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Research Paper

FREQUENCY OF DISTRIBUTION OF APOLIPOPROTEIN B 100 ECoRI GENE POLYMORPHISM AND ITS RELATION TO LIPID PROFILE AND INSULIN RESISTANCE IN OBESE EGYPTIAN CHILDREN COHORT

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Abstract

The prevalence of obesity in childhood is an important public health issue throughout the world. There are several known Apo B-100 polymorphisms claimed to be associated with obesity, hyperlipidemia and cardiovascular diseases. Study the frequency of distribution of Apo B restriction fragment length polymorphism (ECoRI) on lipids, lipoprotein (a) concentrations and BMI in obese Egyptian children, and its possible relation to insulin resistance. Forty obese children and twenty age and sex matched children were included in the present study as a control group. All subjects were subjected to clinical examination and anthropometric measurement. Fasting serum lipids were assayed by routine techniques on Synchron systems. Serum fasting insulin and serum lipoprotein (a) were assayed by ELISA technique. BMI and HOMA-IR were calculated. DNA was extracted from whole blood, amplified and then Apo B 100 ECoRI polymorphism was assayed using PCR-RFLP. All the control group children carried E-/E- polymorphism in addition to only one obese child (35%). E+/E+ was present in 36.67% while heterozygous E+/ E- polymorphism was found in 28.33% of the children. Both the E+/E- and the E+/E+ groups showed a statistically significant higher levels of FBS, fasting insulin, HOMA-IR, total cholesterol, LDL cholesterol, triglycerides and lipoprotein B when compared to the E-/E- separately. The E-/E- group showed an HDL level higher than the other two groups. There is a great association between Apo B 100 ECoRI gene polymorphism and obesity in Egyptian children. Moreover, there is a cumulative association of positive ECoRI genes with atherogenic hyperlipidemia and insulin resistance in this population.

Key words: Obesity, ApoB-100 ECoRI polymorphism, lipoprotein (a) ,hyperlipidemia , insulin resistance.

INTRODUCTION

Obesity is considered a pandemic health hazard. Childhood overweight is associated with a variety of adverse consequence, such as elevated blood pressure or serum insulin levels or dyslipidemia [4].It affects not only developed countries, but also developing countries. Approximately 22 million children under 5 years of age are overweight across the world [9). In Egypt, the number of overweight children and adolescents has doubled in the last two to three

decades, and similar doubling rates are being observed worldwide, including the developing countries [30].

The percentage of children aged 6–11 years in the United States who were obese increased from 7% in 1980 to nearly 18% in 2012. Similarly, the percentage of adolescents aged 12–19 years who were obese increased from 5% to nearly 21% over the same period.1, 2[15,16].

It has been long known that obesity is associated with premature death as obesity increases the risk of a number of diseases including the two major killers; cardiovascular disease and cancer. It is estimated that, on average, obesity reduces life expectancy between 3 and 13 years [8, 22].

Both nutritional and genetic factors are considered to be risk factors for dyslipidemia, overweight and obesity in childhood leading to CVD. In addition, one of the most closely monitored genes which may affect lipid metabolism is the gene for Apolipoprotein B (Apo B) [17, 27].

Lipoprotein (a) [Lp (a)] is a separate class of lipoproteins. Although its physiological function remains unknown, the correlation between raised levels of Lp (a) with increased risk of ischaemic heart disease is well documented. Several attempts have been made to estimate the influence of diet and physical exercise on serum LP (a) levels, yet the results are conflicting and inconclusive [12] .

Apo B is a large, amphipathic glycoprotein playing a central role in human lipoprotein metabolism and is coded by Apo B gene located on chromosome 2. One of the two Apo B forms is Apo B 100, which is required for very low-density lipoprotein (VLDL) production in the liver. In addition to being an essential structural component of VLDL, Apo B 100 is also the ligand for LDL-receptor-mediated endocytosis of LDL particles [10].

There are several known Apo-B polymorphisms that were proven to cause hyperlipidemia and cardiovascular disease. One of such polymorphisms is the ECoRI polymorphism that results in Glu4154Lys amino acid substitution in the 26th exon. In previous studies, a significant direct relationship between ECoRI polymorphism and the serum levels of both cholesterol and triglyceride was found. It was thought that these polymorphisms reduce the binding capacity of Apo B to LDL receptors and so cause a decrement in LDL clearance [3].

Although the increased prevalence of obesity is mainly attributed to the high caloric diet and the sedentary life style, genetic factors are outstanding determinant of obesity and dyslipidemia in children.

AIM OF THE STUDY:

Assess the effect of Apo B restriction fragment length polymorphism (ECoRI) on lipid, Lp (a) concentrations and BMI in obese Egyptian children, and its possible relation to insulin resistance.

MATERIALS AND METHODS

1- Subjects:

The present study was carried out at Clinical Pathology Department, Faculty of Medicine, Menoufia University in the period between May 2014 and November 2015. Sixty subjects were included in this study and divided according to the body mass index (BMI) into two groups:

Obese group (with BMI > 25): involved 40 obese children (20 males and 20 females), their ages ranged between 6 – 12 years. They were randomly selected from primary schools in Menoufia governorate.

20 Apparently healthy age and gender matched children (10 males and 10 females) with BMI < 25 considered as a control group. The study was approved by the ethics committee of our medical faculty and a written informed consent was obtained from the parents of all subjects before study entry.

2- Methods and techniques:

All participants were subjected to the following:

- Anthropometric measurements (weight in kilograms, height in meters and body mass index) to diagnose obesity as:

BMI = Weight in kilograms / (Height in meters) ^{2}

- 2 ml blood sample were collected after 8 hours fasting, under aseptic condition by clean veinupuncture without venous stasis for determination of fasting serum glucose and fasting insulin.
- 8 ml blood sample was collected after 12 hours fasting, under aseptic condition by clean veinupuncture without venous stasis. It was divided into two parts:

a - 4 ml were added to an EDTA-contained sterile tube for the determination of apo B 100 polymorphisms. The sample were stored in the refrigerator for not more than a week till extraction of DNA or stored as a cell pellet on lysate for longer period at -80º C till extraction.
b - 4 ml were added to a sterile plain tube for immediate assessment of lipid profile (total cholesterol, triglycerides, LDL-c and HDL-c), Lp (a) and lipoprotein electrophoresis. The blood was left to clot at 37ºC and rapidly centrifuged at 4000rpm for 10 min.

Biochemical tests for detection of blood glucose and lipid profile were done on Synchron CX9 autoanalyser using kit supplied by Beckman (24).

- Fasting serum insulin was assayed by ELISA technique using momobind Inc. lake forest, CA 92630, USA Product code: 2425-30 Imunoenzymometric assay (18).
- HOMA-IR equation was calculated to diagnose cases with insulin resistance

$$\text{HOMA-IR} = \frac{\text{fasting glucose (mg/dl)} \times \text{fasting insulin (IU/ml)}}{405}$$

- Lipoprotein (a) was assayed by ELISA technique using IBL International GmbH Flughafenstr. 52A, 22335 Hamburg, Germany Imunoenzymometric assay [2].
- Lipoprotein electrophoresis was done by using Helena cellulose acetate strips [9].

-Genotyping: Determination of Apo B100 genotypes by PCR- RFLP:

Blood for genotyping was drawn into EDTA-containing receptacles. Genomic DNA was prepared from peripheral blood leukocytes according to the standard procedure and stored at -20 ^{o}C. To analyze polymorphic sites, we used separate PCR analyses followed by subsequent restriction fragment length polymorphism (RFLP) analysis. The primer sets used were: F: 5-CTGAGAGAAGTGTCTTCGAAG-3 and R: 5-CTCGAA AGGAAGTGTAATCAC-3 for EcoRI locus. Briefly, 300 ng of DNA was added to a PCR mixture (final volume of 50 ul) containing 1.5 mM MgCl2, 50 pmol of each primer, 200M dNTPs, and 1 U of Taq polymerase (Promega, Madison WI, USA) in a reaction buffer recommended by Promega. The PCR conditions for the ECoRI RFLPs were one cycle at 95 ^{o}C for 5 min followed by 30 cycles at 98^{o}C for 1 min and 58 ^{o}C for 1.5 min, with a final elongation at 72 ^{o}C for 10 min. Negative controls (no DNA added) were included in every PCR run to check for contamination. The PCR amplified products were digested for 3 hours with 5-10 U of the appropriate ECoRI restriction enzyme at 37 ^{o}C, and the fragments were analyzed using electrophoresis on 2.0% agarose gel. The DNA bands were visualized on a 302 nm UV transilluminator and photographed. The gel was examined for bands of 112 and 510 bp as determined by the molecular weight markers run at the same time. The alleles are named according to the presence or absence of the restriction site (Figure 1) (31).

According to the ECoRI electrophoresis, all subjects were reclassified into 3 groups:

- Homozygous negative group E-/E-
- Heterozygous positive group E+/E-
- Homozygous positive group E+/E+

STATISTICAL ANALYSIS:

The results were tabulated and statistical analysis was performed using the statistical package IBM SPSS version 21 [28]. Group differences were analyzed by Student's t test, Mann-Whitney test, and X^2 for normally distributed, non-normally distributed, and non-continuous variables respectively.

RESULTS

Comparison between the control and the obese groups (Table 1).

No significant difference was found between both groups as regarding age, gender and height. As expected the obese group showed a significantly higher weight and BMI. The obese group also showed a significant higher FBS, fasting insulin with subsequent higher HOMA-IR. As regarding lipoproteins, the obese group showed a statistically significant higher total cholesterol, LDL cholesterol, triglycerides and Lp (a). On the other hand, the control group showed significant higher HDL cholesterol.

Frequency of distribution of E ECoRI genotypes (Figure 2):

All the control group subjects showed a homozygous negative gene polymorphism (E-/E-). In addition, only one subject of the obese group showed the same polymorphism so the total number of this group is 21 subjects (35%). 22 subjects (36.67%) of the obese group showed a homogenous positive polymorphism while 17 subjects (28.33%) showed a heterogenous gene polymorphism.

Comparison between ECoRI genotypes (Table 2):

No significant difference was found between the 3 groups as regarding the age, gender and height. Both the E+/E- and the E+/E+ groups showed a statistically significant higher levels of FBS, fasting insulin, HOMA-IR, total cholesterol, LDL cholesterol, triglycerides and Lp (a) when compared to the E-/E- separately. The E-/E- group showed an HDL level higher than the other two groups. The E+/E+ showed significantly higher levels of BMI, fasting insulin, HOMA-IR, total cholesterol, LDL cholesterol and Lp (a) when compared to the E+/E- group. on the other hand no significant difference found between both groups as regarding age, sex, FBS, HDL cholesterol and triglycerides levels.

Table 1: Comparison between the control and the patient groups

Parameter	P value	T test	Obese	Control
Age (years)	0.963	1.107	9.57+2.06	9.55+1.76
Gender (M/F)	0.99	0.124	20/20	10/10
Weight (Kg)	**0.00**	19.196	74.47+17.57	36.85+8.61
Height (M)	0.119	0.676	1.39+0.18	1.31+0.16
BMI (KG/M^2)	**0.00**	17.928	38.92+5.54	21.355+1.93
FBS mg/dl	**0.00**	1.340	101.77+8.43	79.7+6.38
F. Insulin IU	**0.00**	13.516	20.11+2.24	8.18+0.77
HOMA-IR	**0.00**	1.049	5.02+0.90	2.23+0.79
T. Cholesterol mg/dl	**0.00**	1.061	288.7+34.51	148.9+25.86
LDL Cholesterol mg/dl	**0.00**	1.210	219.7+32.06	84.83+24.71
HDL Cholesterol mg/dl	**0.00**	66.891	31.88+1.52	43.4+4.74
TG mg/dl	**0.00**	2.195	186+18.66	103.35+7.95
Lipoprotein (a) mg/dl	**0.00**	18.517	30.3+9.37	5.8+2.55

Table 2: Comparison between different polymorphic forms

Parameter	E-/E-	E+/E-	E+/E+	P1	P2	P3
Number	21	17	22			
Age (years)	9.52±1.72	8.94±2.07	10.09±1.99	0.35	0.32	0.08
Gender (M/F)	11/10	7/10	12/10	0.789	0.992	0.765
Weight (Kg)	37.71±9.27	70.76±17.12	78.22±17.5	**0.00****	**0.00****	0.19
Height (M)	1.31±0.16	1.39±0.17	1.39±0.19	0.15	0.14	0.96
BMI (KG/M²)	21.88±3.08	37.03±5.09	40.67±5.41	**0.00****	**0.00****	**0.039***
FBS mg/dl	80.52±7.28	99.76±8.89	103.55±8.0	**0.00****	**0.00****	0.172
F. Insulin IU	8.74±2.68	19.18±1.72	20.83±2.41	**0.00****	**0.00****	**0.022***
HOMA-IR	2.33±0.90	4.64±0.82	5.340.87	**0.00****	**0.00****	**0.015***
T. Cholesterol (mg/dl)	154.57±36.21	270.53±24.31	303.82±35.13	**0.00****	**0.00****	**0.002***
LDL Cholesterol (mg/dl)	90.35±35.22	202±22.22	234.15±32.33	**0.00****	**0.00****	**0.001****
HDL Cholesterol (mg/dl)	42.81±5.35	31.59±1.50	32.14±1.55	**0.00****	**0.00****	0.275
TG (mg/dl)	106.62±16.87	184.713.65	187.64±22.17	**0.00****	**0.00****	0.635
Lipoprotein (a) (mg/dl)	7.33±7.45	26.58±10.71	32.82±7.42	**0.00****	**0.00****	**0.039***

P1: Comparison between the E-/E- and the E+/E- groups P2: Comparison between the E-/E- and the E+/E+ groups P1: Comparison between the E+/E- and the E+/E+ groups

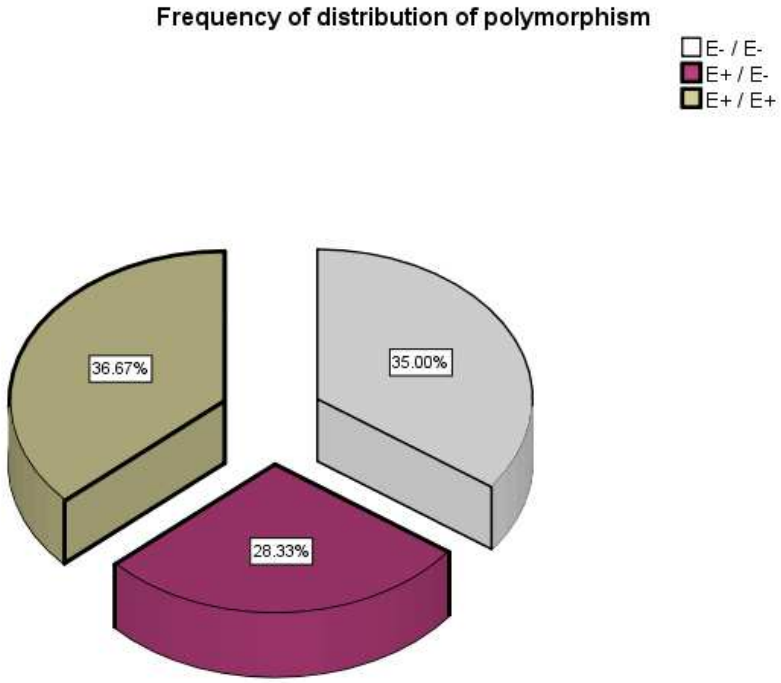

Figure 1: Frequency of distribution of gene polymorphism in the whole group.

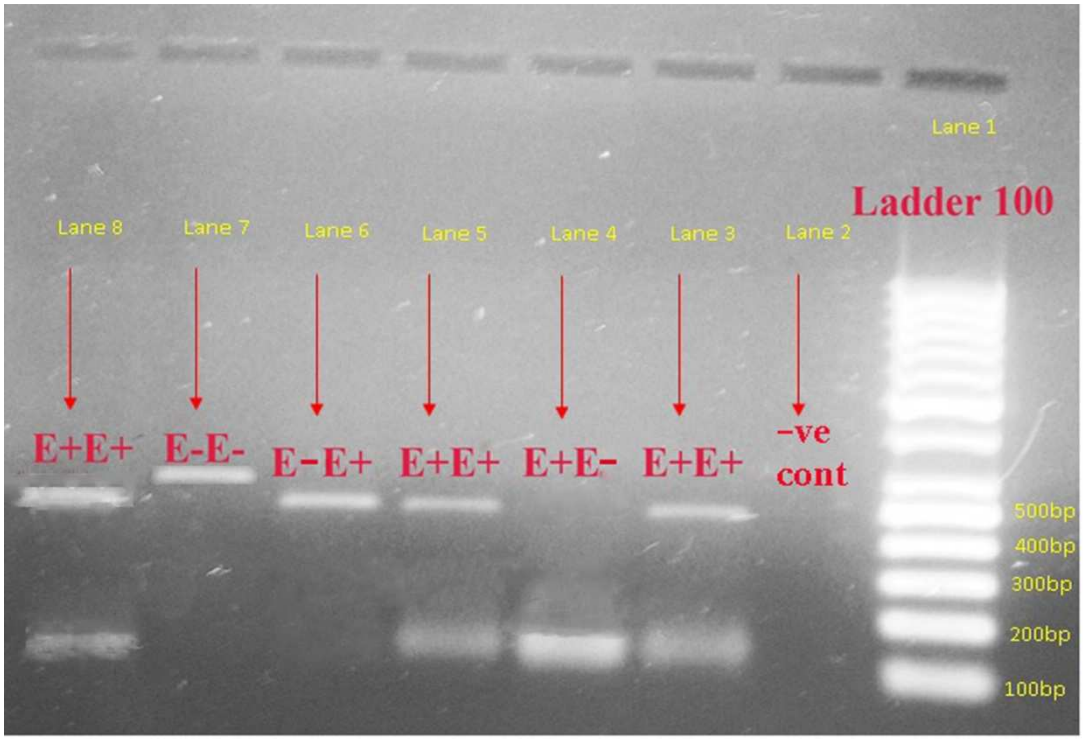

Figure 2 : Agarose gel electrophoresis of PCR product after amplification and digestion by restriction enzyme (ECoRI):

Lane 1 : showed the DNA marker 100 bp (ladder).
Lane 2 : Negative control (no DNA was added).
Lane 3, lane 5 and lane 8: showed positive homozygous polymorphism (E+/E+ alleles) with 2 bands at 112bp and 510bp.
Lane 4 : Positive heterozygous polymorphism with one band at 112bp (E+/E- alleles).
Lane 6 : Positive heterozygous polymorphism with one band at 510bp (E-/E+ alleles).
Lane 7: showed homozygous negative polymorphism E-/E- alleles (no bands neither at 112bp nor at 510bp, with an uncutted DNA at 600 bp).

DISCUSSION

Elevated blood pressure, dyslipidemia, and a higher prevalence of factors associated with insulin resistance and type 2 diabetes appear as frequent comorbidities in the overweight and obese pediatric population [14].

Overweight children are at risk for various chronic conditions in later life and this risk may exist even independent of obesity in adult life. Obesity affects almost every organ of the body. Its effects include metabolic syndrome which is insulin resistance, hyperlipidaemia and hypertension. Moreover, They also have mechanical disorders as osteoarthritis, respiratory problems, sleep apnea and psychosocial disorders[13].

The metabolic syndrome (MS) is one of the greatest challenges to public health throughout the world, due to its association with major risks for cardiovascular diseases and type 2 DM[6].

It was proven that even one amino acid change in the carboxyl end of the Apo B protein can destroy its binding capacity to LDL receptors. It was also shown that Apo B was defective in some hyperlipidemic and/or hypercholesterolemic patients in binding to the receptor [3].

The aim of this work was to evaluate the role of apolipoprotein B-100 ECoRI polymorphism on serum lipid parameters and BMI in obese children and its possible relation to insulin resistance.

The striking finding in this study is that the entire control group showed an E-/E- polymorphism of the Apo B 100 ECoRI gene. On the other hand 39 out of 40 obese children showed positive homozygous or heterozygous gene polymorphism. This finding illustrates

significantly the strong link between obesity and Apo B 100 ECoRI gene polymorphism. This is in accordance with Grundy who found that obesity, metabolic syndrome and cardiovascular disease all are direct complications to increased BMI and ApoB100 EcoRI polymorphism [6].

The metabolic syndrome (MS) is a clustering of components that reflects over nutrition, sedentary lifestyles, and resultant excess adiposity. These components include abdominal obesity, insulin resistance, dyslipidemia and elevated blood pressure [7].

As regard fasting glucose, fasting insulin and HOMA-IR, in the present work, there was a highly significant increase in the mean value of all the 3 parameters in the E+/E+ and the E+/E- when compared with the E-/E- group separately. These results are also in accordance with Grundy who found that obesity, metabolic syndrome and cardiovascular diseases all are linked to increased BMI and Apo B 100 EcoRI polymorphism [6]. This relationship may be a direct effect of the gene polymorphism on the insulin resistance with subsequent elevation of blood glucose and future development of diabetes or it is indirect through increase in body weight in these groups. Moreover, BMI and HOMA-IR were significantly higher in the homozygous (E+/E+) group than in the heterozygous (E+/E-) group. To the best of our knowledge, this is the first work to demonstrate clearly the cumulative effect of the number of Apo B100 EcoRI positive gene polymorphism on insulin resistance.

Although insulin resistance is higher in the (E+/E+) group, blood glucose is not higher than the (E+/E-) group. This is explained on the basis of time factor with insulin resistance preceding derangement of blood glucose by many years. So, we can expect the future development of diabetes and worse outcome in the (E+/E+) group earlier than the (E+/E-) group.

Regarding lipid profile, the obtained data showed that there was a highly significant increase in total cholesterol, LDL-c, and triglycerides and in the obese group more than the control group, while there was a highly significant decrease in HDL-c in the obese group less than the control group. This can explain, partially, the increased cardiovascular risk and adverse outcome in obese population.

Comparison of the lipid profile in the different polymorphic groups showed a highly significant increase in total cholesterol, LDL-c, triglycerides and Lipoprotein B 100 both in the (E+/E+) and the (E+/E-) when compared to the (E-/E-) group. on the other hand HDL-c was higher in the (E-/E-) group. Moreover, the (E+/E+) group showed a higher total cholesterol and LDL-c and than the (E+/E-) group while HDL-c and triglycerides were equal in both groups. These findings magnify the importance of the ECoRI B 100 gene polymorphism on the adverse cardiovascular outcome. These findings are in agreement with Timirci et al., [29] who found nearly similar results in 90 children and adolescents in turkey, but contrary to Saha et al. [25], who found no effect of the ECoRI polymorphism on serum lipid levels in healthy Chinese of Singapore [25,29] .

ECoRI polymorphism of the Apo B 100 gene is one of several Apo B 100 polymorphisms associated with hyperlipidemia. We have studied the ECoRI polymorphism at the 4154 position, causing a glutamine to lysine substitution in the 26th exon. Although the major pathway for removal of LDL from the plasma is through binding of Apo B100 on LDL particles to the LDL receptor, only few studies have investigated the possible mechanisms by which this gene polymorphism affects BMI and serum lipids concentration. For example, studying LDL kinetics in relation to Apo B-100 polymorphism in five different populations showed that LDL fractional catabolic rate was lower in those carrying the EcoRI polymorphism [20]. However, Gallagher and Myant did not encourage this hypothesis. They found no difference between binding affinities to human skin fibroblasts of LDL particles from individuals homozygous for ECoRI polymorphism and those from individuals heterozygous for the EcoRI polymorphism [5].

Lipoprotein (a) has been linked with cardiovascular diseases in children [1]. It is even classified as an independent cardiovascular risk factor in children [19]. In the present work we found a significantly higher level of Lp (a) in the heterozygous state and higher in the homozygous positive group when compared with the homozygous negative group. These finding add to the literature a novel link between obesity and cardiovascular diseases in children. Bailleul et al. recommended Lp (a) level in children as a prognostic risk factor for the future development of

atherosclerosis [26]. On the other hand jugie et al. reached to a different conclusion and they did not find any relation between Lp (a) and obesity in children [11].
Pouliot et al., [17] investigated whether the Apo B 100 ECoRI polymorphism influenced the associations described among obesity, regional adipose tissue distribution, and plasma lipoprotein levels in 56 healthy men. After adjusting for age and BMI rate, they observed that total cholesterol levels were significantly higher in heterozygous individuals compared to homozygous negative individuals. Similar to this study, we found increased total cholesterol and LDL-cholesterol levels in children with positive ECoRI polymorphism compared to negative polymorphism [21, 23]. Moreover, we demonstrated the higher levels in the homozygous positive ECoRI gene polymorphism (E+/E+) than the heterozygous gene polymorphism (E+/E-).

CONCLUSION

There is a great association between Apo B 100 ECoRI gene polymorphism and obesity in Egyptian children. Moreover, there is a cumulative association of positive ECoRI genes with atherogenic hyperlipidemia and insulin resistance in this population.

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