = 0.0005$.

Table 2. Clinical and biological characteristics of the study population

Variables	Population		p-value
	Controls (n= 8)	Diabetics (n= 41)	
	Mean ±SD	Mean±SD	
Age (year)	46 ± 6.77	56 ± 1.85	.1262
Weight (kg)	66.75± 6.30	68.65± 1.95	.9784
Height (cm)	162.25 ±2.44	161.83± 2.14	.1262
Waist (cm)	91.62± 5.83	98.87± 1.63	.0466
Hip circumference (cm)	97.37± 5.06	105.65± 1.76	.0437
BMI (kg · m ²)	25.29± 2.24	27.03± 1.5	.0501
Glycemia (g/L)	.76±.04	1.58±.1	.0005
Total Cholesterol (g/L)	1.85±.23	1.68± .05	.6849
HDL-Cholesterol (g/L)	.50± .07	.46± .02	.3104
LDL-Cholesterol (g/L)	1.13± .02	1.07± .05	.8077
Triglycerides (g/L)	1.04± .18	.67± .05	.0601
Proteins (g/L)	75.3± 1.6	72.11± 1.11	.1516

3.2. Distribution of SNP C161T in Diabetics and Non-diabetics

3.2.1. PCR of Exon 6 of the PPAR γ Gene

After amplification, the products obtained were revealed by electrophoresis on a 1% agarose gel (Figure 2).

Amplicons of 200 pb in size for samples 1 and 5 to 10. No strips were obtained for samples 2, 3 and 4.

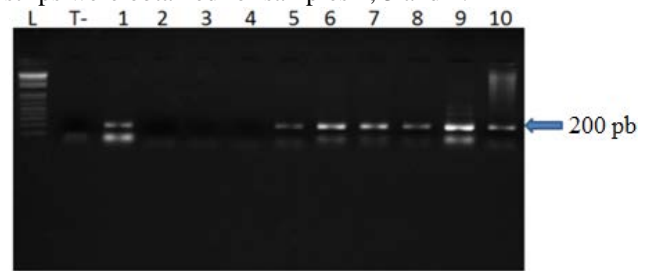


Figure 2. PPAR γ gene amplicons (1-10). (T-: Control, L: Ladder)

3.2.2. Fragments Obtained after the Digestion of PPAR γ Gene Amplicons on Exon 6 by EcoR 72I

Eighteen (18) mutations were obtained; 2 in controls and 16 in diabetics. In fact, the amplicons subjected to the digestion of EcoR 72I appear after electrophoresis at the position 200 bp; 120 bp and 80 bp as shown in Figure 3. In Table 3, we presented the distribution of mutation of PPAR γ gene

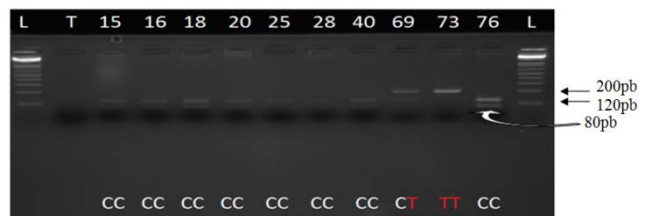


Figure 3. Fragments obtained after digestion of the amplicons by EcoR 72I (CC: 120 and 80pb; CT: 200, 120 and 80pb; TT: 200pb)

Table 3. Distribution of mutation C161T between diabetics and non-diabetics

	Non diabetics (n=8)	Diabetics (n=41)
CC	6 (75%)	25 (60.98%)
CT	2(25%)	15(36.59%)
TT	0	1(2.43%)
CT+TT	2(25%)	16(39.02%)

3.3. Clinical and Biological Characteristics of Diabetics by Genotype

There was no significant statistical difference between clinical and biological characteristics and diabetic genotypes ($p > 0.05$).

3.4. Correlation between PPAR γ Polymorphism and Clinico-biological Parameters in Diabetics

In linear regression (Table 5), considering body mass index (BMI) as an obesity-dependent parameter with clinico-biological parameters, firstly results showed a correlation between BMI, sex, height and weight was found, respectively: $p = 0.05$; $p < 0.00$; $p < 0.00$. Secondly, correlation was found between BMI and lipid parameters (total cholesterol, HDL-cholesterol, triglycerides, LDL-cholesterol) $p = 0.01$.

Table 4. Biometric, lipoprotein and glycaemic distribution in relation to polymorphism C161T of the PPAR γ gene related to type 2 diabetes

Groups	PPAR γ genotypes			
	CC	CT	TT	CT+TT
Variables	n=25	n=15	n=1	n=16
Age (year)	55 \pm 2.42	56 \pm 3.04	69	56 \pm 3
Weight (kg)	67 \pm 2.19	71.06 \pm 3.90	74	71.25 \pm 3.65
Height (cm)	163.48 \pm 1.91	159.4 \pm 4.95	157	159.25 \pm 4.64
Waist (cm)	98.84 \pm 2.17	98 \pm 2.50	113	98.93 \pm 2.51
Hip circumference (cm)	104 \pm 2.30	107.46 \pm 2.75	120	108.25 \pm 2.69
BMI (kg \cdot m 2)	25.41 \pm 0.90	29.53 \pm 4.01	30.03	29.56 \pm 3.75
Glycaemia (g/L)	1.50 \pm .16	1.76 \pm .26	.946	1.70 \pm .23
Total Cholesterol (g/L)	1.63 \pm .08	1.73 \pm .08	2.10	1.76 \pm .08
HDL-Cholesterol (g/L)	.45 \pm .03	.50 \pm .03	.31	.49 \pm .03
LDL-Cholesterol (g/L)	1.04 \pm .07	1.08 \pm .08	1.74	1.12 \pm .08
Triglycerides (g/L)	.66 \pm .07	.73 \pm .06	.17	.69 \pm .07
Proteins (g/L)	72.23 \pm 1.65	72.01 \pm 1.39	71.93	72.55 \pm 1.30

Table 5. Correlation between BMI, clinical and lipid parameters of diabetics

BMI	Coef.	Std. Err.	t	P> t	[95% CI]	
Sex	2.69	1.33	9.73	0.05	-.010	107.924
Height	-.59	.04	-11.91	0.00	-.695	-.492
Weight	.43	.04	9.48	0.00	.344	.532
Total Cholesterol	3093.85	1167.97	2.65	0.01	717.59	5470.10
HDL-Cholesterol	-3088.95	1167.10	-2.65	0.01	-5463.45	-714.46
Triglycerides (TG)	-619.05	233.98	-2.65	0.01	-1095.06	-143.01
LDL-Cholesterol	-3094.81	1168.13	-2.65	0.01	-5471.40	-718.22

Table 6. Correlation between PPAR γ , lipid parameters and BMI

PPAR γ	Coef.	Std. Err.	t	P> t	[95% C.I.]	
BMI	.002	.009	0.30	0.76	-.01	.02
T-chol	90.61	179.44	0.52	0.60	-261.87	443.09
Hdl-chol	-90.08	173.29	-0.52	0.60	-442.26	262.09
TG	-18.05	34.74	-0.52	0.60	-88.65	52.55
Ldl-chol	-90.50	173.46	-0.52	0.60	-443.02	262.02

The analysis of linear regression does not show a correlation between PPAR γ and lipid parameters and BMI as a factor of obesity (Table 6).

4. Discussion

4.1. Socio-demographic Characteristics of Participants in the Study

In our study, the age group most represented in diabetics was \geq 55 years (53.66%). These results are similar to those of [15] at Benin in 2011, which found a predominance of the age group 50-69 in a population of 41 diabetics in a proportion of 63.5%. The same results were obtained by [16], in Mali who found a predominance of the age group 50-60 years in a population of 45 diabetics in 48.9%.

In addition, 80.49% of diabetics were at least 45 years of age. This shows the aging of the population like reported by [17].

58.54% of diabetics were female. This feminine predominance was also found by [15] in Benin (53.63%). However, some studies had found a male predominance in 2005 (57.40%) [18]. These different results could be in the basis of their studies population in one hand, or the year's studies in the other hand.

4.2. Overweight and Obesity

34.15% of the diabetics were overweight (BMI in kg/m 2 [25,30] compared to 25% in non-diabetics. Obesity has been confirmed as a risk factor for type 2 diabetes ($p = 0.05$). Indeed, 19.2% of diabetics compared with 12.5% of non-diabetics were obese (BMI in kg / m 2 \geq 30).

4.3. Relationship between the SNP C161T and Type 2 Diabetes

This study is the first one to evaluate the impact of the C161T polymorphism of the PPAR γ gene on the risk of type 2 diabetes in diabetic patients and control subjects in

Republic of Benin. It included 41 diabetics and 8 diabetics. Results showed that the frequency of the wild-type 'C' allele of the C161T polymorphism was 63.27% and the mutated allele "T" was 36.73%. This corresponds perfectly to the work done by [13] in China who found 78.7% of wild-type allele "C" and 21.3% for the mutated allele "T". This was also the case in Asia [19] and in Brazil [20]; all found the mutated **C161T** allele in their study population.

Other groups revealed that the allele "T" is not associated with the occurrence of type 2 diabetes, but it would have an influence on lipid parameters. In addition, some have shown that the allele "T" would reduce the risk of type 2 diabetes.

Indeed, in China, [13] reported that the allele "T" was found with frequencies of 36.1% and 38.2% respectively in type 2 diabetic patients and non-diabetics, indicating that this mutation is not a predisposing factor for type 2 diabetes.

However, in Asia, [19] reported that SNP C161T decreased the risk of type 2 diabetes. They found the **C161T** allele at a frequency of 42.1% and 34.8% respectively in diabetics and non-diabetics.

What could explain this contradiction?

We can say that the size of the study population would be the main element that would justify the contradiction observed. Indeed, [19] investigated 450 diabetics and 3080 non-diabetics from China, Malaysia and India. As regards to the study of [13], they had worked on 86 diabetics and 89 non-diabetics, all from China. However, [20] studied 207 diabetics and 170 non-diabetics from Portugal, Italy, India, Japan and Africa.

4.4. Relationship between SNP C161T and the Lipid Parameters of Type 2 Diabetics

No statistically significant difference was obtained between the SNP C161T and the lipid variables CT, TG, C-HDL, C-LDL ($p > 0.05$). This was the case in several studies where the researchers did not find an association between the C161T polymorphism of the PPAR γ gene and the serum lipid levels in the different populations. As [21] who did their studies on Asian and non-Asian, [22] on a Brazilian population reported that C161T of the PPAR γ gene was not associated with lipid variables related to type 2 diabetes.

However, other studies tried to show that the TT genotype group had higher levels of TG than the CT and CT + TT genotype groups of the C161T polymorphism [23]. The results of our work showed that the level of TG is lower in the TT carriers than CT and CT + TT (0.17g/L TT vs 0.73 g / L CT vs 0.69g / L CT + TT).

Indeed some authors carried out a retrospective analysis on the studies made on the C161T polymorphisms of the PPAR γ gene; and the participants in these different studies had the same geographic location and practically identical food habits [23]. Thus the diet could be an integral part of these inconsistencies.

5. Conclusion

Diabetes mellitus is increased in the Beninese population, especially type 2 diabetes. The PPAR γ gene is the

essential transcription factor for the regulation of glucose genes and lipid metabolism. So PPAR γ is one of the potential candidate genes for the link between diabetes mellitus and lipid changes. The polymorphism C161T of the PPAR γ gene is one of the mutations that would influence the occurrence of type 2 diabetes and the modification of the lipid parameters associated with it. To assess the state of this in the population of Cotonou, we carried out a case-control study.

Finally, we can conclude that obesity is a risk factor for type 2 diabetes in our study population.

Analysis of the genetic variants of PPAR γ (C161T) allowed to define that the C161T polymorphism is present in the study population.

The absence of a correlation between the polymorphism C161T of PPAR γ gene and the lipid parameters related to the type 2 diabetes showed that this mutation would have no influence on the variables related to type 2 diabetes in the population study.

However, the results of our work showed an influence on lipid parameters of the TT carriers which decreased the level of triglycerides.

For the future, it would be better to increase the population's study in order to observe clearly these inconsistencies which had showed in our work.

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