



Corresponding of Genetic polymorphism the apolipoprotein B R3500Q gene mutation with possible Familial Hypercholesterolemia (FH) pateints in Sulaymaniyah

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Abstract

Familial Hypercholesterolemia (FH) is autosomal codominant disease characterized by elevated LDL Cholesterol and Early Coronary Artery disease. (FH) is commonly caused by mutations in the three genes: The Low-Density Lipoprotein Receptor (LDLR), apolipoprotein B (apoB), Proprotein Convertase Subtilisin / Kexin type 9 (PCSK9). The current study aimed to identify mutations in people with homozygous genotypes that affect protein binding causing defects and to ensure that these conditions are diagnosed through important molecular tests through the early intervention of the apolipoprotein gene (apoB) for the R3500Q mutagenic of healthy individuals not associated with hypercholesterolemia (FH) in Sulaymaniyah through the conduct of the polymer chain reaction system and Restriction enzyme genotyping. The study included determination of the polymorphism of genes associated with familial hypercholesterolemia (FH). The molecular study included the genetic analysis of (50) samples of the R3500Q mutation of the apoB gene, after adding the *ScaI* enzyme, showed there three genotype: were four cases found Homozygous to be one bundle (S+/S+) (143 bp), a one case compound heterozygous (S-/S+) model are two bundle (143 bp, 90 bp) and a forty-five cases had mutant Homozygous (S-/S-) model of the one bundle (90 bp), all the R3500Q mutations were found on the same allele.

the study also included the R3500Q mutation of the apoB gene and Its relation to the studied traits, there was a significant increase in the 0.01 for cholesterol, TG and LDL for patients with hypercholesterolemia was mean (235.61 mg / dl, 321.83 mg / dl and 330.90 mg / dl) respectively , compared to healthy pateints with mean (172.15 mg / dl , 109.88 mg / dl and 77.1 mg / dl).

Introduction

Familial Hypercholesterolemia (FH) is an autosomal dominant disorder, an expression of a defect in the gene responsible for the production of the (Low Density Lipoprotein (LDL)) [1]. The most common causes of (FH) are the genetic defect in the gene that produces the Low Density Lipoprotein (LDLR) gene [2] . In fact, there are more than 1000 different mutations in the LDL receptor (LDLR), apolipoprotein B (apoB), Proprotein Convertase Subtilisin / Kexin type 9 (PCSK9) causing Familial Hypercholesterolemia (FH) [3].

In such a transformation of the disease causing mutation, DNA can be determined by varying percentages of pateints with (FH) , ranging from 20% to more than 90% with highest rates of detection in children that have been carefully selected for height Familial Hypercholesterolemia [4]. The first family defect of apolipoprotein B-100 was described in 1989 by replacing the amino acid arginine with glutamine (R3500Q) [5]. The R3500Q codon has an effect on the binding of apoB-100 to the LDLR receptor, causing an increase in low density lipoprotein

cholesterol (LDL-C) [6]. Clinical diagnosis of (FH) severe due to the accumulation of LDL in plasma cholesterol in the skin with a significant risk of atherosclerosis, which is mainly manifested by Coronary Heart Disease [3].

The frequency of FH is due to defects in the LDLR gene. It is estimated that the heterozygous of FH is 1:500 in most populations in (Europe, North America and Japan) and is thought to be one of the most common human disease caused by mutations in a single gene homozygous is estimated at one million and is of very rare type [1].

Methods

1. Sample Collections:

Sample were collected from whom (39-79) years in the central laboratory in Sulaymaniyah, blood sample were obtained from the 10 ml venous vein in the early morning hours, with some information pertaining to each. Blood samples were placed in test tubes that were centrifuged at 3500 rpm, for 15 minutes to obtain a blood serum (Blood Serum) where it was put in a new plastic test tubes and recorded all the information after it was transferred to the laboratory in the college of Science university of Sulaymaniyah and kept in the fridge (-20 °C).

2. Estimation of Cholesterol in Blood :

Cholesterol was estimated in serum using several analysis kit of the French Biolabo type, the enzyme method [7] was used in estimating the amount of cholesterol, as the esterase cholesterol in the packaging is equipped to measure cholesterol analysis, the indicator in serum to cholesterol and Fatty acids with oxygen and cholesterol enzyme Oxidiz that are working on free cholesterol oxidation as a result of the first reaction (cholesterol 4 one 3) and hydrogen peroxide interacts with 4-aminoantipyrine with an enzyme pyruvates to consist of a composite pink color (quinonemine) and the intensity of the color is proportional to the concentration of cholesterol in the blood serum, measured by the severity Color with the optical spectrometer at a wavelength of 500 nanometer.

3. Estimation of Triglyceride in Blood:

Triglycerides in the serum were estimated using a kit of French Biolabo type analysis on method [8] Used enzymatic method to quantify triglycerides, the lipase enzyme in the packaging that is equipped to measure the triglycerides in the serum to the Glycerol and fatty acids, the resulting Glycerol is traveling by ATP and enzyme Glycerol Kinase to Glycerol-3-phosphate, which is oxidized by an enzyme glycerol-3-phosphate to chlorophenol-4 and hydrogen

peroxide and by means of an enzyme (peroxidase) and (4-amino antipyrine), Pink is a product of a compound (quinoneimine) which is proportional to its color intensity with the concentration of triglycerides in serum in the measurement of the absorption in contrast (Blank) at a wavelength of 500 nanometer.

4. Estimation of Serum HDL-Cholesterol :

High-density Lipoprotein (HDL) is estimated in serum using several French Biolabo type kit [9] The principle of this method is based on direct measurement for High-Density Lipoprotein HDL method is not connected to any variables and does not need centrifuge steps and the method contains a detector The first contains (dextran sulfate) (α -cyclodextrin) for precipitation (VLDL, LDL) and, and the second Chylomicrons contains a PEG enzyme that selects the molecules of HDL, so HDL is the only size of the Lipoprotein .

5. Estimation of Serum LDL-Cholesterol :

Low-density Lipoprotein (LDL) is estimated in serum using several analysis kit of French type Biolabo [8] The principle of this experience depends on the way the color can be LDL level directly in serum in a way to remove that includes two steps in the first step still VLDL, Chylomicron and HDL in the second step after enzymatic reaction and precipitation LDL.

6. DNA Extraction:

DNA was extracted from the serum which preparation of stock Solutions used in DNA extraction and preparation of Buffers (work) solutions according to the method Keller *et al.* [10]. The DNA was measured concentrated and purified using a Nanodrop device. The solutions are prepared in the process Gel electrophoresis to the method described by [11].

7. Specific-primer –PCR :

Interactions were carried out based on Wishart *et al.* [12] using a pairs starting on 106 samples of DNA samples extracted from serum based on purity and concentration of DNA. However, a large preparation of these primers featured packages appear, only 3 prefixes which are the primary target in these interactions.

The master reaction mixture was prepared by mixing the reaction components in a 2 ml Eppendrofe tube sterilize the mixture in the Microfuge for 3-5 minutes to complete the mixing of the reaction reaction components. taking into consideration that the work inside the hood is sterile, the gloves are placed and the tubes are placed inside the ice. As shown in the table (1):

Table (1) : shows the solutions used in the specific premir of mutation R3500Q

No.	Compnents	Final concentration	Size for one sample
1	Green Master Mix	1X	13 μ
2	Forward primer	10 picomols/ μ	1 μ
3	Reverse primer	10 picomls/ μ	1 μ
4	Nuclease free water to	---	8 μ
5	DNA	50 ng	2 μ

The specific primer of R3500Q as show in the table (2). after the reaction time the tubes were lifted from the thermoforming device and kept in the freezer, 5 μ l of the tubes were withdrawn and the mixture is loaded on the pre-prepared agarose gel at 2% concentration with the volume guide marker (100bp – 3000bp) in a special hole on either of the gel, the

relay is then switched on by Voltage 5 volts / cm , then the electrodes must be set to move the samples towards the positive pole to the point of arrival of samples before the end and the operation takes 2 hours. the samples were then carried on the electrophoresis displays for the UV source on the Ultraviolet light and the gel image.

Table (2) : Interaction program the specific primer of mutation R3500Q

Primer	Sequences	Tm (°C)	GC (%)	Product size
Forward	5'-CTTACTTTTCCATTGAGTACTCTACC-3'	63.2	38.46%	143 Base pairs
Reverse	5'-AGTGCCCTGCAGCTTCACTGAGTAC-3'	69.1	56.0%	

No.	Stage	Temperature (°C)	Time	No. of Cycle
1	Initial Denaturation	94 °C	3 min	35 Cycle
2	Denaturation	94 °C	35 sec	
3	Anealing	54 °C	35 sec	
4	Extensation 1	72 °C	35 sec	
5	Extensation 2	72 °C	10 min	

8. Cutting of the genomic DNA :

The DNA is cut by the Restriction enzyme where the reaction is prepared in a (0.2) ml tube and the solutions are placed according to the table (3), The samples are then incubated in a water bath at a

temperature of 37 °C for 4 hours. The samples are then transferred to conform the completion of the cut , during the appearance of DNA in the form of a long the gel and the use of Et Br dye and the concentration of gel 2% agarose .

Table (3) : Components of the genetically modified DNA with Restriction enzyme *ScaI* .

No.	Solution	Microliter / model	Final concentration
1	Distilled sterile water	16.8 μ	
2	10 X digestion buffer	2 μ	1X
3	Actylated BSA	0.2 μ	
4	DNA	20 μ	1 Microgram
5	Restriction Enzyme	1 μ	10 units
6	The final size	40 μ	

9. Statistical program :

Statistical data analysis was carried out according to SPSS Version 20 .

Result & Discussion

1. Physiological study :

apoB R3500Q gene mutation is one of the genetic causes of Familial Hypercholesterolemia, this gene is located on an encoded chromosome (1) of protein fragment LDL [13]. This mutation occurs in the transition from G to A in 10708 nucleotides in Axon 26 of the apoB gene, replace glutamine for arginine in the amino acid of the apolipoprotein B gene and reduce the affinity of apoB to LDL Receptors, the interaction between Low Density Lipoprotein (LDL) and LDL Receptors is essential for regulatory plasma cholesterol, the only protein component of LDL is apoB-100, which acts as a link to LDLR [14]. Many of the mutations in the apolipoprotein B gene alter the functional activity of the protein and reduce the binding of LDLr and delay the removal of LDL molecules and thus the accumulation of Cholesterol in the circulatory system, so far, four mutations have been identified that cause Familial Defective

apolipoprotein B (FDB) the LDL-r link in the apoB gene is: R3480W, R3500Q, R3500W and R3531C [15]. The CGG-CAG in amino acid code 3500 led to the replacement of glutamine to arginine (R3500Q) the most common change which causes FDB [16]. the results in a change in function of the apoB gene, which in turn results in an increase in hypercholesterolemia with an increased risk of atherosclerosis [14,15].

In this study, the results of the statistical analysis of Cholesterol, Triglyceride (TG), and Lipid profile levels (HDL, LDL) for the R3500Q mutagen of the apoB gene showed a significant increase in the mean level of 0.01 except HDL, which was insignificant, the mean of Cholesterol and TG for pateints was (215.61 \pm 5.01 mg / dl and 299.83 \pm 26.2 mg / dl) respectively, compared to healthy pateints with mean (172.15 \pm 5.18 mg / dl and 109.88 \pm 6.55 mg / dl) and Lipid profile (HDL, LDL) the mean (41.87 \pm 9.26 mg / dl and 300.96 \pm 28.731 mg / dl) respectively, while the healthy mean (48.93 \pm 1.67 mg / dl and 77.1 \pm 5.80 mg / dl) respectively, according to the table (4). These results were agreed with Farrokhi and his

group [16] In their study of 30 Iranian people in Chaharmal in Bakhtiari province south western Iran, who were likely to have (FH) , the disease was clinically diagnosed the aim of their study was to study the properties of the LDLR gene and three

common gene mutations of the apoB gene secondary causes of high Cholesterol, such as Diabetes, Hypertension, Smoking habits and family history had Coronary Artery Disease.

Table(4) : Calculations of the concentration of (Cholesterol , TG , HDL , LDL) of mutation R3500Q for (FH) pateints compared to healthy pateints

Studied traits	groups	N	Mean ± SE	t. value	p.value
Cholesterol mg/dl	Pateints	33	215.61 ± 5.01	-8.80	< .000
	healthy	13	172.15 ± 5.18	-7.33	
Triglyceride mg/dl	pateints	29	299.83 ± 26.2	7.82	< .000
	healthy	16	109.88 ± 6.55	-5.90	
HDL mg/dl	pateints	30	41.87 ± 9.26	0.75	< .290
	healthy	14	48.93 ± 1.67	0.51	
LDL mg/dl	pateints	27	300.96 ± 28.7	-8.66	< .002
	healthy	19	77.1 ± 5.80	-7.31	

2. Molecular analysis of mutation R3500Q from apolipoprotein B:

The results of the (PCR) DNA sequnces using the R3500Q Initiator of the apoB gene showed that this initiator was used with a combination of PCR (106) samples of individuals suspected of (FH) in Sulaymaniyah and based on the program which [17] .

After reproducing the PCR genomic DNA samples on 2% agarose gel and imaging with the UV transilluminator, the resulting bundle of the genomic DNA samples emerged, Scientific source indicate that the products of this initiator without the Restriction Enzyme show their molecular size (143 bp) as in images (1) and (2)[18].

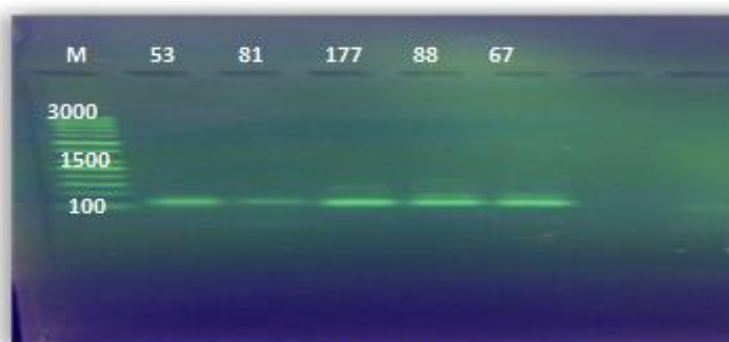


Image (1)

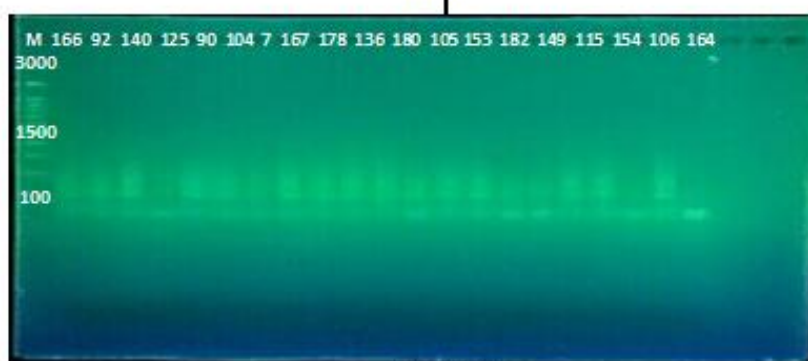


Image (2)

Images (1) and (2) : Product of the Gel Electrophoresis to multiply the R3500Q initiator of the apoB gene without the Restriction enzyme for a group of study samples on 2% agarose gel.

After adding the *ScaI* to the PCR product, three types of genotypes: Homozygous (S+ / S+) was shown as (143 bp), Heterozygous (S+ / S-) were shown as (143,

90) bp and Mutant Homozygous (S- / S-) was shown as (90) bp as in picture (3), (4) and (5).

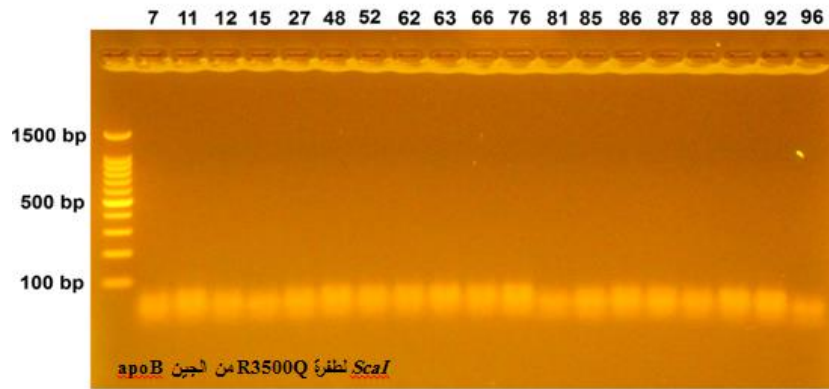


Image (3) : The PCR-PFLP (*ScaI*) electrical relay product for samples of patients with familial hypercholesterolemia on the agarose gel concentration of 2.5% .
All samples : represent the Mutant genotype (S- / S-).

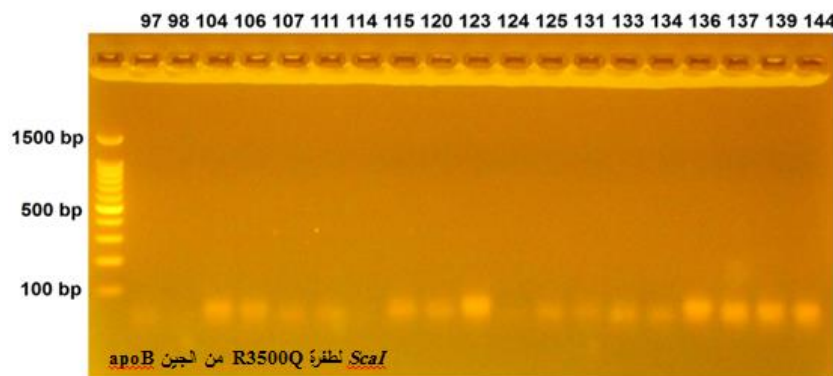


Image (4) : The PCR-PFLP (*ScaI*) electrical relay product for samples of patients with familial hypercholesterolemia on the agarose gel concentration of 2.5% .
Samples: (97,98, 114 124) represent the Homozygous genotype (S + / S +) .
Samples: (104,106,107,111,115,120,123,125,131, 133,134,136,137,139,144) represents the Mutant genotype (S-/ S-) .

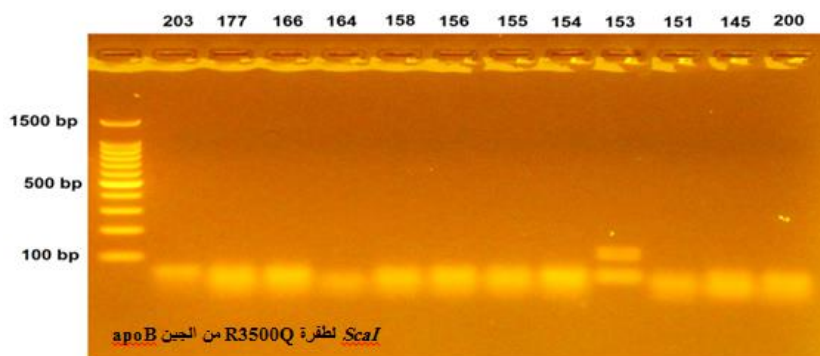


Image (3) : The PCR-PFLP (*ScaI*) electrical relay product for samples of patients with familial hypercholesterolemia on the agarose gel concentration of 2.5% .
The sample: (153) represents the Heterozygous genotype (S + / S-) .
Samples: (203,177,166,164,158,156,155,154,151, 145 200) represent the Mutant genotype (S- / S-) .

That the substitution base of the T to G nuclease at the 445 Site of the Entron 5 of apoB gene . After the addition of the *ScaI* enzyme to the PCR product , the resulting bundle of the molecular size multiplication of the (143bp) will be divided into the first two pieces (143 bp) and the second (90 bp) , Either if the person is not carried with the mutation in both alleles , he will have the same normal genotype (S+ / S+) one bundle of (143 bp) [19].

If a person has a heterozygous (S+ / S-) one of the alleles will be cut to its (90 bp) molecular weight and the other allele will not be cut because it does not have a cut-off location of (143 bp) . Thus , two bundles will appear when the PCR-RFLP the first is (143bp) representing the non-mutated allele harvested with *ScaI* enzyme, if the person has the same genotype (S- / S-) the two alleles will not have a *ScaI*-cut size and thus a single bundle of (90 bp) molecular

size will appear when relaying the PCR-RFLP, all the R3500Q mutation were found on the same allele, product as shown in figures (3, 4 and 5).

This is confirmed by Al-Obeidi [20] when he pointed to the presence of Similar genotypes in his study on the effect of polymorphism *Hind III* and *Ser 447 Ter* polymorphisms of lipoprotein lipase gene on lipid metabolism and concentration in Atherosclerosis patients in Tikrit and Samarra Cities. Al-Jafari and his group [21] when he pointed to the existence of genotype among Saudi Society patients. this was also noted by Shim-Nakanishi and his group [22] when he studied Cerebral Infraction. Ahmadi and his group [23] found the same results when studying the effect of the polymorphism *Hind III* and *S447X* for the Iranian patients with Coronary Artery Disease in Tehran and Shiraz .

There have been studies that showed a very low detection rate of the common R3500Q mutation in 30

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patients, Indicating that the potential mutation was caused by a change in apoB frequencies [15]. The R3500Q mutation was found to be gradually in the populations of Northern Europe, Germany, the United Kingdom and the United States, R3500Q prevalence rates ranged from 1:700 to 1:1500 and as found in most countries in Europe 3-5 % of patients with hypercholesterolemia the cause of this high is the R3500Q mutation of the apoB gene [24].

While there are studies conforming that the R3500Q mutation could not occur in Lebanon, Syria and Turkey, where mutations in the apoB gene were not detected in any of the patients it was concluded that the absence of the R3500Q mutation is likely due to the geographical distribution of the mutation and also to low frequency (0.24% in Northern Finland (1.5 %) in South Africa [25].

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تحديد تعدد الاشكال الوراثي للطفرة الجينية R3500Q للعوائل المحتمل اصابتها بفرط الكوليسترول العالي في السلبيانية

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الملخص

فرط كوليسترول الدم العائلي (FH) Familial Hypercholesterolemia هو جسيمة سائدة مهيمنة تتميز بارتفاع كوليسترول LDL ومرض الشريان التاجي المبكر Early Coronary Artery Disease، هو عادة يحدث بسبب حدوث الطفرات في ثلاثة جينات: Low-Density Lipoprotein Receptor (LDLR) و (apoB) apolipoprotein B و Proprotein Convertase Subtilisin / Kexin type 9 و (PCSK9) وهدفت الدراسة الحالية إلى تحديد الطفرات في الأشخاص الذين يمتلكون النمط الوراثي المتمثل Homozygous والتي تؤثر في ارتباط البروتين مسبقاً مسبباً العيوب وضمان تشخيص هذه الحالات من خلال الأختبارات الجزيئية المهمة من خلال التدخل المبكر لجين لأبوليبوبروتين (apoB) لطفرة R3500Q للأشخاص السلبيين ليس لديهم صلة بمرض فرط كوليسترول الدم العائلي (FH) في السلبيانية من خلال إجراء نظام سلسلة تفاعل البوليمرات Polymerase Chain Reaction والأنزيم القاطع المجهني Restriction enzyme genotyping. وشملت الدراسة تحديد التعدد الشكلي للجينات المقترنة بمرض فرط كوليسترول الدم العائلي (FH) تضمنت الدراسة الجزيئية التحليل الجيني لـ (50) عينة لطفرة R3500Q من جين apoB، فبعد إضافة الأنزيم القاطع *Scal*، ظهرت هناك 3 طرز وراثية وهي: (4) حالات طراز وراثي طبيعي متمثل (S+/S+) بحزمة واحدة (143bp)، وحالة واحدة من الطراز الوراثي غير المتمثل (S+/S-) Heterozygous متمثلة بحزمتين (143 bp, 90 bp) و (45) حالة متمثلة بالطراز الوراثي الطافر المتمثل (S-/S-) Mutant Homozygous متمثلة بحزمة واحدة (90 bp)، تم العثور على جميع طفرات R3500Q على الأليل نفسه.

شملت الدراسة أيضاً طفرة R3500Q من الجين apoB وعلاقتها بالصفات المدروسة وكانت هناك زيادة معنوية عند ($p \leq 0.01$) للكوليسترول و TG و LDL للمرضى فرط كوليسترول الدم كان المتوسط (235.61 mg/dl و 321 mg/dl و 330 mg/dl) مقارنة بالأصحاء إذ بلغ المتوسط الحسابي (172.15 mg/dl و 109.88 mg/dl و 77.1 mg/dl).