MEDICAL
UNIVERSITY – PLEVEN

FACULTY OF MEDICINE

DEPARTMENT OF ANATOMY, HISTOLOGY, CYTOLOGY AND BIOLOGY

Tihomir Rumenov Rashev

Alterations in Orexigenic and Anorexigenic Genes Regulating Energy Homeostasis and Their Role in the Development of Obesity and Metabolic Syndrome

Dissertation Abstract

submitted in partial fulfillment of the requirements for the award of the educational and scientific degree of Doctor of Philosophy (PhD)

Supervisor:

Assoc. Prof. Stefan Venelinov Trifonov, MD, PhD

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The dissertation comprises 164 standard pages and is organized into the following sections: Introduction, Literature Review, Aim, Objectives and Methodological Approaches, Results, Discussion, Conclusion, Conclusions, Contributions, and Future Perspectives. The dissertation is illustrated with 20 tables and 34 figures and includes 5 appendices.

The bibliography comprises 298 references. The laboratory work and molecular analyses were carried out at the Department of Anatomy, Histology, Cytology and Biology, Division of Anatomy, Histology and Cytology, Faculty of Medicine, Medical University – Pleven, and at the University Research Laboratory, Medical University – Pleven.

Three full-text scientific publications and three presentations at national scientific conferences have resulted from this dissertation.

The dissertation was approved and recommended for public defense by the Extended Department Council of the Department of Anatomy, Histology, Cytology and Biology, Faculty of Medicine, Medical University – Pleven, at its meeting held on 26 May 2026.

The public defense of the dissertation will take place on ……, at ……, in Hall ……, Medical University – Pleven, in accordance with the Regulations on the Conditions and Procedures for the Acquisition of Scientific Degrees and the Occupation of Academic Positions at Medical University – Pleven, and pursuant to the Rector's Order No. ……, before the following Scientific Jury:

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The materials related to the dissertation defense are available on the website of Medical University – Pleven:

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Abbreviations

T2DM – Type 2 diabetes mellitus

SNP – Single nucleotide polymorphism

PRDM16 – PR domain containing 16

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

IRF4 – Interferon regulatory factor 4

FGF21 – Fibroblast growth factor 21

BMP7 – Bone morphogenetic protein 7

PKA – Protein kinase A

UCP1 – Uncoupling protein 1

AMPK – AMP-activated protein kinase

IL-6 – Interleukin-6

PAI-1 – Plasminogen activator inhibitor-1

MetS – Metabolic syndrome

TNF-α – Tumor necrosis factor-alpha

FFA – Free fatty acids

ASP – Acylation-stimulating protein

VEGF – Vascular endothelial growth factor

IGF-1 – Insulin-like growth factor 1

LEPR – Leptin receptor

Myf5 – Myogenic factor 5

Ob-R (LR, DR, CD295, HuB219) – Leptin receptor

JAKs – Janus kinases

STATs – Signal transducers and activators of transcription

MC4R – Melanocortin 4 receptor

MT-II – Melanotan II

CaMKK – Calcium/calmodulin-dependent protein kinase kinase

PP2C – Protein phosphatase 2C

LKB1 (STK11) – Liver kinase B1 (serine/threonine kinase 11)

CaMKKβ – Calcium/calmodulin-dependent protein kinase kinase beta

SOCS3 – Suppressor of cytokine signaling 3

PTP1B – Protein tyrosine phosphatase 1B

SH2 domain – Src homology 2 domain

POMC – Pro-opiomelanocortin

NPY – Neuropeptide Y

UPR – Unfolded protein response

GnRH – Gonadotropin-releasing hormone

PRMT2 – Protein arginine methyltransferase 2

Grb2 – Growth factor receptor-bound protein 2

ERK1/2 – Extracellular signal-regulated kinases 1 and 2

PI3K–Akt–FoxO1 – Phosphatidylinositol 3-kinase–protein kinase B–forkhead box protein O1 pathway

IRS – Insulin receptor substrate

PDK1 – Phosphoinositide-dependent protein kinase 1

PTEN – Phosphatase and tensin homolog

mTOR – Mammalian target of rapamycin

PDE3B – Phosphodiesterase 3B

TCPTP – T-cell protein tyrosine phosphatase

LRP2 – Low-density lipoprotein receptor-related protein 2

LEPROT – Leptin receptor overlapping transcript (Endospanin 1)

RNF41 – Ring finger protein 41

ADAM10 – A disintegrin and metalloproteinase 10

RPGRIP1L – Retinitis pigmentosa GTPase regulator-interacting protein 1-like

XBP1s – Spliced X-box binding protein 1

IKKβ–NF-κB – IκB kinase beta–nuclear factor kappa B

GRP78 – Glucose-regulated protein 78

TLR4 – Toll-like receptor 4

AdipoR1 – Adiponectin receptor 1

APPL1 – Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1

CART – Cocaine- and amphetamine-regulated transcript

BMI – Body mass index

TC – Total cholesterol

HDL – High-density lipoprotein cholesterol

LDL – Low-density lipoprotein cholesterol

FPG – Fasting plasma glucose

SD – Standard deviation

CI – Confidence interval

95% CI – 95% confidence interval

Content

I. Introduction

Obesity and its associated metabolic disorders represent one of the greatest challenges facing modern medicine. Over the past decades, the prevalence of obesity has increased dramatically, particularly in developed countries, leading to a substantial rise in the incidence of conditions collectively referred to as the metabolic syndrome. This syndrome encompasses insulin resistance, type 2 diabetes mellitus (T2DM), cardiovascular disease, and non-alcoholic fatty liver disease. These disorders arise from a complex interplay between genetic predisposition, environmental factors, and impaired regulation of energy homeostasis. Type 2 diabetes mellitus is the predominant form of diabetes, accounting for approximately 90–95% of all cases, and is characterized by a broad spectrum of metabolic abnormalities ranging from pronounced insulin resistance to impaired insulin secretion. The increasing prevalence of T2DM closely parallels the global obesity epidemic, highlighting the need for a comprehensive understanding of the pathogenic mechanisms linking these disorders. The etiology is multifactorial, and genome-wide studies have identified numerous susceptibility genes, including those encoding adipokines such as leptin and adiponectin, which play pivotal roles in metabolic regulation.

In this context, adipose tissue is no longer regarded merely as an energy storage depot but rather as a highly active endocrine organ. White adipose tissue is the largest endocrine organ in the human body and secretes a wide range of biologically active molecules, collectively known as adipokines, including leptin, adiponectin, resistin, and pro-inflammatory cytokines such as TNF-α and IL-6. These mediators regulate appetite, energy expenditure, insulin sensitivity, inflammatory responses, and vascular function. Moreover, adipose tissue communicates with multiple organs—including the liver, skeletal muscle, pancreas, and central nervous system—through a complex network of endocrine, paracrine, and autocrine signaling pathways.

Obesity is associated with profound structural and functional alterations in adipose tissue. Expansion of adipose tissue mass leads to adipocyte hypertrophy and hyperplasia, accompanied by macrophage infiltration and the development of chronic low-grade inflammation. This condition is characterized by increased production of pro-inflammatory cytokines and free fatty acids, which impair insulin signaling in peripheral tissues and contribute to the development of insulin resistance.

Adipokines play a central role in these processes. Leptin, produced predominantly by adipocytes, functions as a signal of the body's energy stores and regulates appetite and energy expenditure through its actions on the central nervous system. Under physiological conditions, leptin suppresses food intake while promoting energy expenditure. In obesity, however, leptin resistance develops, whereby elevated circulating leptin concentrations fail to elicit the

expected biological responses. Conversely, adiponectin, an adipokine with insulin-sensitizing, anti-inflammatory, and anti-atherogenic properties, is markedly reduced in obesity, thereby further exacerbating metabolic dysfunction.

The hypothalamus serves as the principal integrative center for peripheral metabolic signals and plays a fundamental role in maintaining energy homeostasis. Hypothalamic nuclei integrate signals derived from adipokines (leptin, adiponectin), hormones (insulin and ghrelin), and nutrient availability. Of particular importance is the arcuate nucleus (ARC), which contains two major neuronal populations: orexigenic neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), which stimulate food intake, and anorexigenic neurons expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which suppress appetite. The balance between these neuronal systems ultimately determines feeding behavior and energy homeostasis.

Leptin and insulin activate anorexigenic neurons while inhibiting orexigenic neurons, whereas ghrelin exerts the opposite effect. In obesity, hypothalamic leptin resistance disrupts these neuronal networks, resulting in persistent hyperphagia and reduced energy expenditure. In addition to the arcuate nucleus, other hypothalamic regions—including the paraventricular nucleus (PVN), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN)—participate in the regulation of energy homeostasis by forming an integrated neural network that coordinates metabolic and behavioral responses.

The paraventricular nucleus integrates signals originating from the arcuate nucleus and regulates autonomic nervous system activity and endocrine function. The ventromedial nucleus is primarily associated with satiety signaling, whereas the lateral hypothalamic area promotes feeding behavior. Together, these interconnected neuronal circuits ensure the precise regulation of whole-body energy balance.

In conclusion, obesity results from a complex interaction between peripheral and central regulatory mechanisms. Adipose tissue, functioning as an endocrine organ, and the hypothalamus, serving as the central regulator of energy homeostasis, constitute an integrated system controlling energy balance. Dysregulation of this system, including alterations in adipokine secretion and hypothalamic signaling, plays a fundamental role in the development of insulin resistance, T2DM, and metabolic syndrome. A comprehensive understanding of these mechanisms is essential for the development of effective preventive and therapeutic strategies.

II. Aim, Objectives, Materials and Methods

1. Aim

- To evaluate the association of **ADIPOQ rs266729**, **LEPR Q223R**, and the **LEP -2548G>A** polymorphism with the risk of obesity, type 2 diabetes mellitus (T2DM), and related cardiometabolic disorders in an ethnically homogeneous population from Northwestern Bulgaria.
- To analyze the genotype and allele frequency distributions of the investigated polymorphisms by comparing patients with metabolic syndrome and healthy controls from the same population.

2. Objectives

- To perform a comparative analysis of the anthropometric, clinical, and biochemical characteristics of patients with metabolic syndrome and healthy controls.
- To determine the genotype and allele frequency distributions of **ADIPOQ rs266729**, **LEPR Q223R**, and **LEP -2548G>A** polymorphisms in both study groups.
- To evaluate the association of these polymorphisms with the risk of metabolic syndrome, obesity, type 2 diabetes mellitus, and arterial hypertension.
- To investigate the influence of the studied genetic polymorphisms on carbohydrate and lipid metabolism through genotype–phenotype association analysis.
- To assess the presence of an allele-dose effect of the risk alleles on major metabolic traits.
- To determine the relative contribution of genetic polymorphisms and phenotypic characteristics to cardiometabolic risk stratification.
- To integrate the genetic, clinical, and biochemical findings in order to clarify the role of **ADIPOQ**, **LEPR**, and **LEP** in the pathogenesis of metabolic syndrome and related cardiometabolic disorders.

3. Materials and Methods

3.1 Study Population

The study included **156 patients** recruited from the **Second Department of Cardiology** at **University Hospital "Dr. Georgi Stranski" – Pleven** and the **Specialized Hospital for Active Treatment in Cardiology – Pleven** between **May 2015 and April 2016**. The study protocol was approved by the **Research Ethics Committee of the Medical University – Pleven**.

All participants were informed about the aims and methodology of the study and provided written informed consent before undergoing genetic and immunological investigations.

The study population was divided into two groups:

Group I (Control group): 74 individuals without obesity or type 2 diabetes mellitus and with normal fasting plasma glucose levels.

Group II (Target group): 82 patients with obesity and/or metabolic abnormalities.

Inclusion criteria

Control group

- Age >21 years;
- BMI ≤25 kg/m²;
- Normal fasting plasma glucose;
- No diagnosis of type 2 diabetes mellitus;
- Normal blood pressure.

Target group

- Age >21 years;
- BMI ≥25 kg/m²;
- Elevated fasting plasma glucose;
- Diagnosed type 2 diabetes mellitus;
- Arterial hypertension;
- Dyslipidemia.

Exclusion criteria

- Age <18 years;
- Pregnancy or within three months postpartum;
- Severe forms of physiological obesity;
- Severe arterial hypertension;
- Advanced renal disease (serum creatinine >250 μmol/L or hemodialysis);
- Disorders affecting calcium–phosphate metabolism (e.g., hyperparathyroidism, Paget's disease);
- Refusal to provide written informed consent.

Demographic characteristics

A total of **157 participants** were included, comprising **82 men (52.6%)** and **75 women (47.4%)**. The mean age of the study population was **69.15 ± 0.87 years** (range: **35–85 years**). The age and sex distribution was as follows:

- Among men, the majority of participants were aged **60–69** and **70–79 years** (35 participants each), followed by the **50–59-year** age group (7 participants). Only one participant was older than 90 years.

- Among women, the largest proportion belonged to the **70–79-year** age group (40 participants), followed by those aged **60–69 years** (18 participants). Seven women were aged **50–59 years**, and no participants were older than 90 years.

3.2 Definition of the Study Variables

3.3 Arterial Hypertension

Arterial hypertension was assessed as a categorical variable and defined as a documented history of systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg requiring antihypertensive treatment, and/or hypertension confirmed during the study. Blood pressure was measured at the brachial artery after a 5-minute rest in the seated position. Three consecutive measurements were obtained at 2-minute intervals, and the mean values were calculated. Hypertension was defined as a mean systolic blood pressure ≥140 mmHg and/or a mean diastolic blood pressure ≥90 mmHg.

3.4 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) was defined as fasting plasma glucose ≥7.0 mmol/L (after at least 8 hours of fasting), random plasma glucose ≥11.1 mmol/L in the presence of typical symptoms of diabetes, and/or current treatment with antidiabetic medication.

3.5 Impaired Fasting Glucose

Impaired fasting glucose (IFG) was defined as fasting plasma glucose >5.6 mmol/L and <7.0 mmol/L in individuals without a previous diagnosis of diabetes mellitus.

Additional assessments, including glycated hemoglobin (HbA1c), oral glucose tolerance test (OGTT), insulin measurements, HOMA-IR, and other indices of insulin secretion or insulin resistance, were not performed in the present study.

3.6 Obesity

Obesity was assessed using the body mass index (BMI). Participants with a BMI ≥25 kg/m² were classified as overweight/obese for the purposes of this study.

3.7 Genetic Profiles

The following genetic polymorphisms were analyzed:

LEPR Q223R polymorphism (rs1137101), located on chromosome **1p31**.

Genotypes were classified as:

- **AA** – homozygous for the wild-type allele;
- **AG (GA)** – heterozygous;
- **GG** – homozygous for the minor allele.

LEP -2548G>A polymorphism (rs7799039), located in the promoter region of the **LEP** gene on chromosome **7q31.3**.

Genotypes were classified as:

- **GG** – homozygous for the wild-type allele;
- **GA (AG)** – heterozygous;
- **AA** – homozygous for the minor allele.

ADIPOQ rs266729 polymorphism, located in the promoter region of the **ADIPOQ** gene on chromosome **3q27**.

Genotypes were classified as:

- **CC** – homozygous for the wild-type allele;
- **CG (GC)** – heterozygous;
- **GG** – homozygous for the minor allele.

3.2.6 Plasma Lipid Profile

Plasma concentrations of HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) were expressed in mmol/L.

Peripheral venous blood samples were collected from all participants into standard vacuum tubes for biochemical analysis. Plasma lipid concentrations were determined using commercially available enzymatic assays.

4. Methods

4.1 Documentary Review

Medical records were reviewed for all participants. Information regarding medical history, comorbidities, hospital discharge reports, and laboratory findings was extracted from the available clinical documentation.

4.2 Clinical Assessment

All participants underwent a detailed medical history interview and physical examination. Additional information regarding the onset and clinical course of obesity and type 2 diabetes mellitus was obtained whenever relevant clinical records were available.

4.3 Biochemical Analysis

Biochemical parameters were determined in fasting venous blood samples collected after an overnight fast of at least 8 hours.

The following variables were measured:

- **Fasting plasma glucose (FPG)** (mmol/L); values >5.6 mmol/L were considered elevated.

- **Total cholesterol (TC)** (mmol/L); values >5.6 mmol/L were considered elevated.
- **Low-density lipoprotein cholesterol (LDL-C)** (mmol/L); values >3.4 mmol/L were considered elevated.
- **High-density lipoprotein cholesterol (HDL-C)** (mmol/L); values <1.0 mmol/L were considered reduced.
- **Triglycerides (TG)** (mmol/L); values >1.7 mmol/L were considered elevated.
- **Serum creatinine** (μmol/L); individuals with serum creatinine concentrations >250 μmol/L were excluded from the study.

4.4 Molecular Genetic Analysis

Genetic analyses were performed at the **University Research Laboratory, Medical University – Pleven**, and included the following procedures:

- Isolation and storage of genomic DNA from all study participants;
- Design of highly specific primer pairs flanking the polymorphic regions;
- *In vitro* validation of the designed primers and optimization of the annealing temperature using gradient polymerase chain reaction (gradient PCR);
- Genotyping by polymerase chain reaction–restriction fragment length polymorphism **(PCR–RFLP)** analysis;
- Separation and visualization of PCR products by horizontal agarose gel electrophoresis.

Genomic DNA Isolation

Genomic DNA was extracted from peripheral venous blood using one of two methods, depending on the quantity and quality of the biological material:

- QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions;
- Conventional salting-out DNA extraction method (Appendix 5).

Primer Design

Primer pairs were designed using **Primer3** software. Approximately 1,000 bp genomic sequences surrounding each polymorphic locus were retrieved from the National Center for Biotechnology Information **(NCBI)** database. Based on these reference sequences, specific primer pairs were designed for amplification of the investigated polymorphic regions.

Table 3. Primer sequences used for amplification of the **LEPR rs1137101 (Q223R)** polymorphism.

Primer	Sequence (5′→3′)
Forward primer	GCTCTTATTTTTCAATATAGGC
Reverse primer	ATCATTACAGTGTTAAGCAAA

*Primers were purchased from **Bioneer** (Daejeon, Republic of Korea).*

Table 4. Primer sequences used for amplification of the **LEP rs7799039 (-2548G>A)** polymorphism.

Primer	Sequence (5′→3′)
Forward primer	TTTCCTGTAATTTTCCCGTGAG
Reverse primer	AAAGCAAAGACAGGCATAAAAA

*Primers were purchased from **Metabion International AG** (Planegg, Germany).*

Table 5. Primer sequences used for amplification of the **ADIPOQ rs266729** polymorphism.

Primer	Sequence (5′→3′)
Forward primer	GGTGGACTTGACTTTACTGG
Reverse primer	TAGAAGCAGCCTGGAGAA

*Primers were purchased from **Metabion International AG** (Planegg, Germany).*

Optimization of PCR Conditions by Gradient PCR

Prior to genotyping, the primer pairs were validated *in vitro*, and the optimal annealing temperatures were determined using gradient polymerase chain reaction (gradient PCR).

Genomic DNA was diluted to a working concentration of 5 ng/µL, as determined using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 6. PCR reaction mixture and amplification conditions used for gradient PCR

PCR reaction mixture	PCR step	Temperature	Time
Genomic DNA (5 ng/µL)	Initial denaturation	94°C	5 min
200 µM dNTPs	Denaturation	94°C	30 s
0.3 µM Forward primer	Annealing (gradient)	45–65°C	30 s
0.3 µM Reverse primer	Extension	72°C	45 s
0.5 U Hot Start Taq DNA polymerase	Final extension	72°C	5 min

Final reaction volume: 20 µL	Number of cycles	**35**	

The PCR reaction mixture contained 0.3 µM of each primer, 200 µM dNTPs (Thermo Fisher Scientific, USA), 1.5 mM $MgCl_2$, 0.5 U Hot Start Taq DNA polymerase (Canvax Biotech, Spain), and 10 mM Tris-HCl (pH 7.4) in a final reaction volume of 20 µL.

PCR amplification was performed using a qTOWER³ G thermal cycler (Analytik Jena, Germany). Cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45–65°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

The optimal annealing temperature for each primer pair was determined by applying a temperature gradient ranging from 45°C to 65°C, and the temperature producing the highest amplification specificity and yield was selected for subsequent PCR–RFLP analyses.

Table 6. PCR reaction mixture and thermal cycling conditions used for gradient PCR

PCR reaction mixture	PCR step	Temperature	Time
Genomic DNA (5 ng/µL)	Initial denaturation	94°C	5 min
200 µM dNTPs	Denaturation	94°C	30 s
0.3 µM Forward primer	Annealing (gradient)	45–65°C	30 s
0.3 µM Reverse primer	Extension	72°C	45 s
0.5 U Hot Start Taq DNA polymerase	Final extension	72°C	5 min
Final reaction volume: 20 µL	**Number of cycles**	**35**	

PCR amplification was performed using a qTOWER³ G thermal cycler (Analytik Jena, Germany). Hot Start Taq DNA polymerase (Canvax Biotech, Spain) was used for amplification, and primers were synthesized by Metabion International AG (Planegg, Germany).

PCR Reaction Conditions

The PCR reaction mixture contained 0.3 µM of each forward and reverse primer, 200 µM dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), 1.5 mM $MgCl_2$, 0.5 U Hot Start Taq DNA polymerase (Canvax Biotech, Spain), and 10 mM Tris-HCl (pH 7.4) in a final reaction volume of 20 µL.

PCR amplification was performed using a qTOWER³ G thermal cycler (Analytik Jena, Jena, Germany).

The amplification protocol consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45–65°C for 30 s, and extension at 72°C

for 45 s, with a final extension at 72°C for 5 min. The optimal annealing temperature for each primer pair was determined using a 20°C temperature gradient (45–65°C), and the temperature providing the highest amplification specificity and yield was selected for subsequent PCR–RFLP analyses.

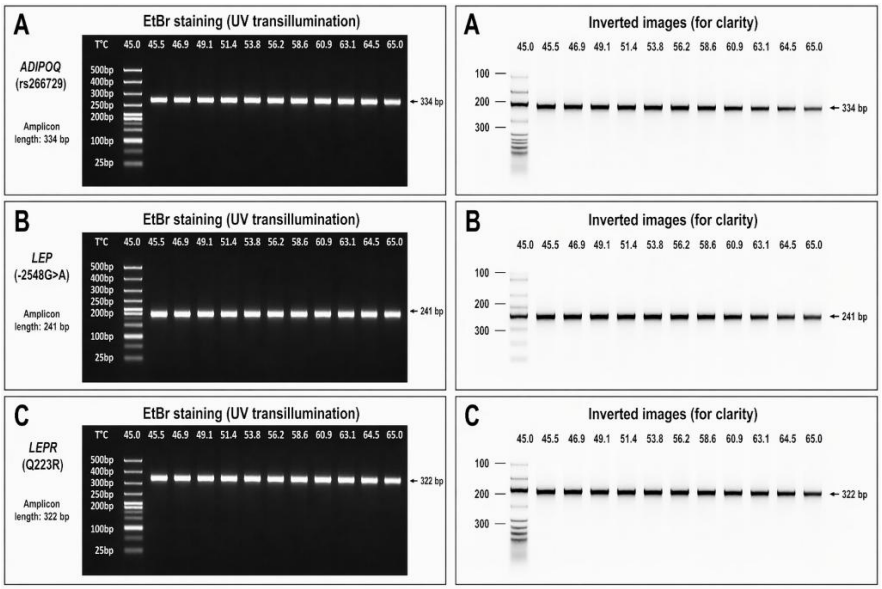


Figure 12. Gradient PCR demonstrating the optimization of the annealing temperature for the *de novo*-designed primer pairs following separation by horizontal agarose gel electrophoresis. HyperLadder™ 25 bp and 100 bp DNA Ladder (Bioline, Germany) were used as molecular size markers.

Restriction Fragment Length Polymorphism (PCR–RFLP) Analysis

Genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis. This technique detects sequence variations based on differences in the lengths of restriction fragments generated by endonuclease digestion. Single nucleotide polymorphisms (SNPs) may create or abolish restriction enzyme recognition sites, resulting in genotype-specific restriction fragment patterns following agarose gel electrophoresis (Appendix 6).

Restriction Digestion Conditions

The restriction digestion conditions used for the three investigated polymorphisms were as follows:

LEPR rs1137101 Genotyping

For amplification of the LEPR rs1137101 polymorphism, PCR was performed in a final reaction volume of 20 µL containing 5 ng/µL genomic DNA, 0.3 µM of each forward and reverse primer, 200 µM dNTPs (Thermo Fisher Scientific, USA), 1.5 mM $MgCl_2$, 0.5 U thermostable Taq DNA polymerase, and 10 mM Tris-HCl (pH 7.4).

PCR amplification was carried out under the following cycling conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 45 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

Table 7. PCR reaction components and thermal cycling conditions for LEPR rs1137101 genotyping

Reaction mixture components	PCR step	Temperature	Time / Number of cycles
1 µL genomic DNA (5 ng/µL)	Initial denaturation	94°C	5 min
200 µM dNTPs	Denaturation	94°C	30 s
0.3 µM Forward primer	Annealing	60°C	45 s
0.3 µM Reverse primer	Extension	72°C	45 s
0.5 U Taq DNA polymerase	Final extension	72°C	5 min
Final reaction volume: 20 µL	**Total number of cycles**	—	**35 cycles**

PCR amplification was performed using a qTOWER³G thermal cycler (Analytik Jena, Germany). Primers were synthesized by Bioneer (Daejeon, Republic of Korea). Taq DNA polymerase and dNTPs were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Table 8. PCR reaction components and thermal cycling conditions for LEP rs12112075 genotyping

Reaction mixture components	PCR step	Temperature	Time / Number of cycles
1 µL genomic DNA (5 ng/µL)	Initial denaturation	94°C	5 min
200 µM dNTPs	Denaturation	94°C	30 s
0.3 µM Forward primer	Annealing	54°C	45 s
0.3 µM Reverse primer	Extension	72°C	45 s
0.5 U Taq DNA polymerase	Final extension	72°C	5 min

Final reaction volume: 20 µL	**Total number of cycles**	—	**35 cycles**

PCR amplification was performed using a qTOWER³G PCR thermal cycler (Analytik Jena, Germany). Primers were synthesized by Bioneer (Daejeon, Republic of Korea). Taq DNA polymerase and dNTPs were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The PCR amplification generated a 241-bp amplicon encompassing the investigated polymorphic region.

ADIPOQ rs266729 Genotyping

For amplification of the ADIPOQ rs266729 polymorphism located in the promoter region of the ADIPOQ gene, PCR was performed in a final reaction volume of 20 µL containing 5 ng/µL genomic DNA, 0.3 µM of each forward and reverse primer, 200 µM dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), 1.5 mM $MgCl_2$, 0.5 U thermostable Taq DNA polymerase, and 10 mM Tris-HCl (pH 7.4).

PCR amplification was carried out using the following cycling conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 45 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

Table 9. PCR reaction components and thermal cycling conditions for ADIPOQ rs266729 genotyping

Reaction mixture components	PCR step	Temperature	Time / Number of cycles
1 µL genomic DNA (5 ng/µL)	Initial denaturation	94°C	5 min
200 µM dNTPs	Denaturation	94°C	30 s
0.3 µM Forward primer	Annealing	57°C	45 s
0.3 µM Reverse primer	Extension	72°C	45 s
0.5 U Taq DNA polymerase	Final extension	72°C	5 min
Final reaction volume: 20 µL	Total number of cycles	—	**35 cycles**

PCR amplification was performed using a qTOWER³G PCR thermal cycler (Analytik Jena, Germany). Primers were synthesized by Bioneer (Daejeon, Republic of Korea). Taq DNA polymerase and dNTPs were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

The PCR amplification generated a 334-bp amplicon encompassing the investigated polymorphic region.

Following PCR amplification, the amplicons were subjected to restriction enzyme digestion using highly specific restriction endonucleases.

LEPR rs1137101 Restriction Fragment Length Polymorphism (PCR–RFLP) Analysis

PCR products containing the LEPR rs1137101 polymorphism were digested with MspI restriction endonuclease (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of the amplified products were incubated with MspI at 37°C for 12 h.

The restriction fragments were separated by 2.5% agarose gel electrophoresis and visualized following ethidium bromide staining.

Genotypes were identified according to the restriction fragment pattern:

- AA genotype (wild-type homozygote): a single undigested fragment of 334 bp.*
- AG genotype (heterozygote): three fragments of 334 bp, 192 bp, and 154 bp.*
- GG genotype (minor allele homozygote): two restriction fragments of 192 bp and 154 bp.

*Fragment sizes should be verified against the original laboratory protocol, as the reported amplicon size and restriction fragment lengths should be internally consistent.

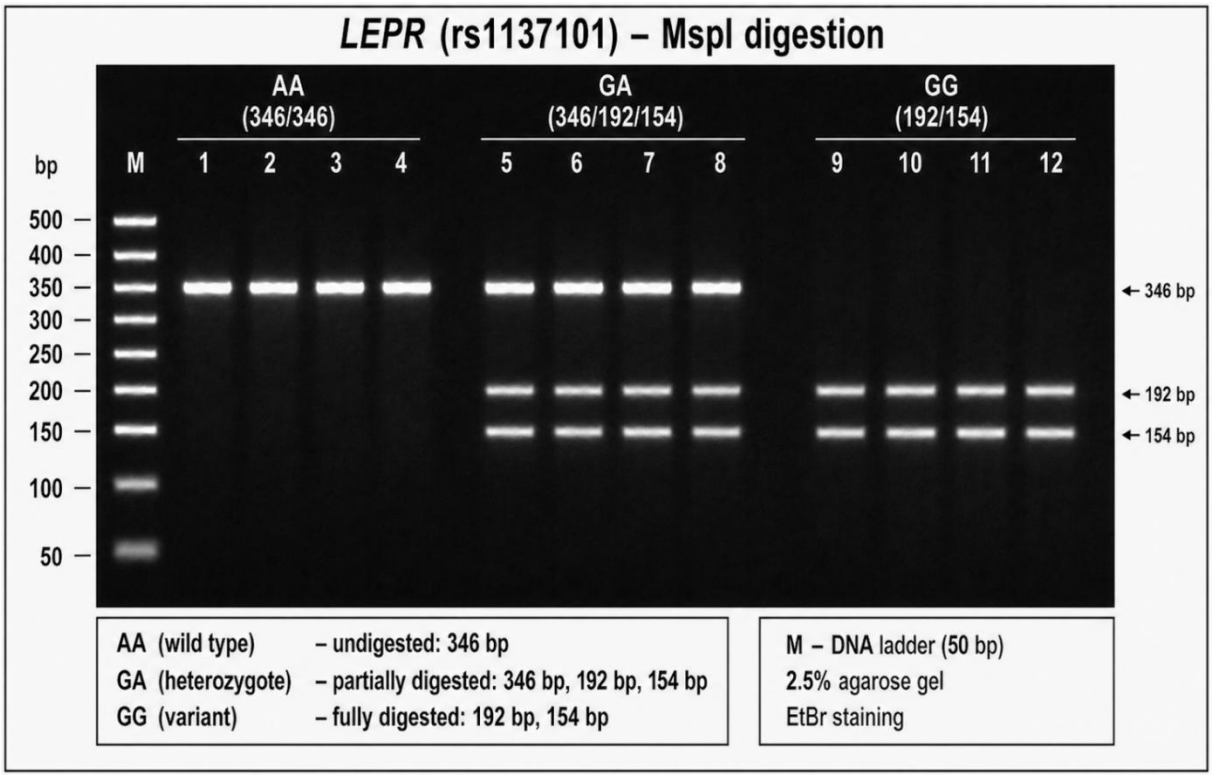


Figure 13. Visualization of the restriction digestion products for the LEPR rs1137101 polymorphism following PCR–RFLP analysis.

Panel A (AA genotype, wild-type homozygote): All samples exhibited a single 346-bp fragment, corresponding to the undigested PCR product.

Panel B (GA genotype, heterozygote): Three fragments were observed in each sample, corresponding to 346 bp, 192 bp, and 154 bp, indicating the presence of both the wild-type and variant alleles.

Panel C (GG genotype, variant homozygote): Two restriction fragments of 192 bp and 154 bp were detected, indicating complete digestion of the PCR product.

DNA fragments were separated by 2.5% agarose gel electrophoresis and visualized using HyperLadder™ 50 bp DNA Marker (Bioline, Germany).

Leptin (LEP) gene polymorphism rs12112075

Restriction digestion was performed using HhaI restriction endonuclease (Thermo Scientific, USA). Aliquots of the PCR products were incubated with HhaI at 37°C for 12 h. The resulting restriction fragments for the LEP rs12112075 polymorphism (182 bp and 59 bp) were separated by 2.5% agarose gel electrophoresis and visualized following ethidium bromide staining (Analytik Jena, Germany).

For individuals carrying the wild-type homozygous genotype (GG), a single 241-bp fragment corresponding to the undigested PCR product was observed.

Heterozygous carriers (GA/AG) exhibited three fragments of 241 bp, 182 bp, and 59 bp, indicating the presence of both wild-type and variant alleles.

Individuals homozygous for the variant allele (AA) displayed two restriction fragments of 182 bp and 59 bp, indicating complete digestion of the PCR product.

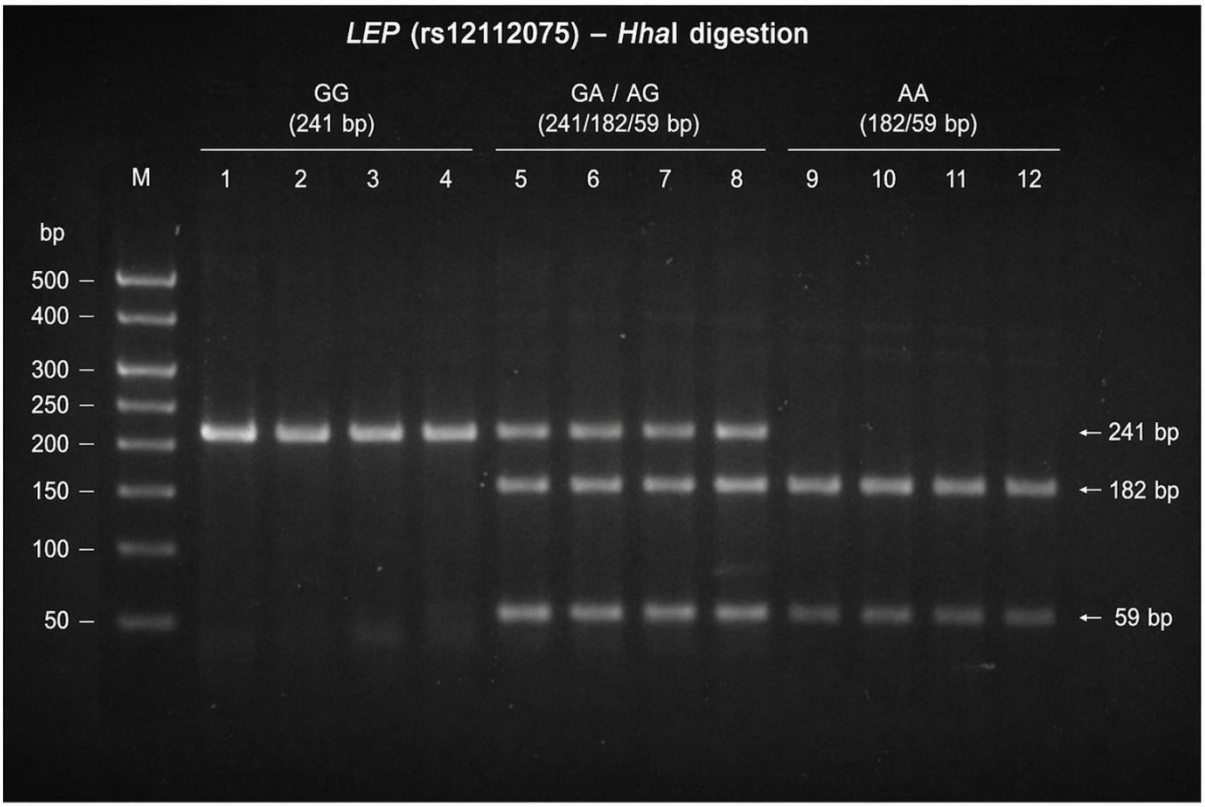


Figure 14. Representative agarose gel electrophoresis of the PCR–RFLP products for the LEP rs12112075 polymorphism following HhaI restriction enzyme digestion. *Panel A: GG genotype (wild type) – all samples display a single 241-bp fragment. Panel B: GA genotype (heterozygous) – three fragments are observed in each sample (241 bp, 182 bp, and 59 bp). Panel C: AA genotype (variant homozygous) – samples display two fragments (182 bp and 59 bp). Molecular weight marker: HyperLadder™ 50 bp (Bioline, Germany).*

AdipoQ rs266729 promoter polymorphism

PCR products encompassing the AdipoQ rs266729 promoter polymorphism were digested with HhaI restriction endonuclease (Thermo Scientific, USA). Aliquots of the amplified PCR products were incubated with HhaI at 37°C for 12 h. The resulting restriction fragments, with expected sizes of 213 bp and 121 bp, were separated by 2.5% horizontal agarose gel electrophoresis and visualized following ethidium bromide staining (Analytik Jena, Germany). Samples carrying the wild-type homozygous genotype (CC) exhibited a single 334-bp DNA fragment, indicating the absence of the restriction site.

Samples with the heterozygous genotype (CG) displayed three DNA fragments of 334 bp, 213 bp, and 121 bp, reflecting the presence of both digested and undigested PCR products.

Samples carrying the variant homozygous genotype (GG) showed two DNA fragments of 213 bp and 121 bp, indicating complete digestion of the PCR amplicon by HhaI.

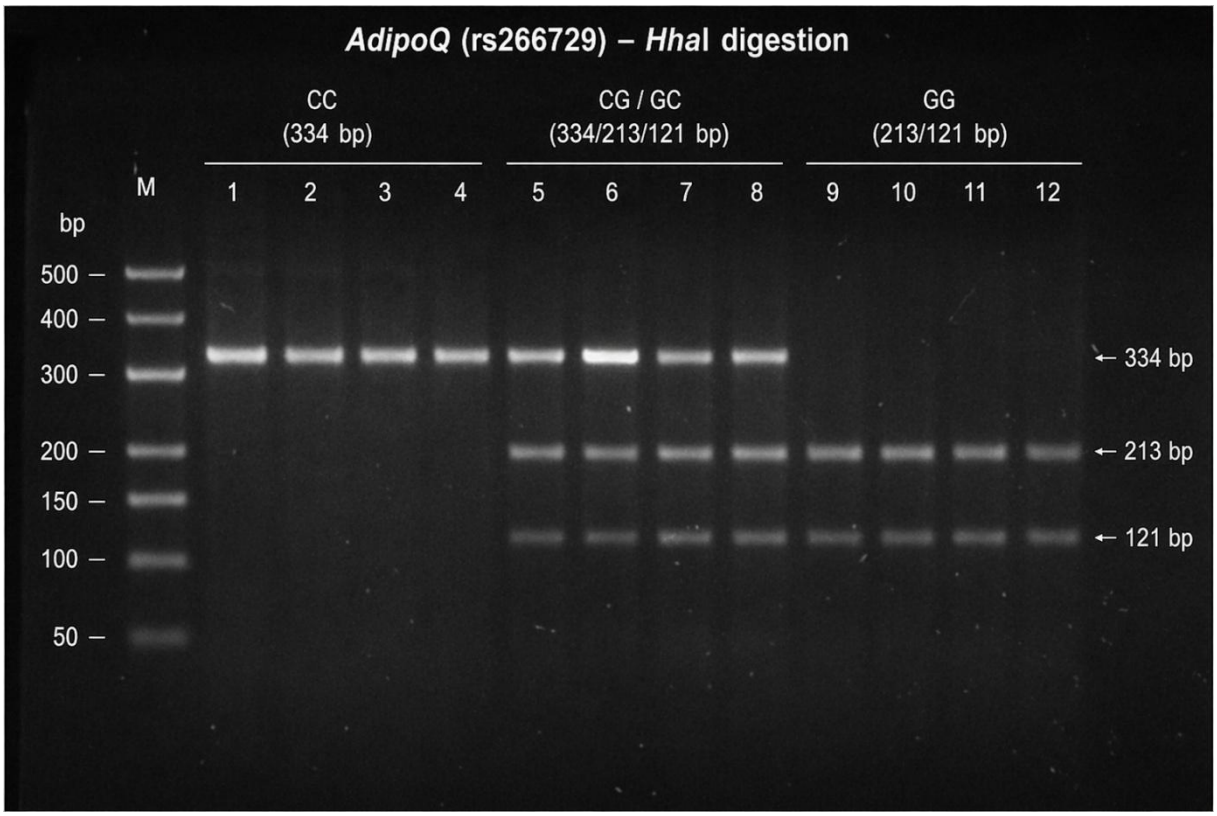


Figure 15. Visualization of the restriction fragment length polymorphism (RFLP) analysis of the AdipoQ rs266729 polymorphism following HhaI digestion.

Panel A (CC genotype – wild type): All samples show a single DNA fragment of approximately 334 bp.Panel B (CG genotype – heterozygous): Each sample displays three DNA fragments of approximately 334 bp, 213 bp, and 121 bp.Panel C (GG genotype – homozygous variant): Samples exhibit two DNA fragments of approximately 213 bp and 121 bp. Molecular weight marker: HyperLadder™ 50 bp DNA Ladder (Bioline, Germany).

5. Statistical Analysis

The results obtained from all **157 study participants** were entered into a Microsoft Excel database and subsequently analyzed. The dataset included the following variables:

- Demographic characteristics (sex, age, and age group);
- Arterial hypertension;
- Type 2 diabetes mellitus (T2DM);
- Obesity;
- Chronic kidney disease;
- Previous statin therapy;
- Fasting plasma glucose (FPG);
- Lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides);

- Serum creatinine;
- Genotype of **LEPR rs1137101**;
- Genotype of **LEP rs12112075**;
- Genotype of **ADIPOQ rs266729**.

Statistical analyses were performed using **IBM SPSS Statistics version 23.0** (IBM Corp., Armonk, NY, USA). Statistical significance was accepted at a two-sided p-value < 0.05.

The following statistical methods were applied:

- Descriptive statistics were used to summarize demographic, clinical, biochemical, and genetic characteristics. Continuous variables are presented as mean ± standard deviation (SD), whereas categorical variables are presented as absolute numbers and percentages.
- The normality of continuous variables was assessed using the Shapiro–Wilk test.
- Comparisons between two independent groups were performed using the Student's t-test for normally distributed variables or the Mann–Whitney U test for non-normally distributed variables.
- Comparisons among three genotype groups were performed using one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, as appropriate.
- Associations between categorical variables, including genotype and allele frequencies, were evaluated using the Pearson chi-square (χ^2) test or Fisher's exact test, where appropriate.
- The strength of associations between genetic polymorphisms and obesity, T2DM, arterial hypertension, and other cardiometabolic traits was estimated by calculating odds ratios (ORs) with corresponding 95% confidence intervals (95% CIs).
- Allelic, genotypic, dominant, recessive, and additive genetic models were analyzed where appropriate.
- A Cochran–Armitage trend test (allele-dose trend analysis) was applied to evaluate dose-dependent effects of the risk alleles on metabolic phenotypes.
- Correlations between continuous variables were assessed using Pearson's or Spearman's correlation coefficients, depending on data distribution.

All statistical tests were two-tailed, and a p-value of <0.05 was considered statistically significant.

The following statistical methods were applied:

5.1 Descriptive analysis was used to summarize the frequency distribution of the investigated variables according to the study groups.

5.2 Descriptive statistics included measures of central tendency and dispersion.

5.3 Graphical analysis was performed to visualize the distribution of the study variables and the obtained results.

5.4 Comparative analysis was used to compare proportions between study groups.

5.5 Pearson's chi-square (χ^2) test and **Fisher's exact test** were applied to assess associations between categorical variables.

5.6. One-way analysis of variance (one-way ANOVA) was used to compare continuous variables among three or more independent groups.

5.7 Student's independent-samples t-test was used to compare continuous variables between two independent groups.

5.8 Binary and multinomial logistic regression analyses were performed to quantify the effects of the investigated variables on the studied outcomes.

Comparisons of categorical variables between study groups were performed using the Pearson chi-square (χ^2) test. Continuous variables are presented as mean ± standard deviation (SD) or median (interquartile range [IQR], 25th–75th percentile), depending on the distribution of the data. Differences between groups with normally distributed continuous variables were assessed using the Student's t-test or one-way ANOVA, as appropriate. A two-sided p-value < 0.05 was considered statistically significant.

III. Results

Table 10. Anthropometric and Biochemical Characteristics of Obese and Non-Obese Participants

Table 10. Anthropometric and Biochemical Characteristics of Obese and Non-Obese Participants

Phenotypic characteristic	Obese with T2DM (n = 82)	Non-obese controls (n = 74)	p-value
Age (years)	69.15 ± 0.87	72.35 ± 0.84	0.009
BMI (kg/m²)	34.23 ± 0.21	22.42 ± 0.15	<0.001
Total cholesterol (mmol/L)	4.55 ± 0.15	5.05 ± 0.15	0.018
HDL cholesterol (mmol/L)	1.22 ± 0.05	1.38 ± 0.03	0.014
LDL cholesterol (mmol/L)	2.61 ± 0.12	3.02 ± 0.13	0.025
Fasting plasma glucose (mmol/L)	6.58 ± 0.26	6.16 ± 0.20	0.205
Hypertension, n (%)	47.4	21.8	<0.001

Type 2 diabetes mellitus, n (%)	26.3	10.9	<0.001

Data are presented as mean ± standard deviation (SD) or percentage, as appropriate. Statistical significance was defined as $p < 0.05$. *Values are presented as mean ± standard deviation (SD) for continuous variables and as percentages for categorical variables. The number of participants in each group is shown in parentheses. Continuous variables were compared using Student's independent-samples t-test, whereas categorical variables were analyzed using the Pearson chi-square (χ^2) test. Natural logarithmic transformation was applied prior to statistical analysis where appropriate. A p-value < 0.05 was considered statistically significant. BMI, body mass index; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FPG, fasting plasma glucose.*

The study cohort comprised 156 individuals, who were divided into two groups: 82 obese participants (BMI ≥25 kg/m²) and 74 non-obese controls. Participants were aged between 35 and 85 years, and all belonged to an ethnically homogeneous population from Northern Bulgaria. The study employed a case–control design, which is appropriate for investigating associations between genetic polymorphisms and disease phenotypes but does not permit conclusions regarding causal relationships.

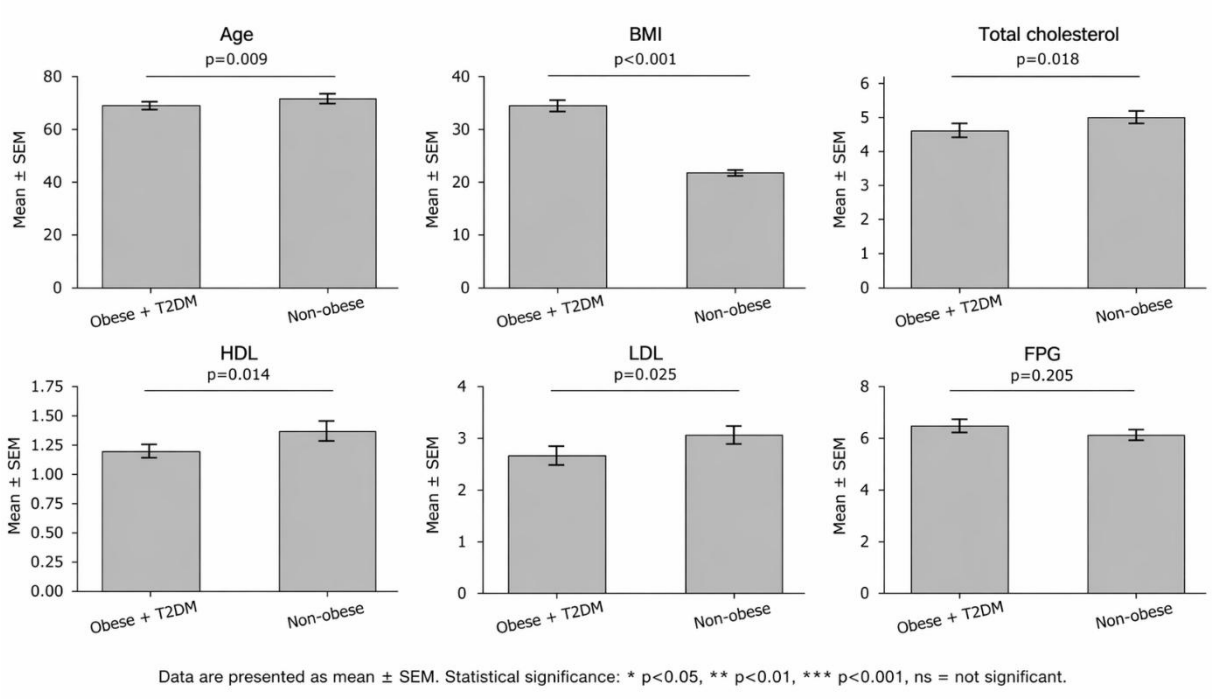


Figure 16. Phenotypic characteristics of obese individuals with type 2 diabetes mellitus (T2DM) and non-obese controls. *Data are presented as mean ± standard error of the mean (SEM). Statistical significance is indicated by the corresponding p-values. Graphs were generated using GraphPad Prism.*

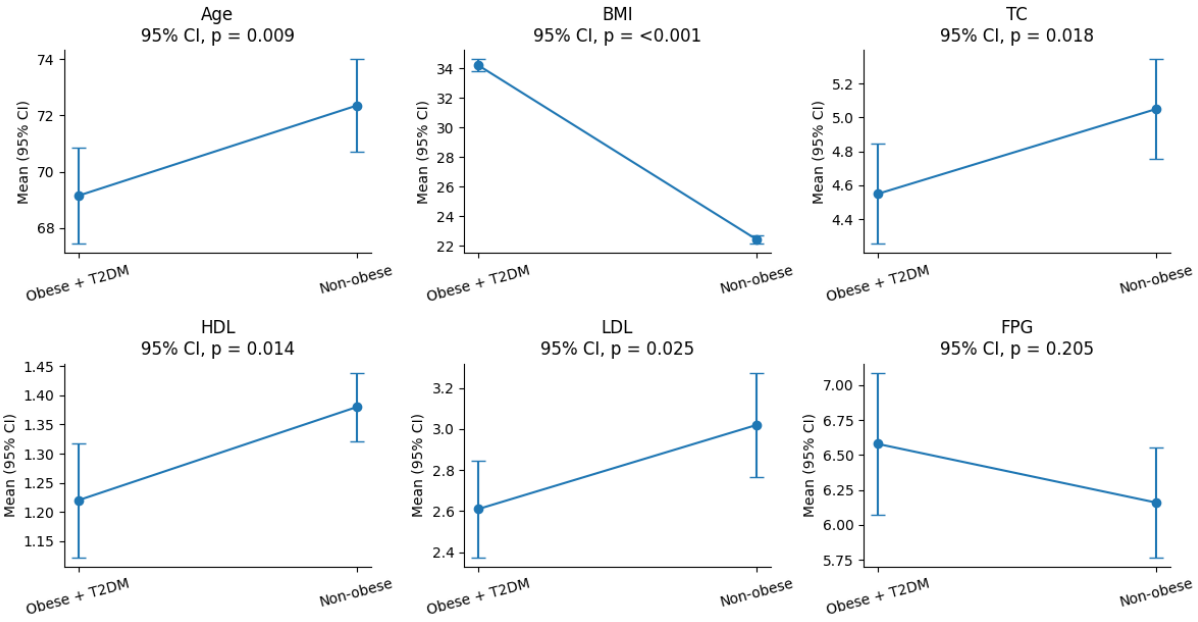


Figure 17. Comparison of phenotypic characteristics between obese individuals with type 2 diabetes mellitus (T2DM) and non-obese controls. *Data are presented as mean values with 95% confidence intervals (95% CI). Differences between groups were evaluated using one-way analysis of variance (one-way ANOVA), and the corresponding p-values are indicated for each parameter.*

The phenotypic and clinical characteristics of the study population are summarized in Table 10. As expected, the obese participants with T2DM exhibited significantly higher BMI, total cholesterol, LDL-cholesterol, and prevalence of hypertension and type 2 diabetes mellitus, together with significantly lower HDL-cholesterol levels compared with the non-obese control group. In contrast, no statistically significant difference was observed in fasting plasma glucose (FPG) levels between the two groups ($p = 0.205$).

Overall, the observed metabolic profile is consistent with the characteristics of metabolic syndrome. The absence of a significant difference in FPG may reflect either an early stage of dysglycaemia or heterogeneity in diabetes severity among the study participants. These findings further emphasize the importance of genetic analyses, as phenotypic characteristics alone may not be sufficient to discriminate individuals with different cardiometabolic risk profiles.

1. Adiponectin

Table 11. Genotype and Allele Frequencies of the ADIPOQ rs266729 Polymorphism and Its Association with Type 2 Diabetes Mellitus (T2DM) in the Study Population

Genotype rs266729 (C/G)	Control n=74	Obese, T2DM=82	Unjustified OR (95%CI) *p* value	Adjusted OR for age and BMI (95%CI) *p* value
CC	56 (75%)	46 (56%)	Reference	
CG	14 (19%)	23 (28%)	2.40 (1.4-4.18) 0.002	2.30 (1.40-4.22) 0.001
GG	4 (6%)	13 (16%)	3.55 (1.30-10.70) 0.01	3.62 (1.28-10.70) 0.01
Frequency of G allele	22 (15%)	49 (30%)	2.39 (1.50-3.72) 0.001	

Data are presented as the number of individuals, with percentages shown in parentheses. Associations between genotype and allele frequencies were evaluated using the Pearson chi-square (χ^2) test or Fisher's exact test, as appropriate. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to estimate the strength of the associations. OR, odds ratio; CI, confidence interval.

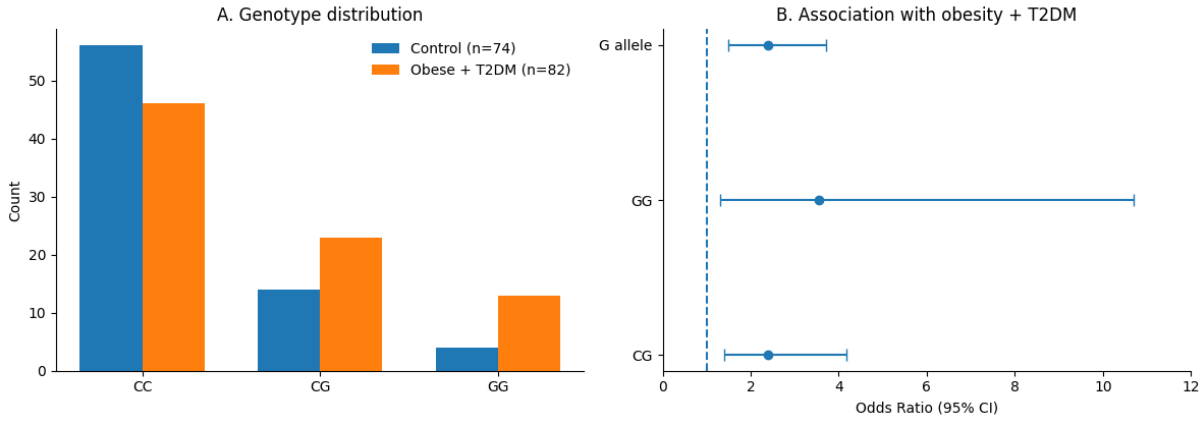


Figure 18. Association analysis of the ADIPOQ rs266729 polymorphism with obesity and type 2 diabetes mellitus (GraphPad Prism, multipanel plot).
(A) Distribution of the CC, CG, and GG genotypes in the control group and in patients with obesity and type 2 diabetes mellitus (T2DM).
(B) Forest plot showing the odds ratios (ORs) and 95% confidence intervals (95% CIs) for the CG and GG genotypes and the G allele, using the CC genotype as the reference category. The dashed vertical line indicates OR = 1.0 (no association). Statistical analysis was performed using GraphPad Prism, with odds ratios calculated by binary logistic regression and **group comparisons performed using the χ^2 (chi-square) test.

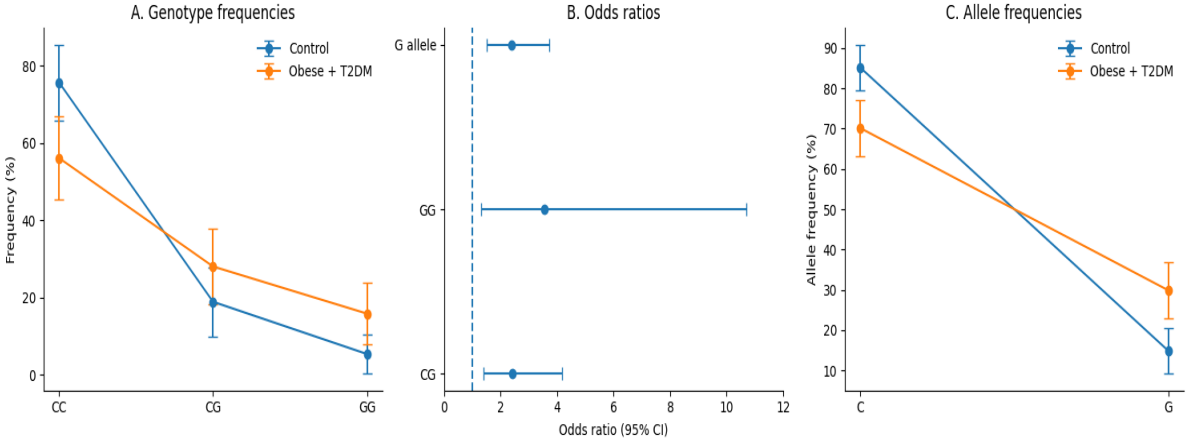


Figure 19. Association of the ADIPOQ rs266729 genotype and allele frequencies with obesity and type 2 diabetes mellitus (T2DM). (A) Distribution of the CC, CG, and GG genotypes (%) in the control group and the obesity + T2DM group. (B) Odds ratios (ORs) with 95% confidence intervals (95% CIs) for the CG and GG genotypes and the G allele relative to the reference CC genotype. The dashed vertical line indicates OR = 1.0. (C) Distribution of the C and G allele frequencies (%) in the two study groups. Graphs were generated using GraphPad Prism.

Genotype association analysis of the ADIPOQ rs266729 polymorphism demonstrated a significant relationship between the polymorphic variants and obesity with type 2 diabetes mellitus (T2DM). The GG genotype (homozygous variant), CG genotype (heterozygous), and CC genotype (wild-type homozygous) were evaluated.

The principal genetic finding was a significantly higher frequency of the G allele among obese individuals with T2DM compared with the control group (p = 0.001). Both the GG and CG genotypes were significantly associated with obesity and T2DM.

Risk estimation showed that individuals carrying the GG genotype had an approximately three-fold higher risk of elevated fasting plasma glucose (FPG) compared with the control group, whereas carriers of the CG genotype exhibited an approximately two-fold increased risk.

These findings indicate a dose-dependent genetic effect, with the risk increasing progressively across genotypes (CC < CG < GG), suggesting that the G allele contributes additively to susceptibility to impaired glucose homeostasis and obesity-associated T2DM.

Table 12. Association of the ADIPOQ rs266729 Polymorphism with Metabolic Parameters in Obese Patients with Type 2 Diabetes Mellitus (T2DM)

Parameter	Genotype	Mean ± SE	95% CI for mean	ANOVA *p*-value
TC (mmol/L)	GG	4.64 ± 0.52	3.53-5.76	0.618
	CG	4.64 ± 0.20	4.24-5.04	
	CC	4.31 ± 0.19	3.91-4.70	
HDL-C (mmol/L)	GG	1.58 ± 0.16	1.33-1.94	<0.001
	CG	1.08 ± 0.07	0.98-1.18	
	CC	0.70 ± 0.07	0.51-0.23	
LDL-C (mmol/L)	GG	2.83 ± 0.45	1.86-3.79	0.444
	CG	2.66 ± 0.16	2.35-2.98	
	CC	2.38 ± 0.15	2.07-2.69	
FPG (mmol/L)	GG	4.53 ± 0.17	4.16-4.89	<0.001
	CG	5.92 ± 0.15	5.62-6.22	
	CC	9.20 ± 0.54	8.08-10.32	

Table 12. Association of the ADIPOQ rs266729 Polymorphism with Metabolic Parameters in Obese Individuals (BMI >30 kg/m²) with Type 2 Diabetes Mellitus (T2DM).

Data are presented as mean ± standard deviation (SD). Comparisons among genotype groups were performed using one-way analysis of variance (one-way ANOVA). TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FPG, fasting plasma glucose; CI, confidence interval.

Figure 20. Comparison of biochemical parameters according to ADIPOQ rs266729 genotypes. Data are presented as grouped columns (mean ± SEM). Comparisons among the CC, CG, and GG genotype groups were performed using one-way analysis of variance (one-way ANOVA). Statistical significance is indicated by *p*-values. Graphs were generated using GraphPad Prism.

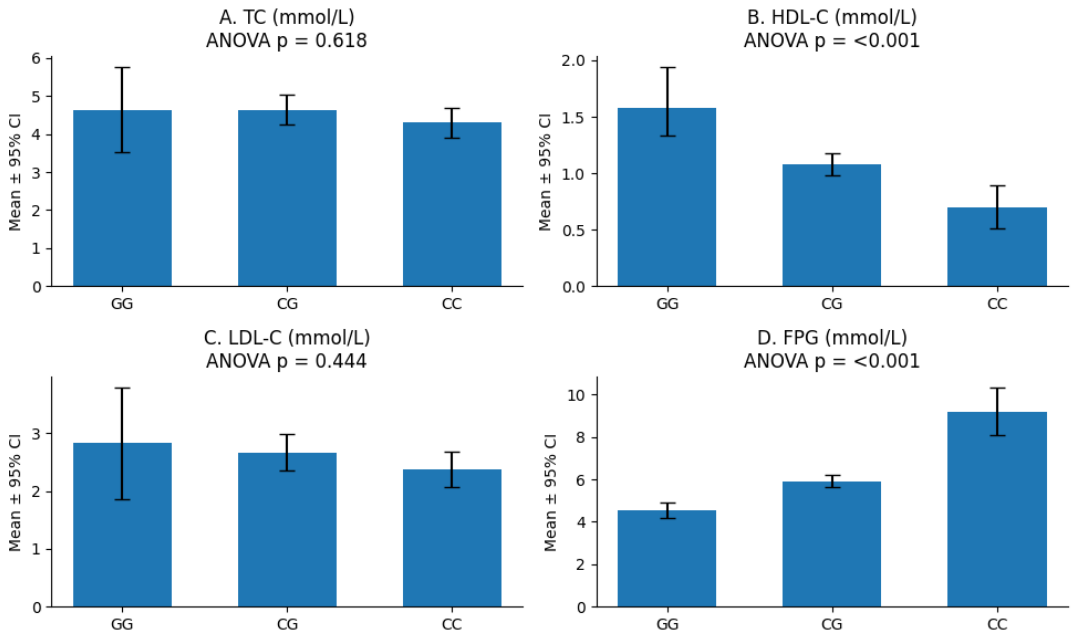


Figure 20. Comparison of biochemical parameters according to ADIPOQ rs266729 genotypes. Data are presented as grouped columns (mean ± SEM). Comparisons among the CC, CG, and GG genotype groups were performed using one-way analysis of variance (one-way ANOVA). Statistical significance is indicated by *p*-values. Graphs were generated using GraphPad Prism.

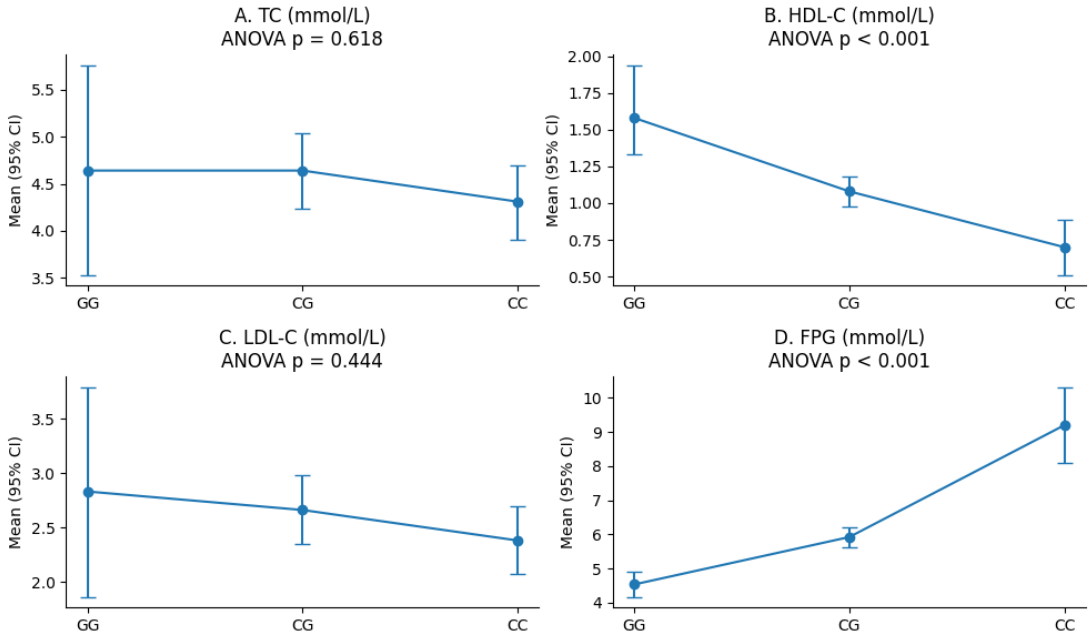


Figure 21. Dot plots with error bars and connected mean values illustrating biochemical parameters according to ADIPOQ rs266729 genotypes. Multi-panel graphs show the mean values of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) across the GG, CG, and CC genotypes of the ADIPOQ rs266729 polymorphism. Data are presented as mean ± SEM.

Statistical comparisons among genotypes were performed using one-way analysis of variance (one-way ANOVA). Graphs were generated using GraphPad Prism.

Analysis of the biochemical parameters revealed a significant association between the ADIPOQ rs266729 genotype and fasting plasma glucose (FPG) as well as HDL-cholesterol levels (p = 0.001), whereas no significant association was observed with total cholesterol (TC) or LDL-cholesterol levels. These findings suggest that the rs266729 polymorphism primarily influences glucose homeostasis and the protective HDL lipoprotein fraction, rather than overall lipid metabolism.

This observation is consistent with the established physiological functions of adiponectin, including its role in:

- enhancing insulin sensitivity;
- exerting anti-inflammatory effects; and
- providing anti-atherogenic protection.

Collectively, these findings support a model in which the G allele of rs266729 alters ADIPOQ gene expression, leading to reduced adiponectin activity, impaired insulin sensitivity, elevated fasting plasma glucose, and decreased HDL-cholesterol levels. These metabolic alterations may contribute to an increased susceptibility to type 2 diabetes mellitus (T2DM) and metabolic syndrome, in agreement with the well-established role of adiponectin in obesity, insulin resistance, and glucose metabolism.

2. Leptin Receptor (LEPR)

Table 13. Genotype Distribution and Allele Frequencies of the LEPR Q223R Polymorphism in the Study Groups

Genotype	Obese (n=82)	Non-obese (n=74)	OR (CI)	*p*-value
AA	15 (18.3 %)	18 (24.3 %)	0.697 (0.322-1.507)	0.358
AG	44 (53.7 %)	37 (50 %)	1.158 (0.617-2.173)	0.648
GG	23 (28 %)	19 (25.7 %)	1.128 (0.555-2.295)	0.739
A	74 (45.1 %)	73 (49.3 %)		
G	90 (54.9 %)	75 (50.7 %)		

Table 13. Data are presented as the number of individuals, with percentages shown in parentheses. Genotype and allele frequencies were compared using the Pearson chi-square (χ^2) test or Fisher's exact test, as appropriate. Odds ratios (ORs) and 95% confidence intervals (95%

CIs) were calculated to estimate the strength of the associations. OR, odds ratio; CI, confidence interval.

The data presented in Table 13 summarize the genotype distribution and allele frequencies of the LEPR Q223R polymorphism in obese and non-obese individuals, together with the corresponding odds ratios (ORs), 95% confidence intervals (95% CIs), and *p*-values used to assess the statistical significance of the observed associations.

The genotype distribution showed that the AA genotype was present in 18.3% of obese individuals and 24.3% of non-obese controls. The calculated OR of 0.697 (95% CI: 0.322–1.507) suggests a lower odds of obesity among carriers of the AA genotype. However, this association did not reach statistical significance ($p = 0.358$). Therefore, no definitive conclusion can be drawn regarding either a protective or a risk effect of the AA genotype in relation to obesity.

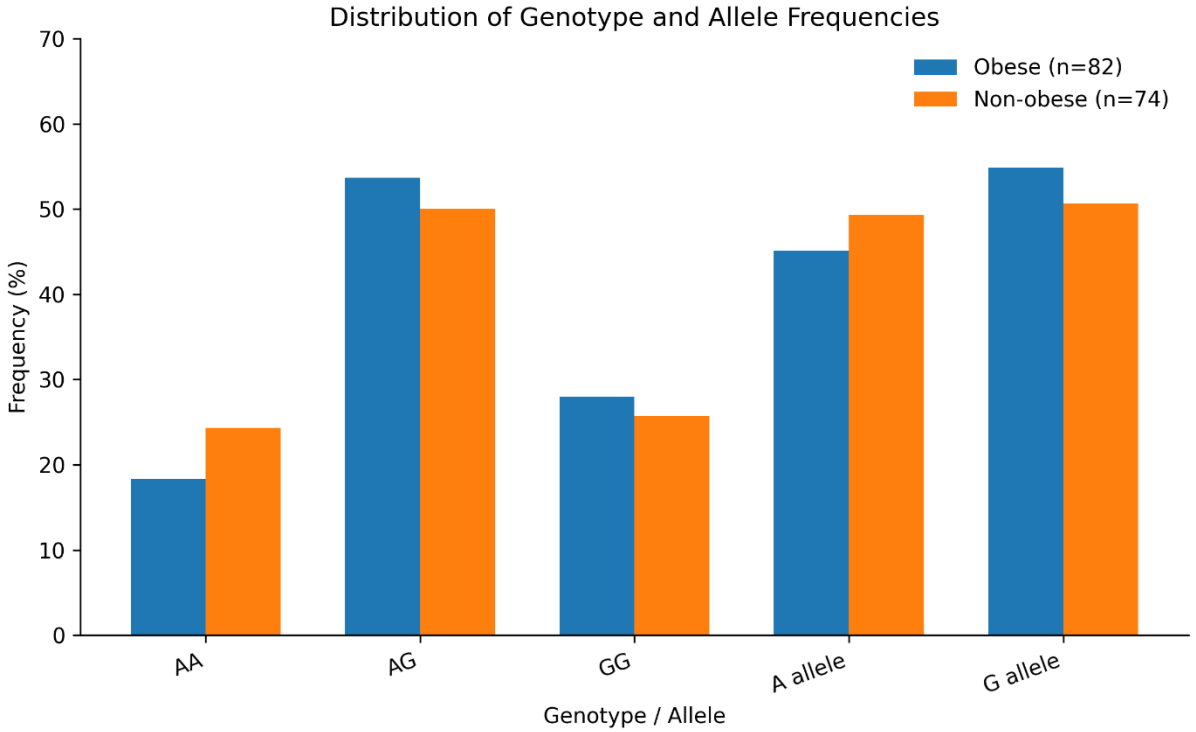


Figure 22. Distribution of LEPR Q223R genotype and allele frequencies. Distribution of the AA, AG, and GG genotypes (%) and A and G allele frequencies (%) in the obese and non-obese study groups. Data are presented as percentages. Graphs were generated using GraphPad Prism.

The AG genotype was the most frequent genotype in both study groups, occurring in 53.7% of obese individuals and 50.0% of non-obese controls. The calculated odds ratio (OR = 1.158; 95% CI: 0.617–2.173) indicated a slight, but statistically non-significant, increase in the odds of obesity among heterozygous individuals. This finding was further supported by the high *p*-value ($p = 0.648$), indicating the absence of a statistically significant association.

Similarly, the GG genotype was observed in 28.0% of obese individuals and 25.7% of non-obese controls. The estimated OR of 1.128 (95% CI: 0.555–2.295) suggested a modest increase in the odds of obesity; however, this association was also not statistically significant ($p = 0.739$). Moreover, the wide 95% confidence interval, which included the null value (OR = 1.0), further supports the lack of a reliable association between the GG genotype and obesity in the study population.

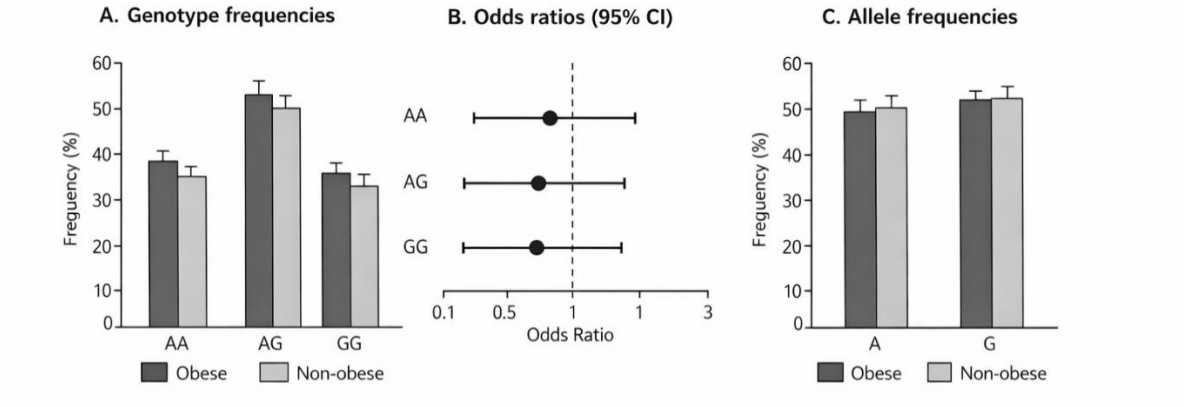


Figure 23. Distribution of LEPR Q223R genotypes and allele frequencies in obese and non-obese individuals. *(A) Genotype distribution (%) of the AA, AG, and GG genotypes. (B) Odds ratios (ORs) with 95% confidence intervals (95% CIs) for the AG and GG genotypes relative to the reference AA genotype. (C) Distribution of A and G allele frequencies (%) in the two study groups. No statistically significant associations were observed (p ≥ 0.05). Graphs were generated using GraphPad Prism.*

At the allele level, the A allele was observed with a frequency of 45.1% in obese individuals and 49.3% in non-obese controls, whereas the G allele occurred at frequencies of 54.9% and 50.7%, respectively. Although the G allele was slightly more frequent among obese individuals, no statistically significant difference in allele frequencies was observed between the two groups.

Overall, the results demonstrate no statistically significant differences in either genotype distribution or allele frequencies between obese and non-obese individuals. All estimated odds ratios (ORs) were close to 1.0, and the corresponding p-values were well above the predefined threshold for statistical significance ($p < 0.05$). These findings indicate that the LEPR Q223R polymorphism is not significantly associated with obesity in the studied Northern Bulgarian population.

Table 14. Association of the LEPR Q223R Polymorphism with Metabolic Parameters in the Study Population (Control and Obesity/T2DM Groups)

Parameters	Genotype	Mean ± SE	95 % CI for mean	ANOVA *p*-value
TC (mmol/L)	AA	4.85 ± 0.27	4.29-5.41	0.314
	AG	4.89 ± 0.15	4.60-5.19	
	GG	4.52 ± 0.17	4.17-4.87	
HDL (mmol/L)	AA	1.37 ± 0.08	1.20-1.54	0.094
	AG	1.32 ± 0.45	1.23-1.41	
	GG	1.18 ± 0.05	1.08-1.29	
LDL (mmol/L)	AA	2.96 ± 0.23	2.48-3.43	0.331
	AG	2.86 ± 0.12	2.60-3.10	
	GG	2.59 ± 0.15	2.29-2.89	
FPG (mmol/L)	AA	5.01 ± 0.15	4.71-5.32	<0.001
	AG	5.82 ± 0.39	5.58-6.06	
	GG	8.55 ± 0.39	7.77-9.34	

Table 14. Data are presented as mean ± standard deviation (SD). Comparisons of metabolic parameters among LEPR Q223R genotype groups were performed using one-way analysis of variance (one-way ANOVA). TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FPG, fasting plasma glucose; CI, confidence interval.

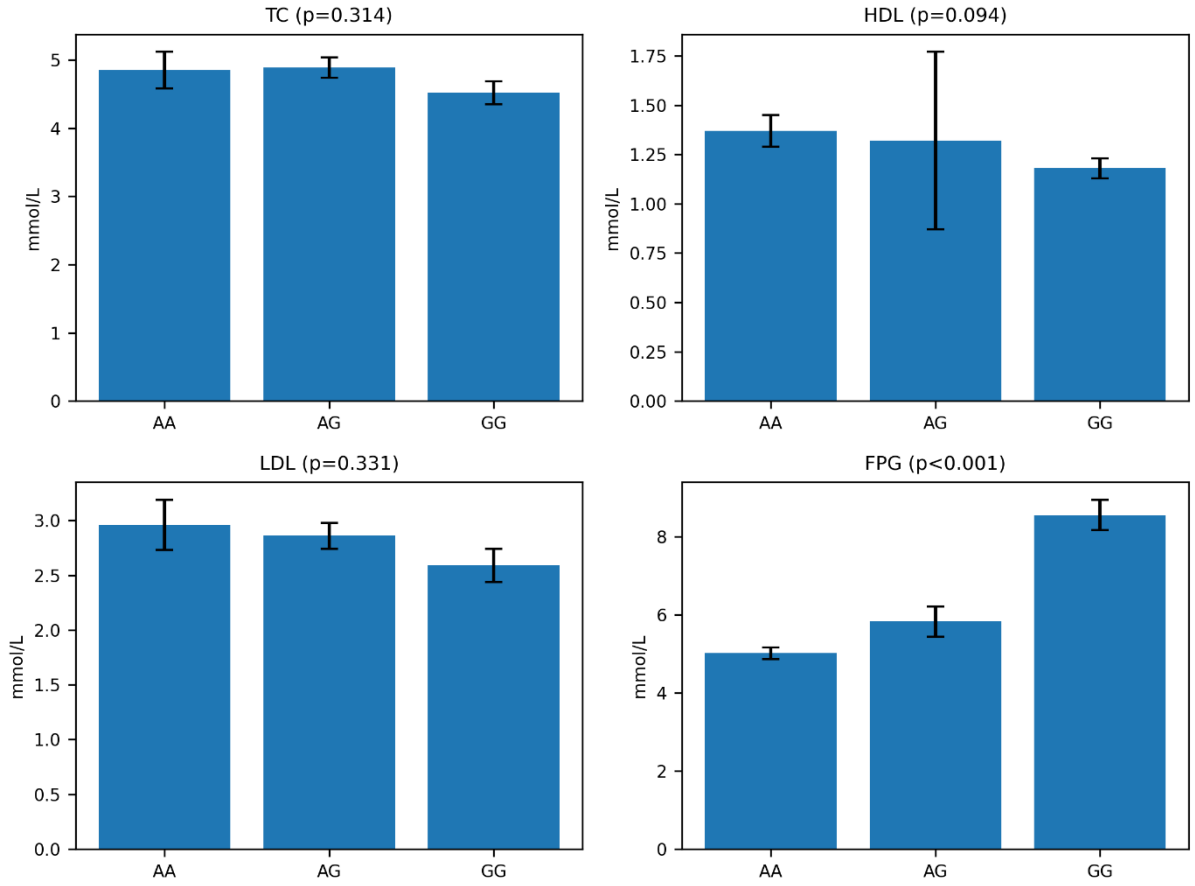


Figure 24. Biochemical parameters according to LEPR Q223R genotype distribution. *(A) Total cholesterol (TC), (B) high-density lipoprotein cholesterol (HDL-C), (C) low-density lipoprotein cholesterol (LDL-C), and (D) fasting plasma glucose (FPG). Data are presented as mean ± standard error of the mean (SEM). No statistically significant differences were observed for the lipid parameters ($p > 0.05$), whereas fasting plasma glucose showed a statistically significant increase across the genotype groups ($p < 0.001$). Graphs were generated using GraphPad Prism, and comparisons among genotype groups were performed using one-way analysis of variance (one-way ANOVA).*

Table 14 presents a comparative analysis of the main biochemical parameters, including total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG), according to the LEPR Q223R genotypes (AA, AG, and GG). Data are expressed as mean ± standard error of the mean (SEM) and accompanied by 95% confidence intervals (95% CIs). Differences among genotype groups were evaluated using one-way analysis of variance (one-way ANOVA).

For total cholesterol (TC), mean values showed only minor variation across the genotype groups. The mean TC level was 4.85 ± 0.27 mmol/L (95% CI: 4.29–5.41) in AA carriers, 4.89 ± 0.15 mmol/L (95% CI: 4.60–5.19) in AG carriers, and 4.52 ± 0.17 mmol/L (95% CI: 4.17–

4.87) in GG carriers. Although a slight reduction was observed in the GG genotype, no statistically significant differences were detected ($p = 0.314$), indicating that the LEPR Q223R polymorphism had no significant effect on total cholesterol levels.

A similar pattern was observed for HDL-C, with a trend toward lower values among GG carriers. Mean HDL-C concentrations were 1.37 ± 0.08 mmol/L (95% CI: 1.20–1.54) for AA, 1.32 ± 0.05 mmol/L (95% CI: 1.23–1.41) for AG, and 1.18 ± 0.05 mmol/L (95% CI: 1.08–1.29) for GG. Despite the gradual decline in HDL-C with increasing numbers of G alleles, the differences did not reach statistical significance ($p = 0.094$).

Likewise, LDL-C levels exhibited a decreasing trend across the genotype groups, with mean values of 2.96 ± 0.23 mmol/L (95% CI: 2.48–3.43) for AA, 2.86 ± 0.12 mmol/L (95% CI: 2.60–3.10) for AG, and 2.59 ± 0.15 mmol/L (95% CI: 2.29–2.89) for GG. However, these differences were not statistically significant ($p = 0.331$), suggesting that genotype variation does not substantially influence LDL-C concentrations.

In contrast to the lipid parameters, fasting plasma glucose (FPG) showed a clear and statistically significant association with genotype. Mean FPG values increased progressively from 5.01 ± 0.15 mmol/L (95% CI: 4.71–5.32) in AA carriers to 5.82 ± 0.39 mmol/L (95% CI: 5.58–6.06) in AG carriers, reaching 8.55 ± 0.39 mmol/L (95% CI: 7.77–9.34) in individuals with the GG genotype. The difference among genotype groups was highly significant ($p < 0.001$), demonstrating a clear allele-dose effect, with fasting plasma glucose increasing according to the number of G alleles. The minimal overlap between the 95% confidence intervals further supports the robustness of this association.

Overall, no statistically significant genotype-dependent differences were observed for the lipid parameters (TC, HDL-C, and LDL-C), despite modest trends across genotype groups. In contrast, fasting plasma glucose was strongly associated with the LEPR Q223R polymorphism, with GG homozygotes exhibiting markedly higher glucose levels than carriers of the AA or AG genotypes. These findings suggest that the LEPR Q223R polymorphism may exert a more specific effect on glucose homeostasis than on lipid metabolism.

Table 15. Association of the LEPR Q223R Polymorphism with Metabolic Parameters in Obese Individuals (BMI ≥ 25 kg/m²).

Parameters	Genotype	Mean ± SE	95 % CI for mean	ANOVA *p*-value
TC (mmol/L)	AA	4.64 ± 0.52	3.53 - 5.76	0.618
	AG	4.64 ± 0.20	4.24 - 5.04	
	GG	4.31 ± 0.19	3.91 - 4.70	

HDL (mmol/L)	AA	1.28 ± 0.16	0.93 - 1.63	0.217
	AG	1.28 ± 0.07	1.14 - 1.42	
	GG	1.07 ± 0.07	0.92 - 1.23	
LDL (mmol/L)	AA	2.83 ± 0.45	1.86-3.79	0.444
	AG	2.66 ± 0.16	2.35 - 2.98	
	GG	2.38 ± 0.15	2.07 - 2.69	
FPG (mmol/L)	AA	4.53 ± 0.17	4.16 - 4.89	< 0.001
	AG	5.92 ± 0.15	5.62 - 6.22	
	GG	9.20 ± 0.54	8.08 - 10.32	

Table 15. *Data are presented as mean ± standard deviation (SD). Comparisons of metabolic parameters among LEPR Q223R genotype groups were performed using one-way analysis of variance (one-way ANOVA). TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FPG, fasting plasma glucose; CI, confidence interval.*

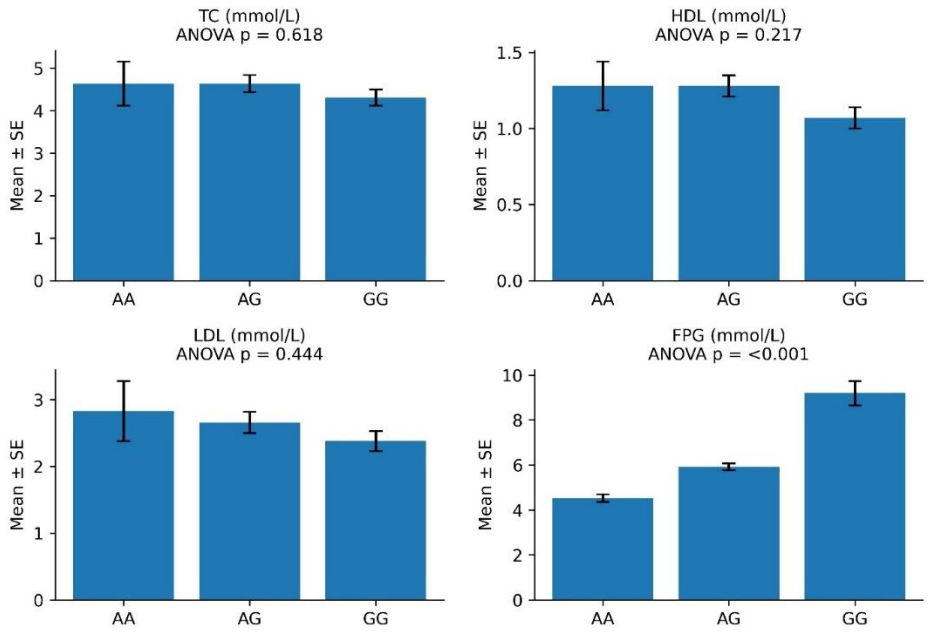


Figure 25. Biochemical parameters according to LEPR Q223R genotype. *(A) Total cholesterol (TC), (B) high-density lipoprotein cholesterol (HDL-C), (C) low-density lipoprotein cholesterol (LDL-C), and (D) fasting plasma glucose (FPG). Data are presented as mean ± standard error of the mean (SEM). No statistically significant differences were observed for the lipid*

parameters (p > 0.05), whereas fasting plasma glucose exhibited a significant genotype-dependent increase (p < 0.001). Statistical comparisons among genotype groups were performed using one-way analysis of variance (one-way ANOVA). Graphs were generated using GraphPad Prism.

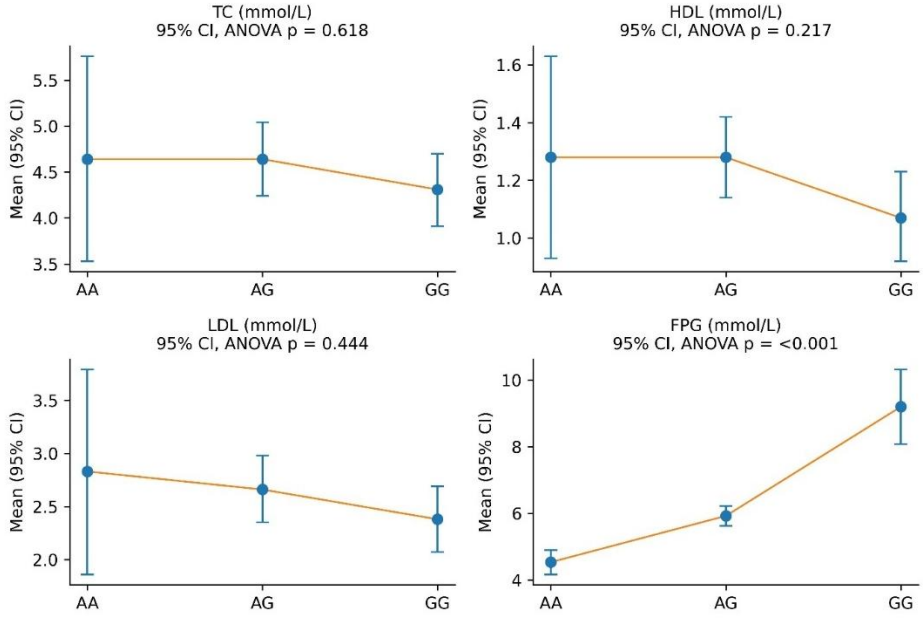


Figure 26. Mean biochemical parameters according to LEPR Q223R genotypes with 95% confidence intervals (95% CIs). Mean values of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) are shown for the AA, AG, and GG genotypes. Statistical comparisons among genotype groups were performed using one-way analysis of variance (one-way ANOVA). Fasting plasma glucose (FPG) differed significantly among the genotypes ($p < 0.001$), whereas no statistically significant differences were observed for TC, HDL-C, or LDL-C ($p > 0.05$).

Table 15 presents a comparative analysis of the principal biochemical parameters, including total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG), according to the LEPR Q223R genotype (AA, AG, and GG) in obese individuals. Data are expressed as mean ± standard error of the mean (SEM) with 95% confidence intervals (95% CIs). Statistical significance among genotype groups was assessed using one-way analysis of variance (one-way ANOVA).

For total cholesterol (TC), the mean values were relatively homogeneous across the three genotype groups, with no substantial variation. Individuals carrying the AA genotype had a mean TC concentration of 4.64 ± 0.52 mmol/L (95% CI: 3.53–5.76), reflecting greater variability within this group. The AG genotype exhibited an identical mean value (4.64 ± 0.20 mmol/L; 95% CI: 4.24–5.04), whereas GG homozygotes showed a slightly lower mean TC

concentration (4.31 ± 0.19 mmol/L; 95% CI: 3.91–4.70). Despite this modest downward trend, the differences were not statistically significant ($p = 0.618$), indicating that the LEPR Q223R polymorphism does not significantly influence serum total cholesterol levels in the studied population.

A similar pattern was observed for HDL-C, a major anti-atherogenic lipoprotein. Individuals with the AA genotype had a mean HDL-C level of 1.28 ± 0.16 mmol/L (95% CI: 0.93–1.63), whereas carriers of the AG genotype showed a comparable mean value (1.28 ± 0.07 mmol/L; 95% CI: 1.14–1.42). The GG genotype was associated with a lower mean HDL-C concentration (1.07 ± 0.07 mmol/L; 95% CI: 0.92–1.23), suggesting a potential unfavorable trend in the lipid profile. However, this difference did not reach statistical significance ($p = 0.217$).

Likewise, LDL-C demonstrated a gradual decrease across the genotype groups. Mean LDL-C concentrations were 2.83 ± 0.45 mmol/L (95% CI: 1.86–3.79) for the AA genotype, 2.66 ± 0.16 mmol/L (95% CI: 2.35–2.98) for AG, and 2.38 ± 0.15 mmol/L (95% CI: 2.07–2.69) for GG. Although LDL-C levels tended to decline with an increasing number of G alleles, the differences were not statistically significant ($p = 0.444$), indicating no convincing association between the LEPR Q223R polymorphism and LDL-C concentrations.

In contrast, fasting plasma glucose (FPG) exhibited a clear and highly significant association with genotype ($p < 0.001$). Individuals with the AA genotype had a mean FPG level of 4.53 ± 0.17 mmol/L (95% CI: 4.16–4.89), which was within the normal glycemic range. In AG heterozygotes, the mean FPG increased to 5.92 ± 0.15 mmol/L (95% CI: 5.62–6.22), approaching the threshold for impaired fasting glucose. The highest values were observed in GG homozygotes, with a mean FPG of 9.20 ± 0.54 mmol/L (95% CI: 8.08–10.32), consistent with marked hyperglycemia.

Importantly, FPG demonstrated a distinct genotype–phenotype relationship, characterized by a progressive increase in glucose levels with increasing numbers of G alleles (AA < AG < GG). This gradient strongly suggests an allele-dose effect of the LEPR Q223R polymorphism on glucose homeostasis. In contrast, the lipid parameters (TC, HDL-C, and LDL-C) showed no statistically significant genotype-dependent differences, despite modest and consistent trends.

Overall, these findings indicate that the LEPR Q223R polymorphism is not associated with significant alterations in the lipid profile but demonstrates a strong, statistically significant, and biologically plausible association with fasting plasma glucose. This suggests that the genetic variant exerts a more specific influence on glucose homeostasis than on lipid metabolism, consistent with the established role of the leptin receptor in the regulation of energy balance, glucose metabolism, and insulin sensitivity.

3. Leptin (LEP)

Table 16. Genotype and Allele Distribution of the LEP -2548G>A Polymorphism in Obese and Non-Obese Individuals.

Genotype / Allele	Obese	Non-obese	OR (95% CI)	p-value
GG	16 (19.5%)	20 (27%)	0.65 (0.31–1.38)	0.342
GA	40 (48.8%)	37 (37%)	0.95 (0.51–1.79)	1.000
AA	26 (31.7%)	17 (17%)	1.56 (0.76–3.18)	0.282
G	72 (44%)	77(52%)	0.72 (0.46–1.13)	0.173
A	92 (56%)	71 (48%)	1.39 (0.89–2.17)	0.173

Table 16 summarizes the distribution of LEP -2548G>A genotype and allele frequencies among obese and non-obese individuals. The GA genotype was the most frequently observed genotype in both study groups, occurring in 48.8% of obese individuals and 37.0% of non-obese controls.Genotype and allele frequencies were compared using one-way analysis of variance (one-way ANOVA).

Table 16 summarizes the genotype and allele distributions of the LEP -2548G>A polymorphism in obese and non-obese individuals. The GA genotype was the most prevalent genotype in both study groups, occurring in 48.8% of obese individuals and 37.0% of non-obese controls.

The AA genotype was more frequent among obese individuals (31.7%) than among non-obese controls (17.0%). The estimated odds ratio (OR) of 1.56 (95% CI: 0.76–3.18) suggests a trend toward increased odds of obesity among AA genotype carriers. However, this association did not reach statistical significance ($p = 0.282$), as the confidence interval included the null value (OR = 1.0). In contrast, the GG genotype was observed more frequently in the non-obese group (27.0%) than in obese individuals (19.5%). The calculated OR of 0.65 (95% CI: 0.31–1.38) suggests a possible protective effect against obesity, although this association was likewise not statistically significant ($p = 0.342$). Similarly, the GA genotype showed no significant association with obesity (OR = 0.95, 95% CI: 0.51–1.79; $p = 1.000$).

At the allele level, the A allele was more frequent among obese individuals (56.0%) than among non-obese controls (48.0%), with an estimated OR of 1.39 (95% CI: 0.89–2.17). Conversely, the G allele occurred slightly more frequently in the non-obese group (52.0%) than in the obese group (44.0%), corresponding to an OR of 0.72 (95% CI: 0.46–1.13). Neither allele demonstrated a statistically significant association with obesity ($p = 0.173$).

Overall, no statistically significant differences in genotype or allele distributions were observed between obese and non-obese individuals. Although the AA genotype and A allele exhibited a non-significant trend toward increased obesity risk, whereas the GG genotype and G allele

showed a tendency toward a protective effect, all 95% confidence intervals included the null value and all *p*-values exceeded the threshold for statistical significance. These findings indicate that the LEP -2548G>A polymorphism was not significantly associated with obesity in the studied Northern Bulgarian population. Nevertheless, the observed trends warrant further investigation in larger cohorts with greater statistical power.

Table 17. Comparison of Biochemical Parameters Among LEP -2548G>A Genotypes in the Control Group.

Control Group (BMI<25kgm^2)				
Parameter	Genotype	Mean±SE	95% Confidence interval for mean	ANOVA p-value
TC (mmol/L)	GG	5.03±0.26	4.47-5.58	0.480
	GA	5.21±0.21	4.77-5.64	
	AA	4.78±0.29	4.15-5.40	
HDL-C (mmol/L)	GG	1.45±0.07	1.30-1.60	0.410
	GA	1.38±0.05	1.28-1.48	
	AA	1.32±0.06	1.20-1.44	
LDL-C (mmol/L)	GG	3.06±0.22	2.61-3.52	0.744
	GA	3.09±0.19	2.68-3.5	
	AA	2.85±0.27	2.29-3.41	
FPG (mmol/L)	GG	5.42±0.19	5.01-5.82	<0.001
	GA	5.70±0.20	5.30-6.10	
	AA	7.78±0.52	6.70-8.90	

Table 17 compares lipid profile parameters and fasting plasma glucose (FPG) among the different LEP -2548G>A genotypes (GG, GA, and AA) in the control group with a normal body mass index (BMI < 25 kg/m²). Data are presented as mean ± standard error of the mean (SEM) together with 95% confidence intervals (95% CIs) for each mean value. The statistical significance of differences among the three genotype groups was assessed using one-way analysis of variance (one-way ANOVA), with the corresponding *p*-values indicating whether significant differences were present.

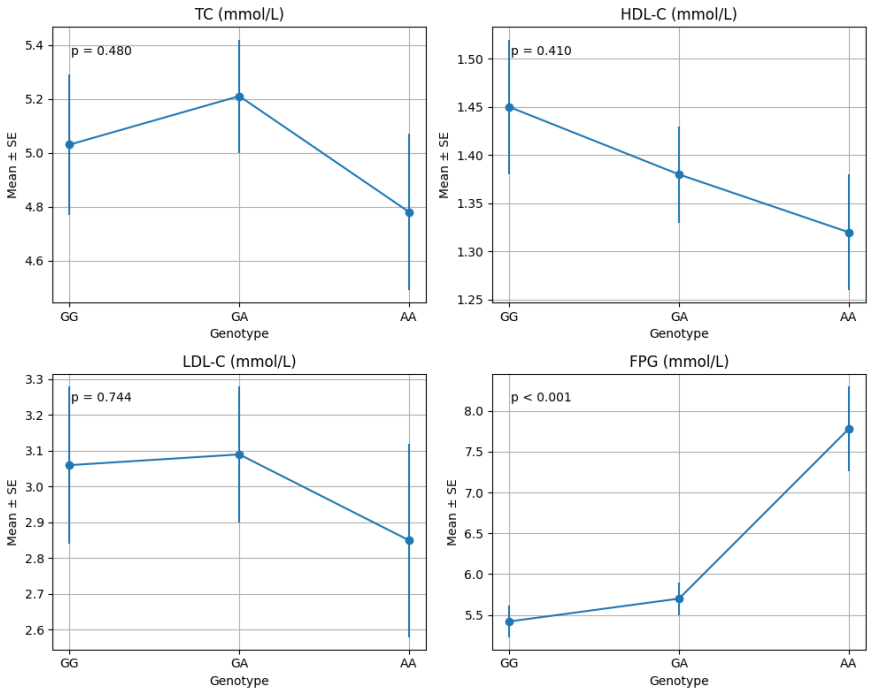


Figure 27. Comparison of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) among the GG, GA, and AA genotypes in the control group. Statistical comparisons among genotype groups were performed using one-way analysis of variance (one-way ANOVA).

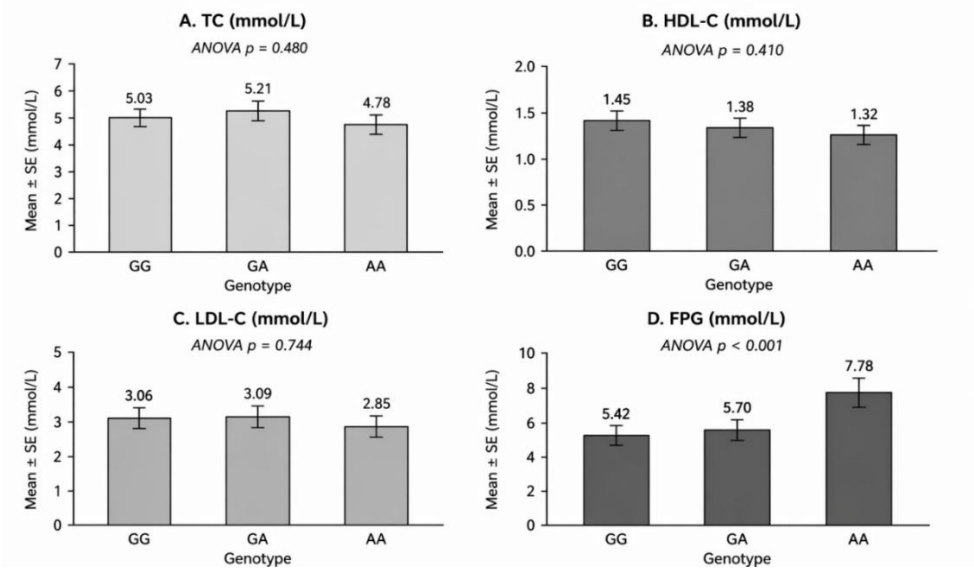


Figure 28. Comparison of metabolic parameters according to LEP -2548G>A genotypes in the control group. Data are presented according to the GG, GA, and AA genotypes. Graphs were generated using GraphPad Prism.

In the control group (BMI < 25 kg/m²), biochemical parameters were compared among the three LEP -2548G>A genotypes (GG, GA, and AA). For total cholesterol (TC), the mean values were 5.03 ± 0.26 mmol/L for the GG genotype, 5.21 ± 0.21 mmol/L for GA, and 4.78 ± 0.29 mmol/L for AA. One-way ANOVA showed no statistically significant differences among the genotype groups ($p = 0.480$).

Similarly, HDL-C levels were comparable across the three genotypes, with mean values of 1.45 ± 0.07 mmol/L for GG, 1.38 ± 0.05 mmol/L for GA, and 1.32 ± 0.06 mmol/L for AA, with no statistically significant differences ($p = 0.410$).

Likewise, LDL-C concentrations did not differ significantly among the genotype groups. Mean values were 3.06 ± 0.22 mmol/L for GG, 3.09 ± 0.19 mmol/L for GA, and 2.85 ± 0.27 mmol/L for AA ($p = 0.744$).

In contrast to the lipid parameters, fasting plasma glucose (FPG) demonstrated a marked difference among the genotypes. Mean FPG levels were 5.42 ± 0.19 mmol/L for GG, 5.70 ± 0.20 mmol/L for GA, and a substantially higher value of 7.78 ± 0.52 mmol/L for the AA genotype. This difference was highly statistically significant ($p < 0.001$), indicating that the AA genotype of the LEP -2548G>A polymorphism is associated with elevated fasting plasma glucose levels in the control group, whereas no significant associations were observed with the lipid profile parameters.

Table 18. Association of the LEP -2548G>A Polymorphism with Hypertension and Type 2 Diabetes Mellitus (T2DM) in the Control Group.

		Non-hypertensive	Hypertensive	p-value
Hypertension	GG	11 (14.9%)	7 (9.5%)	0.049
	GA	15 (20.3%)	22 (29.7%)	
	AA	14 (18.9%)	5 (6.8%)	
		Non-diabetic	Diabetic	p-value
Diabetes Mellitus	GG	16 (21.6%)	2 (2.7%)	0.058
	GA	30 (40.5%)	7 (9.5%)	
	AA	11 (14.9%)	8 (10.8%)	

With respect to hypertension, genotype frequencies among normotensive individuals were GG: 11 (14.9%), GA: 15 (20.3%), and AA: 14 (18.9%). Among hypertensive participants, the corresponding frequencies were GG: 7 (9.5%), GA: 22 (29.7%), and AA: 5 (6.8%). The association between LEP -2548G>A genotype distribution and hypertension status was

statistically significant ($p = 0.049$), suggesting that variation in the LEP gene may be associated with the presence of hypertension in the control group. Notably, the GA genotype was more frequently observed among hypertensive individuals, whereas the AA and GG genotypes occurred less frequently in this subgroup.

Regarding type 2 diabetes mellitus (T2DM), genotype frequencies among non-diabetic controls were GG: 16 (21.6%), GA: 30 (40.5%), and AA: 11 (14.9%). In the diabetic subgroup, the corresponding frequencies were GG: 2 (2.7%), GA: 7 (9.5%), and AA: 8 (10.8%). Although the association between genotype distribution and T2DM did not reach conventional statistical significance ($p = 0.058$), the results suggest a borderline trend. The AA genotype appeared to be relatively overrepresented among diabetic individuals, whereas the GG genotype was the least frequent in this subgroup. The GA genotype remained the most common genotype in both diabetic and non-diabetic participants.

Overall, the findings indicate that the LEP -2548G>A polymorphism is significantly associated with hypertension in the control population and demonstrates a borderline association with T2DM. Although the relationship with diabetes did not achieve statistical significance, the observed genotype distribution suggests a potential biological association that warrants further investigation in larger cohorts with greater statistical power.

Table 19. Comparison of Biochemical Parameters According to LEP -2548G>A Genotype in the Target Group (BMI > 30 kg/m²).

Obese Group (BMI>30kgm2)				
Parameter	Genotype	Mean±SE	95% Confidence interval for mean	ANOVA p-value
TC (mmol/L)	GG	4.7±0.24	4.19-5.21	0.336
	GA	5.01±0.21	4.59-5.43	
	AA	5.4±0.38	4.61-6.18	
HDL-C (mmol/L)	GG	1.40±0.1	1.19-1.6	0.447
	GA	1.40±0.05	1.30-1.50	
	AA	1.30±0.05	1.18-1.41	
LDL-C (mmol/L)	GG	2.56±0.24	2.05-3.07	0.129
	GA	3.04±0.18	2.67-3.40	
	AA	3.43±0.34	2.73-4.14	

FPG (mmol/L)	GG	5.93±0.41	5.05-6.81	0.065
	GA	6.43±0.29	5.84-7.03	
	AA	5.42±0.20	5.00-5.84	

With respect to total cholesterol (TC), mean concentrations showed a gradual increase across the genotype groups, reaching 4.70 ± 0.24 mmol/L for the GG genotype, 5.01 ± 0.21 mmol/L for GA, and 5.40 ± 0.38 mmol/L for AA. Despite this upward trend, the difference did not reach statistical significance (one-way ANOVA, $p = 0.336$), indicating no significant association between the LEP -2548G>A genotype and total cholesterol levels in the obese group.

Similarly, HDL-C concentrations were comparable among the genotype groups, with mean values of 1.40 ± 0.10 mmol/L for GG, 1.40 ± 0.05 mmol/L for GA, and a slightly lower value of 1.30 ± 0.05 mmol/L for AA. The observed variation was not statistically significant ($p = 0.447$), suggesting that HDL-C levels were not influenced by LEP genotype in obese individuals.

For LDL-C, the mean concentrations were 2.56 ± 0.24 mmol/L for GG, 3.04 ± 0.18 mmol/L for GA, and 3.43 ± 0.34 mmol/L for AA. Although a gradual increase from GG to AA was observed, the difference did not reach statistical significance ($p = 0.129$). These findings suggest a possible trend toward higher LDL-C levels among AA homozygotes, although the available data are insufficient to establish a statistically significant association.

Regarding fasting plasma glucose (FPG), the mean values were 5.93 ± 0.41 mmol/L for GG, 6.43 ± 0.29 mmol/L for GA, and 5.42 ± 0.20 mmol/L for AA. The GA genotype exhibited the highest mean FPG level, whereas AA homozygotes showed the lowest value. The observed difference approached, but did not reach, statistical significance ($p = 0.065$), suggesting a borderline trend toward genotype-dependent differences in glucose metabolism among obese individuals. Confirmation of this observation will require studies with larger sample sizes.

Overall, no statistically significant genotype-dependent differences were identified for lipid profile parameters or fasting plasma glucose in the obese group. Although LDL-C and FPG demonstrated suggestive trends, all *p*-values exceeded the threshold for statistical significance ($p > 0.05$). These findings indicate that the LEP -2548G>A polymorphism is not significantly associated with lipid metabolism or fasting glucose levels within the present cohort of obese individuals. Nevertheless, the observed trends for LDL-C and FPG may become significant in larger studies with greater statistical power. Collectively, these results suggest that obesity itself may exert a stronger influence on metabolic parameters than the LEP genotype alone.

Table 20. Association of the LEP -2548G>A Polymorphism with Hypertension and Type 2 Diabetes Mellitus (T2DM) in the Target Group (Obese Individuals).

		Non-hypertensive	Hypertensive	p-value
Hypertension	GG	14 (17.1%)	1 (1.2%)	<0.001
	GA	28 (34.1%)	16 (19.5%)	
	AA	6 (7.3%)	17 (20.7%)	
		Non-diabetic	Diabetic	p-value
Diabetes Mellitus	GG	13 (15.9%)	2 (2.4%)	0.324
	GA	31 (37.8%)	13 (15.9%)	
	AA	19 (23.2%)	4 (4.9%)	

In the obese group (BMI > 30 kg/m²), the distribution of LEP -2548G>A genotypes was analyzed in relation to hypertension and type 2 diabetes mellitus (T2DM).

A highly significant association was observed between genotype distribution and hypertension status ($p < 0.001$). Among normotensive individuals, genotype frequencies were GG: 14 (17.1%), GA: 28 (34.1%), and AA: 6 (7.3%). In contrast, among hypertensive individuals, the corresponding frequencies were GG: 1 (1.2%), GA: 16 (19.5%), and AA: 17 (20.7%). Notably, the AA genotype was markedly overrepresented in hypertensive patients, whereas the GG genotype was rare. This distribution strongly suggests an association between the AA genotype and an increased risk of hypertension in obese individuals, while the GG genotype may exert a relatively protective effect.

Regarding T2DM, genotype distribution showed no statistically significant association ($p = 0.324$). Among non-diabetic individuals, genotype frequencies were GG: 13 (15.9%), GA: 31 (37.8%), and AA: 19 (23.2%), whereas among diabetic patients the corresponding frequencies were GG: 2 (2.4%), GA: 13 (15.9%), and AA: 4 (4.9%). The GA genotype remained the most prevalent genotype in both groups, and no consistent genotype-dependent pattern associated with diabetes risk was identified.

Overall, these findings indicate that the LEP -2548G>A polymorphism is strongly associated with hypertension, but not with T2DM, in obese individuals. The markedly increased frequency of the AA genotype among hypertensive patients supports a potential role of this genetic variant in cardiovascular risk associated with obesity. Given the established involvement of leptin in sympathetic nervous system activation and blood pressure regulation, these findings are biologically plausible and suggest that the AA genotype may contribute to hypertension through

altered leptin signaling or leptin resistance. However, further studies in larger cohorts, together with functional investigations, are required to clarify the underlying molecular mechanisms.

IV. Discussion of the Results

1. Adiponectin (ADIPOQ rs266729)

1. Adiponectin (ADIPOQ rs266729)

The present study demonstrated that the ADIPOQ rs266729 polymorphism, located within the promoter region of the ADIPOQ gene, is associated with an increased risk of obesity and type 2 diabetes mellitus (T2DM) in a Northern Bulgarian population. Specifically, the G allele and the CG and GG genotypes were associated with an unfavorable metabolic profile characterized by an increased cardiometabolic risk, reduced HDL-cholesterol concentrations, and impaired glucose homeostasis.

These findings are biologically plausible and consistent with the established physiological role of adiponectin as a key regulator of insulin sensitivity, glucose metabolism, and lipid homeostasis. Reduced adiponectin activity has been shown to promote insulin resistance, endothelial dysfunction, and chronic low-grade inflammation, all of which contribute to the development of obesity, T2DM, and metabolic syndrome. Since rs266729 is located within the promoter region of ADIPOQ, the observed associations may reflect altered transcriptional activity resulting in decreased adiponectin expression.

Our results are in agreement with numerous previous studies reporting an association between the G allele of rs266729 and an increased susceptibility to obesity, insulin resistance, metabolic syndrome, and T2DM across different populations. Furthermore, the significant association with reduced HDL-C levels observed in the present study supports the proposed anti-atherogenic role of adiponectin and its contribution to reverse cholesterol transport.

In contrast, no significant associations were identified between rs266729 and either total cholesterol or LDL-cholesterol concentrations. This finding suggests that the metabolic effects of this polymorphism may be more selective, predominantly influencing HDL metabolism and glucose homeostasis rather than the overall lipid profile. Such observations are consistent with the recognized physiological functions of adiponectin, which primarily modulates insulin sensitivity, fatty acid oxidation, and anti-inflammatory pathways.

Several limitations should be acknowledged. The relatively modest sample size may have reduced the statistical power to detect weaker genetic associations. In addition, circulating adiponectin concentrations were not measured, precluding direct evaluation of the relationship between genotype and protein expression. Future studies involving larger cohorts and

functional analyses, including serum adiponectin measurements, are warranted to further clarify the biological mechanisms underlying the observed associations.

Despite these limitations, the present findings support the potential utility of the ADIPOQ rs266729 polymorphism as a genetic marker of cardiometabolic risk and suggest that this variant may contribute to the genetic susceptibility to obesity and T2DM in the Northern Bulgarian population.

2. LEPR (rs1137101)

The present study demonstrated that the LEPR rs1137101 (Q223R) polymorphism was not significantly associated with obesity or lipid profile parameters in the studied Northern Bulgarian population. No statistically significant differences in genotype or allele distributions were observed between obese and non-obese individuals, indicating that this variant is unlikely to represent a major genetic determinant of obesity susceptibility in this cohort.

In contrast, LEPR rs1137101 exhibited a significant association with fasting plasma glucose (FPG). A clear allele-dose effect was observed, with progressively increasing FPG concentrations across the AA, AG, and GG genotypes (AA < AG < GG). This genotype–phenotype relationship suggests that the G allele may contribute to impaired glucose homeostasis and an increased susceptibility to type 2 diabetes mellitus (T2DM).

These findings are consistent with the established biological role of the leptin receptor in the regulation of energy balance, insulin signaling, and glucose metabolism. Experimental and clinical studies have demonstrated that impaired leptin receptor function may reduce insulin sensitivity, alter hypothalamic regulation of glucose homeostasis, and promote hyperglycemia independently of body weight. The absence of significant associations with total cholesterol, HDL-C, and LDL-C further suggests that the metabolic effects of LEPR rs1137101 are more pronounced in glucose regulation than in lipid metabolism.

Our results are in agreement with previous studies indicating that the Q223R polymorphism has a stronger influence on glucose homeostasis and insulin sensitivity than on obesity per se. The observed genotype-dependent increase in fasting plasma glucose supports the hypothesis that LEPR genetic variation contributes primarily to disturbances in glucose metabolism rather than to alterations in body weight or lipid profile.

Several limitations should be considered when interpreting these findings. The relatively modest sample size may have limited the statistical power to detect weaker associations, particularly with obesity and lipid-related traits. Furthermore, measurements of insulin concentrations and indices of insulin resistance, such as the Homeostatic Model Assessment for

Insulin Resistance (HOMA-IR), were not available, precluding a direct evaluation of the relationship between LEPR genotype and insulin sensitivity.

Despite these limitations, the present findings identify LEPR rs1137101 (Q223R) as a potential genetic marker of impaired glucose regulation. Further studies involving larger, ethnically diverse populations and functional analyses are warranted to confirm these observations and to clarify the molecular mechanisms linking LEPR genetic variation to glucose homeostasis and the development of T2DM.

3. LEP (-2548G>A)

The present study demonstrated that the LEP -2548G>A (rs12112075) polymorphism exerts a BMI-dependent effect on metabolic traits. Among individuals with a normal body mass index, the AA genotype was associated with higher fasting plasma glucose (FPG) levels and an increased prevalence of arterial hypertension, whereas no significant association was observed with lipid profile parameters. In contrast, among obese individuals, no statistically significant differences in lipid profile or fasting glucose were detected among the different genotypes, suggesting that the metabolic effects of this polymorphism may be influenced by the underlying metabolic status.

The most notable finding of the present study was the strong association between the LEP -2548G>A polymorphism and hypertension in obese individuals, with the AA genotype being significantly overrepresented among hypertensive patients. These findings are consistent with the well-established role of leptin in blood pressure regulation through activation of the sympathetic nervous system, impaired renal sodium handling, endothelial dysfunction, and vascular remodeling. The observed association may be explained by the phenomenon of selective leptin resistance, in which the anorexigenic effects of leptin are attenuated, whereas its sympathoexcitatory and pressor actions remain preserved, thereby contributing to the development of hypertension.

In contrast, no statistically significant association was identified between LEP -2548G>A and type 2 diabetes mellitus (T2DM) in obese individuals. This lack of association may reflect the predominant influence of obesity-related insulin resistance and other metabolic disturbances that overshadow the relatively modest contribution of this single genetic variant. Furthermore, the limited sample size may have reduced the statistical power to detect weaker genotype-dependent effects.

Overall, the present findings suggest that the biological effects of the LEP -2548G>A polymorphism are BMI-dependent. The variant appears to exert a greater influence on glucose

homeostasis in individuals with normal body weight, whereas its association with arterial hypertension is more pronounced in obese individuals. These observations support the role of leptin as an important mediator of cardiometabolic risk and indicate that the phenotypic consequences of LEP genetic variation are strongly influenced by the metabolic context.

More broadly, metabolic disorders arise from complex interactions between environmental exposures and polygenic genetic susceptibility. Adiponectin and leptin are key adipokines involved in the regulation of insulin sensitivity, energy homeostasis, glucose metabolism, and cardiovascular function. Consequently, genetic variation within the ADIPOQ, LEPR, and LEP genes may contribute to inter-individual differences in susceptibility to obesity, insulin resistance, metabolic syndrome, and type 2 diabetes mellitus.

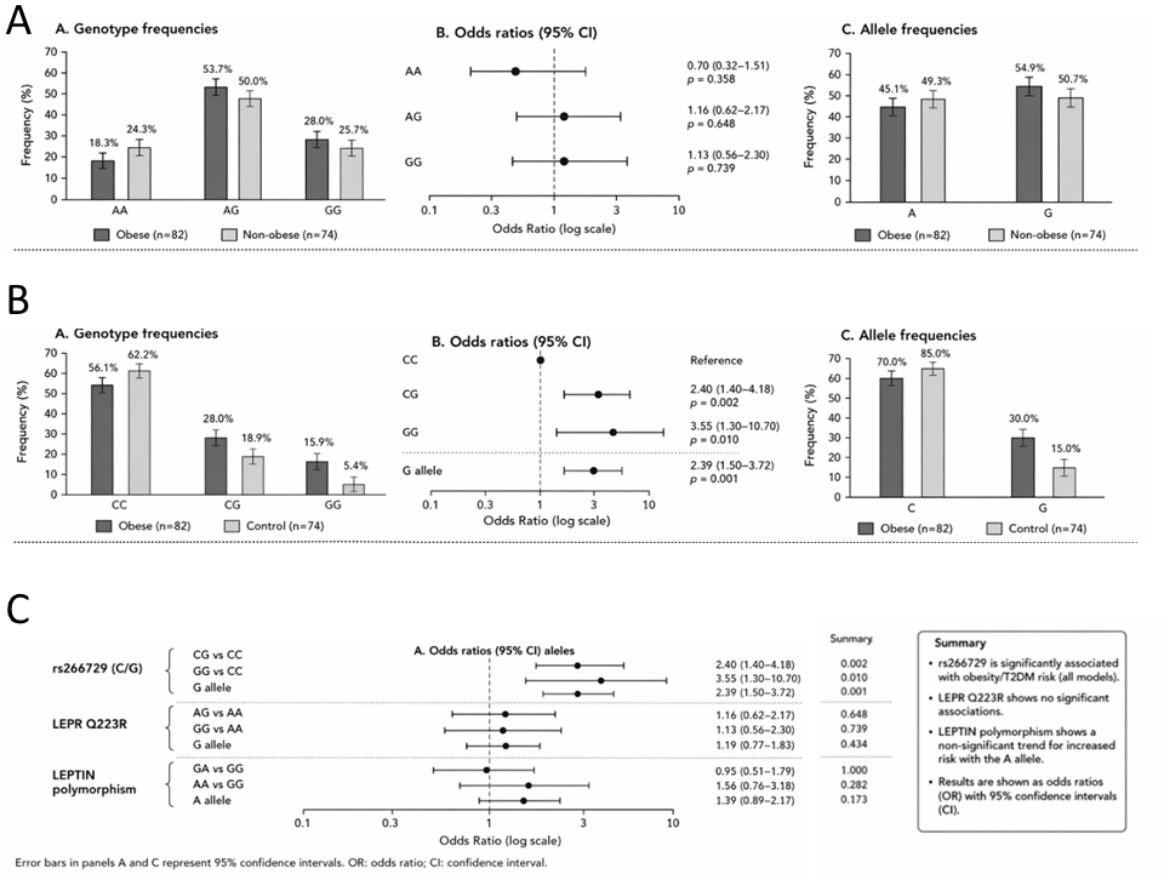


Figure 30. Comparative analysis of genotype and allele frequencies and odds ratios (ORs) for the investigated polymorphisms. *(A) LEPR Q223R (rs1137101), (B) ADIPOQ rs266729 (C>G), and (C) a summary forest plot comparing the genetic associations of the three investigated polymorphisms. The forest plot demonstrates that ADIPOQ rs266729 exhibits a consistent and statistically significant association across all genetic models, whereas LEPR Q223R shows no significant association. The LEP -2548G>A (rs12112075) polymorphism demonstrates only a*

non-significant trend toward increased cardiometabolic risk. Statistical comparisons were performed using one-way analysis of variance (one-way ANOVA).

The comparative analysis presented in Figure 30 demonstrates distinct patterns of genetic association among the investigated polymorphisms. For the LEPR Q223R (rs1137101) polymorphism, no significant differences in genotype or allele distributions were observed between obese individuals and controls. The frequencies of the AA, AG, and GG genotypes were comparable in both groups, suggesting that this genetic variant is not associated with obesity susceptibility in the studied population. Consistent with these findings, the odds ratios (ORs) for the AG and GG genotypes relative to the reference AA genotype were close to unity, and the corresponding 95% confidence intervals (CIs) included the null value, indicating a lack of statistical significance ($p > 0.05$). Likewise, allele frequencies did not differ significantly between the two groups.

In contrast, the ADIPOQ rs266729 (C>G) polymorphism demonstrated a clear and statistically significant association with obesity. The CC genotype was more prevalent among controls, whereas the CG and GG genotypes occurred more frequently in obese individuals. Carriers of the CG genotype exhibited an approximately 2.4-fold increased risk of obesity (OR ≈ 2.40, $p = 0.002$), while individuals carrying the GG genotype had more than a 3.5-fold higher risk (OR ≈ 3.55, $p = 0.010$) compared with CC homozygotes. Furthermore, the G allele itself was significantly associated with obesity susceptibility (OR ≈ 2.39, $p = 0.001$). The progressive increase in risk from CC to CG and GG indicates a clear allele-dose effect, supporting the biological relevance of this promoter variant.

The summary forest plot (Figure 30C) further confirms these observations by demonstrating a consistent and statistically significant effect of ADIPOQ rs266729 across all evaluated genetic models. In contrast, LEPR Q223R showed no significant association with obesity, whereas the LEP -2548G>A (rs12112075) polymorphism exhibited only a non-significant trend toward increased cardiometabolic risk.

Overall, these findings indicate that the genetic determinants of obesity appear to be pathway-specific. Variants affecting adiponectin signaling and peripheral metabolic regulation, such as ADIPOQ rs266729, exert a significant influence on obesity susceptibility, whereas variation in LEPR, which primarily affects central leptin signaling and appetite regulation, does not appear to contribute substantially to obesity risk in the present cohort. These observations support adiponectin as a key regulator of metabolic homeostasis and identify ADIPOQ rs266729 as a potential genetic marker of obesity and related cardiometabolic disorders.

Although ADIPOQ rs266729 was significantly associated with obesity susceptibility, its metabolic effects were more pronounced for glucose homeostasis than for lipid metabolism. No significant genotype-dependent differences were observed for total cholesterol, HDL-C, or LDL-C, whereas fasting plasma glucose demonstrated a significant association with genotype. This finding suggests that the functional consequences of rs266729 primarily involve insulin sensitivity rather than generalized lipid metabolism or adiposity.

Adiponectin is a major regulator of metabolic homeostasis through activation of AMP-activated protein kinase (AMPK), resulting in enhanced peripheral glucose uptake, increased fatty acid oxidation, and suppression of hepatic gluconeogenesis. Reduced circulating adiponectin concentrations and functional variation within ADIPOQ have consistently been linked to insulin resistance and the development of T2DM. Because rs266729 is located within the promoter region of the gene, this variant may alter transcriptional activity and adiponectin expression, thereby contributing to impaired glucose regulation.

Previous meta-analyses have reported a moderate but significant association between ADIPOQ rs266729 and susceptibility to T2DM, although considerable heterogeneity among populations has been noted. These studies have consistently demonstrated stronger associations with insulin resistance and glucose homeostasis than with obesity or lipid abnormalities, supporting the findings of the present investigation.

A similar pattern of metabolic specificity was observed for LEPR Q223R. Although no significant association with obesity or lipid profile was identified, fasting plasma glucose demonstrated a highly significant genotype-dependent gradient ($p < 0.001$), with progressively increasing values across the AA, AG, and GG genotypes. This allele-dose relationship suggests that the G allele exerts an unfavorable effect on glucose homeostasis and further supports the hypothesis that LEPR genetic variation primarily influences insulin signaling and glucose metabolism rather than body weight regulation.

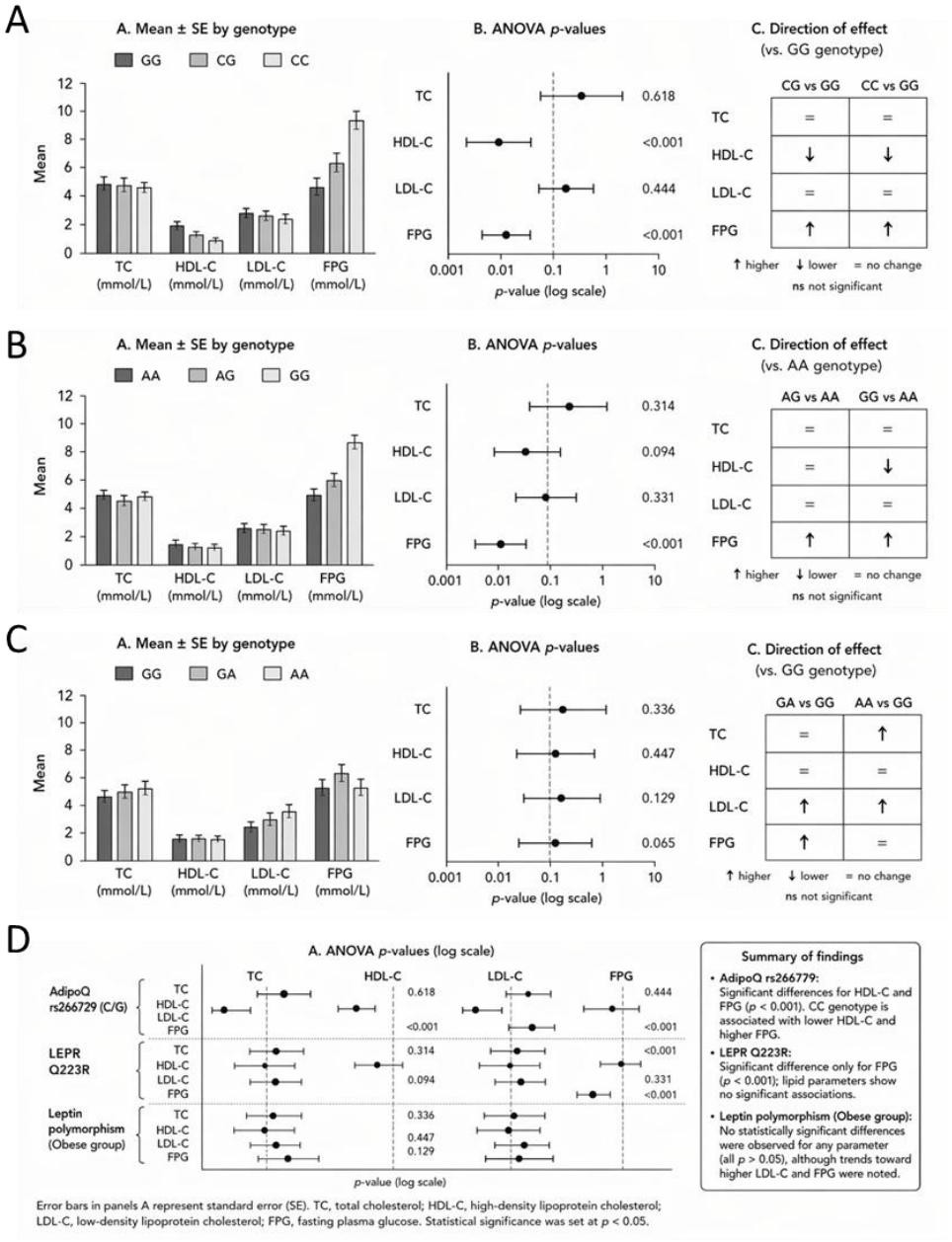


Figure 31. Comparative analysis of the metabolic effects of the investigated genetic polymorphisms. (A) ADIPOQ rs266729. *(A) Mean ± standard error of the mean (SEM) values for total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) according to genotype. (B) One-way ANOVA p-values presented on a logarithmic scale, demonstrating statistically significant differences for HDL-C and FPG ($p < 0.001$). (C) Direction of the genotype effect relative to the reference GG genotype.*

(B) LEPR Q223R (rs1137101). (A) Metabolic parameters according to genotype. (B) Statistically significant differences were observed only for FPG ($p < 0.001$). (C) Direction of the genotype effect relative to the reference AA genotype.

(C) LEP -2548G>A (rs12112075). (A) Metabolic parameters according to genotype in obese individuals (BMI > 30 kg/m²). (B) No statistically significant differences were observed for any of the investigated metabolic parameters ($p > 0.05$). (C) Direction of the genotype effect relative to the reference GG genotype.

(D) Comparative analysis of the metabolic effects of the investigated polymorphisms using one-way ANOVA p-values displayed on a logarithmic scale. The analysis includes ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP -2548G>A

(rs12112075) in obese individuals, evaluating four major metabolic traits: total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and fasting plasma glucose (FPG).
Error bars represent the standard error of the mean (SEM). TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FPG, fasting plasma glucose. Statistical significance was defined as $p < 0.05$.

The analysis of metabolic parameters presented in Figure 31 demonstrates distinct genotype-dependent effects for the investigated polymorphisms. ADIPOQ rs266729 was significantly associated with HDL-cholesterol (HDL-C) and fasting plasma glucose (FPG) ($p < 0.001$ for both), whereas no significant associations were observed with total cholesterol (TC) or LDL-cholesterol (LDL-C) ($p > 0.05$). A clear genotype-dependent pattern was evident, with carriers of the GG genotype exhibiting the lowest HDL-C concentrations and the highest FPG levels, consistent with an allele-dose effect (CC < CG < GG) and an unfavorable metabolic profile.
Similarly, the LEPR Q223R (rs1137101) polymorphism demonstrated a significant association only with FPG ($p < 0.001$). Fasting plasma glucose increased progressively across the genotype groups, with the highest values observed among GG homozygotes, whereas TC, HDL-C, and LDL-C did not differ significantly among the genotypes ($p > 0.05$). These findings suggest that LEPR Q223R primarily influences glucose homeostasis rather than lipid metabolism.
In contrast, the LEP -2548G>A (rs12112075) polymorphism showed no statistically significant associations with any of the investigated metabolic parameters in obese individuals (BMI > 30 kg/m²), as all comparisons yielded *p*-values > 0.05. Nevertheless, modest trends toward higher LDL-C and FPG values were observed in certain genotype groups, although these differences did not reach statistical significance.
Overall, Figure 31 highlights fasting plasma glucose (FPG) as the metabolic parameter most strongly influenced by genetic variation among the investigated polymorphisms. In contrast, the effects on the lipid profile were comparatively modest and appeared to depend on the specific genetic variant. While ADIPOQ rs266729 significantly affected both HDL-C and FPG, LEPR Q223R exerted a selective effect on glucose metabolism, and the LEP -2548G>A polymorphism showed no significant metabolic associations in obese individuals.

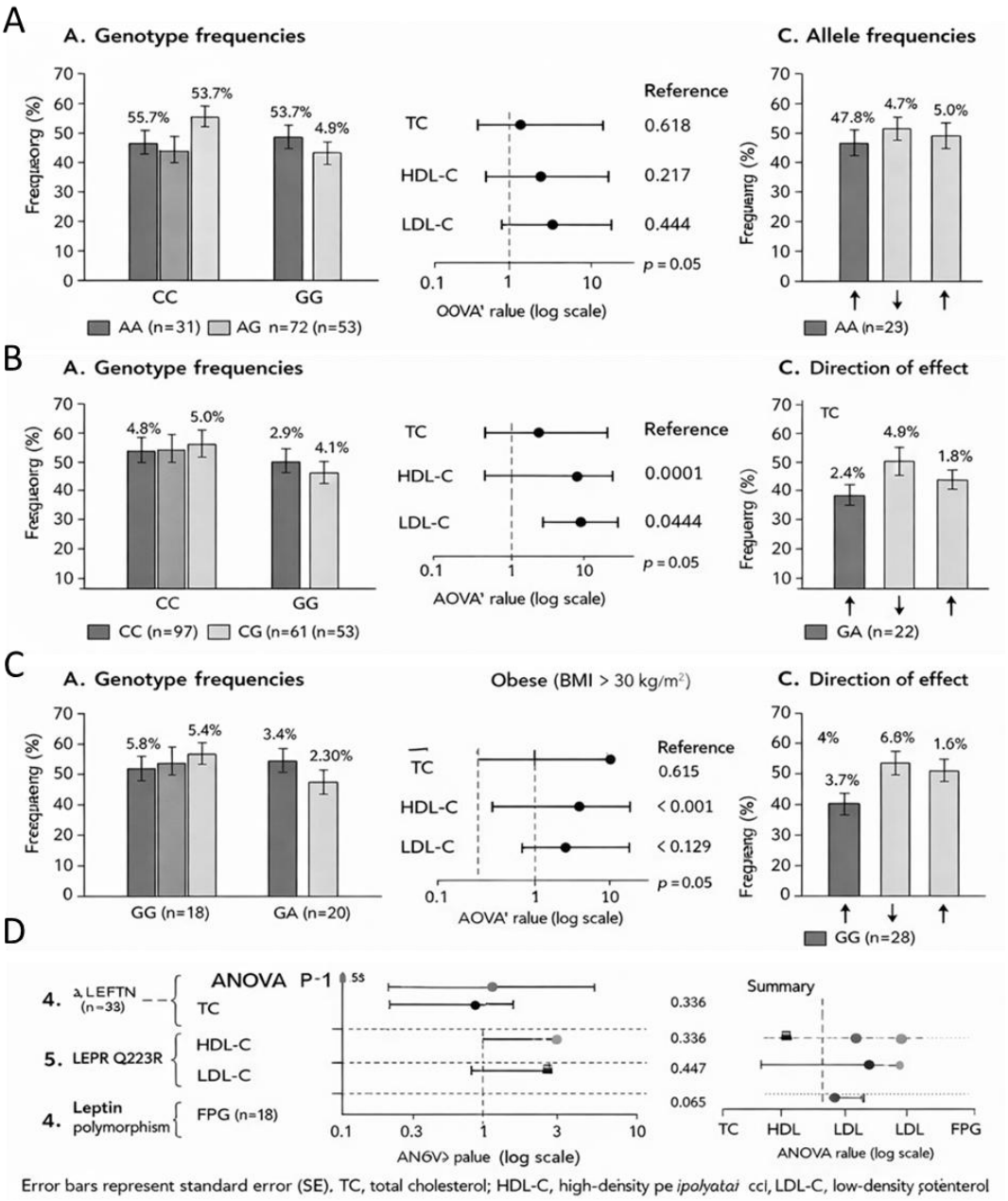


Figure 32. ***Association of the ADIPOQ rs266729 (C>G), LEPR Q223R (rs1137101), and LEP -2548G>A (rs12112075) polymorphisms with metabolic parameters in obese individuals.*** *(A) LEPR Q223R (rs1137101). (A) Mean ± standard error of the mean (SEM) values for total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG) according to genotype. (B) One-way ANOVA p-values presented on a logarithmic scale, demonstrating a statistically significant difference only for FPG ($p < 0.001$). (C) Direction of the genotype effect relative to the reference AA genotype.*

(B) ADIPOQ rs266729 (C>G). (A) Mean ± SEM values of metabolic parameters according to genotype. (B) One-way ANOVA p-values demonstrating statistically significant differences for HDL-C and FPG ($p < 0.001$). (C) Direction of the genotype effect relative to the reference GG genotype.

(C) LEP -2548G>A (rs12112075). (A) Metabolic parameters in obese individuals (BMI > 30 kg/m²) presented as mean ± SEM. (B) No statistically significant genotype-dependent differences were observed for any of the investigated metabolic parameters (all $p > 0.05$). (C) Direction of the genotype effect relative to the reference GG genotype.

(D) Comparative analysis of one-way ANOVA p-values for TC, HDL-C, LDL-C, and FPG across the three investigated polymorphisms. The dashed horizontal line indicates the threshold for statistical significance (p = 0.05).

Overall, the present findings indicate that ADIPOQ rs266729 exhibits the strongest and most consistent association with metabolic disturbances, followed by the more selective effect of LEPR Q223R (rs1137101) on glucose metabolism, whereas LEP -2548G>A (rs12112075) showed no significant overall association within the studied population (Figure 32).

Previous meta-analyses have demonstrated that the association between LEPR Q223R and obesity is generally weak and heterogeneous, whereas its relationship with type 2 diabetes mellitus (T2DM) and glycemic traits appears to be more consistent. These observations are in agreement with the present study, in which LEPR Q223R was not associated with obesity or lipid profile parameters but showed a significant association with fasting plasma glucose (FPG). Similarly, ADIPOQ rs266729 demonstrated its most pronounced effects on glucose homeostasis and HDL-cholesterol rather than on the overall lipid profile, suggesting that both polymorphisms primarily influence glucose regulation and insulin sensitivity rather than adiposity itself.

From a mechanistic perspective, adiponectin and leptin signaling converge on several common metabolic pathways, including AMP-activated protein kinase (AMPK), the IRS-1/PI3K signaling cascade, and GLUT4-mediated glucose transport. Dysregulation of these pathways results in impaired insulin sensitivity, increased hepatic gluconeogenesis, reduced peripheral glucose uptake, and ultimately hyperglycemia. Consistent with these observations, contemporary genome-wide association studies (GWAS) indicate that obesity and glycemic regulation have partially distinct genetic architectures, with glycemic phenotypes being more strongly influenced by endocrine and metabolic regulatory genes.

The LEP -2548G>A (rs12112075) polymorphism appears to exert a BMI-dependent effect. Among individuals with normal body weight, the AA genotype was associated with elevated fasting plasma glucose and an increased prevalence of arterial hypertension. In contrast, these associations were not evident among obese individuals, possibly because the effects of the polymorphism are masked by hyperleptinemia and leptin resistance, which characterize obesity. Nevertheless, the persistent association between the AA genotype and hypertension in obese participants supports the concept of selective leptin resistance, whereby leptin's anorexigenic effects are diminished while its sympathoexcitatory and pressor actions remain largely preserved.

From a clinical perspective, these findings suggest that ADIPOQ rs266729 and LEPR Q223R may represent potential genetic biomarkers of impaired glucose regulation and increased

susceptibility to T2DM, whereas LEP -2548G>A appears to contribute predominantly to cardiovascular risk, particularly hypertension, in obese individuals. Such polymorphisms may therefore improve genetic risk stratification for cardiometabolic disorders when considered alongside conventional clinical risk factors.

Several limitations should be acknowledged. The relatively modest sample size may have limited the statistical power to detect weaker genetic effects. In addition, fasting insulin concentrations and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) were not available, preventing direct assessment of insulin resistance. Furthermore, potential gene–environment interactions, including dietary habits, physical activity, and other lifestyle factors, could not be evaluated and should be addressed in future investigations.

In conclusion, the integrated analysis demonstrates that genetic variation within the ADIPOQ, LEPR, and LEP genes contributes to different components of the metabolic syndrome. Among the investigated variants, ADIPOQ rs266729 exhibited the strongest and most consistent association with adverse metabolic traits, LEPR Q223R primarily influenced glucose homeostasis, and LEP -2548G>A displayed BMI-dependent effects on glucose metabolism and arterial hypertension. Collectively, these findings emphasize the central role of adipokine signaling in the early pathogenesis of type 2 diabetes mellitus and support the potential utility of these polymorphisms as genetic biomarkers of cardiometabolic risk.

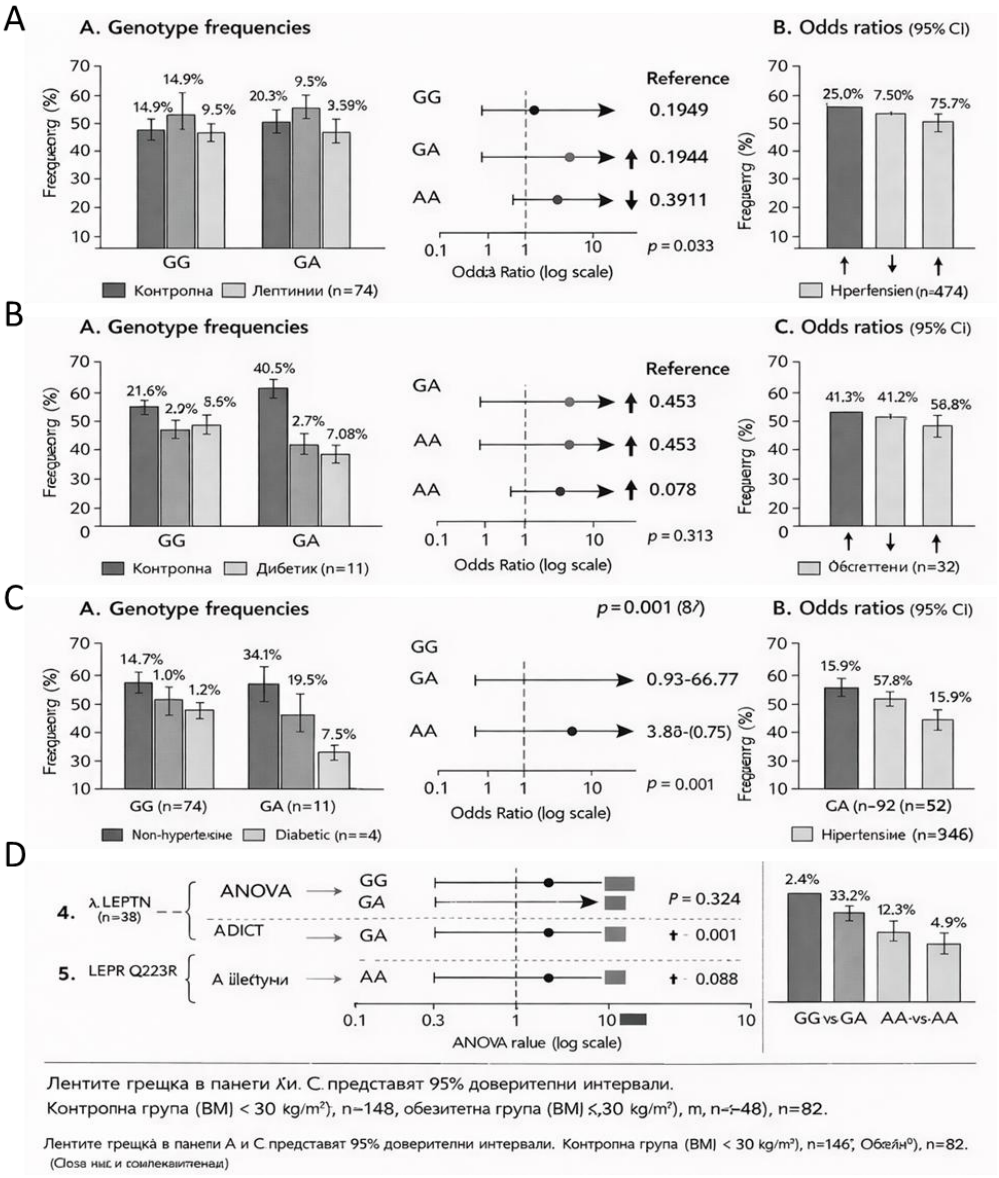


Figure 33. Association of the LEP -2548G>A polymorphism with hypertension and type 2 diabetes mellitus (T2DM) in the control and obese groups. *(A) Control group. (A) Genotype frequencies (%) among participants with and without hypertension. (B) Graphical representation of the association, with the dashed line indicating the threshold for statistical significance. A borderline statistically significant difference was observed (p = 0.049). (C) Genotype distribution among participants with and without T2DM, showing no statistically significant association (p = 0.058).*

(B) Obese group. (A) Genotype frequencies (%) among participants with and without hypertension, demonstrating a highly significant association (p < 0.001). (B) Genotype distribution among participants with and without T2DM, with no statistically significant association (p = 0.324). (C) Graphical representation of the direction of the genotype effect.

(C) Comparative analysis of the control and obese groups with respect to genotype frequencies and associated clinical conditions. The dashed horizontal line indicates the threshold for statistical significance (p = 0.05). Data are presented as percentage frequencies (%).

(D) Comparative analysis of the statistical significance of the metabolic effects associated with the investigated genetic polymorphisms, including the LEP -2548G>A polymorphism and LEPR Q223R (rs1137101). Statistical analysis was performed using one-way analysis of variance (one-way ANOVA), with p-values displayed on a logarithmic scale to facilitate visualization of statistically significant and non-significant effects.

The results presented in Figure 33 demonstrate the association of the LEP -2548G>A (rs12112075) polymorphism with arterial hypertension and type 2 diabetes mellitus (T2DM) in both the control and obese groups.

In the control group, a borderline statistically significant association was observed between genotype distribution and hypertension ($p = 0.049$), indicating that the LEP genotype may influence susceptibility to elevated blood pressure even in individuals without obesity. In contrast, the association with T2DM did not reach statistical significance ($p = 0.058$), although the distribution of genotypes suggested a possible trend that warrants further investigation.

Among obese individuals, the association between LEP -2548G>A and hypertension was markedly stronger, reaching high statistical significance ($p < 0.001$). The AA genotype was substantially more frequent among hypertensive patients, whereas the GG genotype was relatively uncommon, suggesting that the AA genotype may confer an increased susceptibility to hypertension in the presence of obesity. Conversely, no significant association was observed between genotype distribution and T2DM ($p = 0.324$).

The comparative analysis further indicates that the effects of the LEP -2548G>A polymorphism are phenotype-specific. The absence of a significant association with obesity or T2DM does not imply a lack of biological relevance but rather suggests that this variant preferentially influences blood pressure regulation rather than adiposity or glucose metabolism. These observations are consistent with findings from large-scale DIAGRAM and MAGIC consortia, which have demonstrated that glycemic regulation and obesity are characterized by partially distinct genetic architectures.

From a mechanistic perspective, the association between the AA genotype and hypertension is biologically plausible. Leptin is known to increase sympathetic nervous system activity, impair renal sodium handling, and promote endothelial dysfunction, all of which contribute to elevated blood pressure. In obesity, these effects may persist despite the development of selective leptin resistance, whereby leptin's anorexigenic actions are diminished while its cardiovascular effects remain largely intact.

From a clinical perspective, these findings suggest that LEP -2548G>A may serve as a genetic marker of cardiovascular risk, particularly hypertension in obese individuals, whereas ADIPOQ rs266729 and LEPR Q223R appear to be more informative markers of impaired glucose regulation and susceptibility to type 2 diabetes mellitus. The observed BMI-dependent effects of the LEP polymorphism further support the concept of personalized genetic risk assessment based on an individual's metabolic status.

The present study has several limitations, including the relatively modest sample size, the absence of fasting insulin and HOMA-IR measurements for direct assessment of insulin resistance, and the inability to evaluate gene–environment interactions. These limitations should be addressed in future studies involving larger and more diverse populations.

In conclusion, the integrated analysis of ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP -2548G>A (rs12112075) demonstrates that genetic variation within the adipokine signaling pathway primarily affects glucose homeostasis, insulin sensitivity, and cardiovascular regulation, whereas its influence on obesity and lipid metabolism is comparatively modest. Among the investigated variants, ADIPOQ rs266729 exhibited the most consistent metabolic effects, LEPR Q223R was predominantly associated with fasting plasma glucose, and LEP -2548G>A showed a BMI-dependent association with arterial hypertension. Together, these findings expand our understanding of the molecular basis of T2DM and cardiometabolic disease and support the application of an integrated genetic approach for cardiometabolic risk assessment.

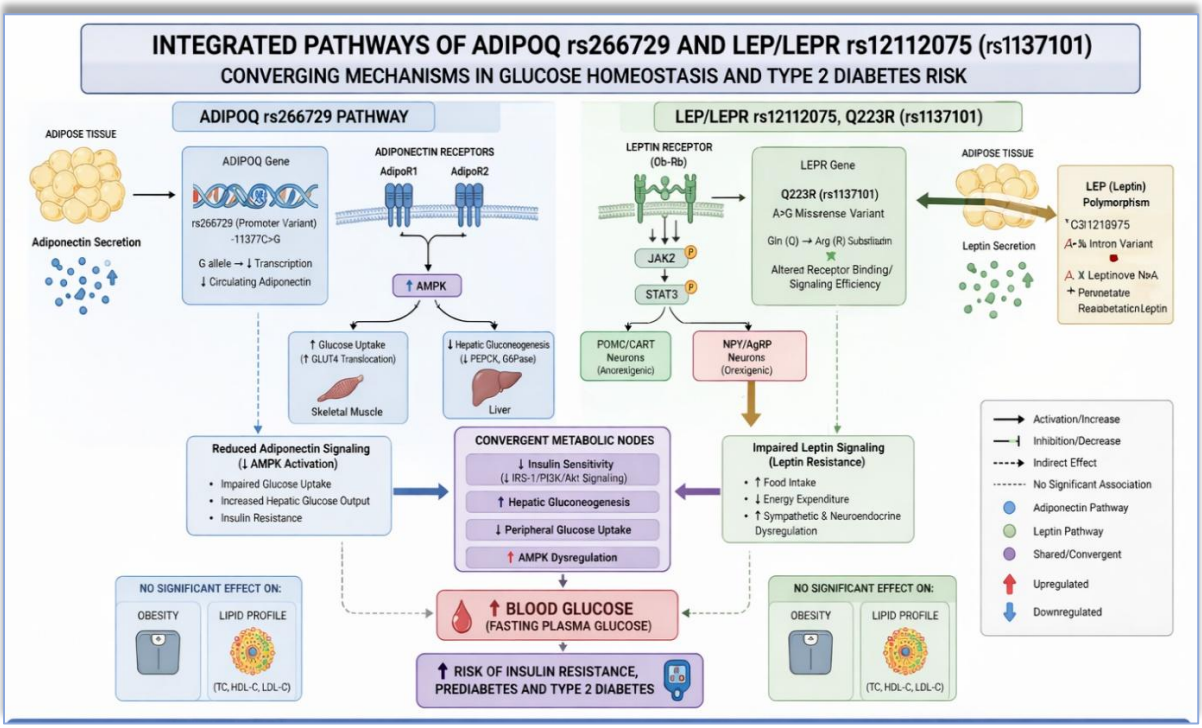


Figure 34. Integrated signaling pathways linking ADIPOQ rs266729, LEP −2548G>A (rs7799039), and LEPR Q223R (rs1137101) polymorphisms with cardiometabolic risk.

V. Conclusion

The present study investigated the relationship between phenotypic characteristics and genetic variation within the adipokine signaling pathway, including ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP −2548G>A (rs7799039), in the context of obesity and type 2 diabetes mellitus (T2DM) in a Northern Bulgarian population.

The phenotypic analysis identified a metabolic profile characteristic of metabolic syndrome, including increased body mass index (BMI), reduced serum HDL-cholesterol concentrations, and a higher prevalence of arterial hypertension and T2DM among obese individuals. The absence of a significant difference in fasting plasma glucose (FPG) between the study groups likely reflects the heterogeneous glycemic status of the investigated population.

Genetic analysis demonstrated that ADIPOQ rs266729 was significantly associated with an increased risk of obesity and T2DM. Carriers of the G allele and the CG and GG genotypes exhibited a significantly higher risk, together with lower HDL-cholesterol levels and elevated fasting plasma glucose, suggesting that this promoter variant contributes to impaired insulin sensitivity and glucose homeostasis.

In contrast, LEPR Q223R (rs1137101) showed no significant association with obesity or lipid profile parameters. However, a clear genotype-dependent relationship with fasting plasma glucose was observed (AA < AG < GG), indicating an additive effect of the G allele on glucose regulation and supporting its role in glucose homeostasis rather than adiposity.

The LEP −2548G>A (rs7799039) polymorphism was not significantly associated with obesity or lipid profile parameters. Nevertheless, genotype-dependent differences in fasting plasma glucose were identified within the control group, together with a significant association with arterial hypertension among obese individuals, indicating that the biological effects of this variant are BMI-dependent and influenced by the underlying metabolic context.

The integrated analysis demonstrated that genetic variation within the adipokine signaling pathway exerts a more consistent influence on glucose homeostasis than on obesity or lipid metabolism. These findings support the hypothesis that disturbances in adipokine signaling contribute more substantially to the development of insulin resistance and T2DM than to adiposity itself.

The results should be interpreted in light of several study limitations, including the relatively modest sample size, the case–control study design, the absence of direct measures of insulin resistance (fasting insulin and HOMA-IR), and the lack of evaluation of gene–environment interactions.

In conclusion, ADIPOQ rs266729 emerged as the polymorphism most strongly associated with an increased risk of obesity and T2DM, whereas LEPR Q223R (rs1137101) was primarily associated with impaired glucose homeostasis. The LEP −2548G>A (rs7799039) polymorphism demonstrated more limited, BMI-dependent effects, predominantly influencing the risk of arterial hypertension in obese individuals. Collectively, these findings expand current knowledge of the role of adipokine signaling in the pathogenesis of metabolic disorders and support the need for future studies in larger, independent populations to validate these associations and further elucidate their underlying biological mechanisms.

VI. Conclusions

- Obese individuals in the studied population were characterized by a significantly higher body mass index (BMI), lower serum HDL-cholesterol concentrations, and a higher prevalence of arterial hypertension and type 2 diabetes mellitus (T2DM) compared with the control group. These findings are consistent with the phenotypic characteristics of metabolic syndrome.
- The obtained data suggest that the ADIPOQ rs266729 polymorphism is associated with an increased susceptibility to obesity and T2DM, with carriers of the G allele exhibiting a significantly higher risk in the studied population.
- The observed genotype-dependent trend (CC < CG < GG), together with the association of ADIPOQ rs266729 with fasting plasma glucose and HDL-cholesterol levels, suggests that this polymorphism may contribute to impaired glucose homeostasis and lipid metabolism.
- No convincing evidence was found for an association between the LEPR Q223R (rs1137101) polymorphism and obesity or lipid profile parameters. However, its association with fasting plasma glucose, together with the genotype-dependent trend (AA < AG < GG), is consistent with a potential role of this variant in the regulation of glucose metabolism.
- The LEP −2548G>A (rs7799039) polymorphism was not significantly associated with obesity, T2DM, or lipid profile parameters. Nevertheless, the observed genotype-dependent differences in fasting plasma glucose within the control group and its significant association with arterial hypertension among obese individuals suggest that the biological effects of this variant may depend on the underlying metabolic context.

- Within the present study, the associations between the investigated genetic polymorphisms and glucose homeostasis were more consistent than those observed for lipid profile parameters or the degree of obesity.
- The findings support the hypothesis that genetic variation within the ADIPOQ, LEPR, and LEP genes may contribute to inter-individual differences in cardiometabolic risk, potentially through mechanisms involving adipokine signaling, insulin sensitivity, and glucose homeostasis.
- Considering the limitations of the present study, including the relatively modest sample size and the case–control study design, the observed associations should be interpreted with caution and require confirmation in larger, independent populations and functional studies.

VII. Scientific Contributions

1. Original Scientific Contributions

- For the first time, a comprehensive analysis of the association between the ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP −2548G>A (rs7799039) polymorphisms and the phenotypic manifestations of metabolic syndrome has been performed in an ethnically homogeneous population from Northwestern Bulgaria.
- Novel data have been generated on the genotype and allele frequencies of the investigated polymorphisms in the studied population, expanding the available knowledge of the genetic architecture of the Bulgarian population with respect to these adipokine-related variants.
- A statistically significant association was identified between ADIPOQ rs266729 and an increased susceptibility to obesity and type 2 diabetes mellitus (T2DM), as well as with fasting plasma glucose and HDL-cholesterol levels, suggesting that this polymorphism may contribute to individual predisposition to cardiometabolic disorders.
- The findings demonstrate that the LEPR Q223R (rs1137101) polymorphism is not associated with obesity or lipid profile parameters in the studied population but may contribute to inter-individual variability in fasting plasma glucose, supporting its potential role in glucose homeostasis.
- Evidence was obtained suggesting a context-dependent (BMI-dependent) effect of the LEP −2548G>A (rs7799039) polymorphism, reflected by differential associations with glucose homeostasis and arterial hypertension according to the metabolic phenotype of the investigated individuals.

- Through an integrated analysis of clinical, biochemical, and genetic data, the relative contribution of the investigated polymorphisms to individual components of metabolic syndrome was evaluated. The findings suggest that these genetic variants are more consistently associated with disturbances in glucose homeostasis than with lipid profile parameters or the degree of obesity.

2. Applied Scientific Contributions

- The findings of the present study expand the available knowledge on the distribution of the ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP −2548G>A (rs7799039) polymorphisms in a Northern Bulgarian population and may serve as reference data for future genetic and epidemiological studies.
- The identified associations between the investigated genetic variants and specific indicators of glucose homeostasis suggest that the integration of genetic information with conventional clinical and biochemical parameters may contribute to a more accurate assessment of individual cardiometabolic risk.
- The results highlight the importance of a comprehensive approach to the evaluation of patients with obesity and metabolic disorders, based on the combined analysis of phenotypic, biochemical, and genetic characteristics.
- The data generated in the present study provide a foundation for the future validation of ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP −2548G>A (rs7799039) as potential components of polygenic risk models for cardiometabolic diseases.
- The findings provide a basis for future prospective, multicenter, and functional studies aimed at further elucidating the role of adipokine signaling in the pathogenesis of obesity, type 2 diabetes mellitus (T2DM), and related cardiometabolic disorders.

VIII. Future Directions and Perspectives

- Conduct studies involving larger, multicenter, and multiethnic cohorts to validate the observed associations and increase the statistical power and generalizability of the findings.
- Include additional metabolic biomarkers, such as fasting insulin, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), and glycated hemoglobin (HbA1c), to enable a more comprehensive assessment of insulin resistance and glucose homeostasis.

- Investigate gene–environment interactions, including the influence of dietary habits, physical activity, and lifestyle factors on the phenotypic effects of ADIPOQ, LEPR, and LEP polymorphisms.
- Perform functional and molecular studies to elucidate the biological mechanisms through which ADIPOQ rs266729, LEPR Q223R (rs1137101), and LEP −2548G>A (rs7799039) influence adipokine signaling pathways and metabolic regulation.
- Develop and validate polygenic risk scores (PRSs) incorporating the investigated polymorphisms to improve the prediction of type 2 diabetes mellitus (T2DM) and overall cardiometabolic risk.
- Conduct longitudinal prospective studies to evaluate the temporal relationships between these genetic variants and the development and progression of metabolic disorders.
- Integrate genetic, clinical, and biochemical data to facilitate the development of personalized strategies for the prevention, early diagnosis, and management of cardiometabolic diseases.
- Investigate the potential of adiponectin and leptin signaling pathways as therapeutic targets for type 2 diabetes mellitus and related cardiometabolic disorders.

IX. Publications, Scientific Meetings, and Research Project Related to the Dissertation

Publications

1. K. Kovacheva, P. Nikolova, V. Hristov, D. Pendicheva, S. Marchev, **T. Rashev**. Preliminary data of a study on polymorphism rs4244285 of P4502C19 cytochrome gene in patients with acute coronary syndrome, undergoing treatment with dual antiplatelet therapy with Clopidogrel and Aspirin. Journal of Biomedical and Clinical Research, 2016, 9(1): 65-71; ISSN: 1313-6917; doi: 10.1515/jbcr-2016-0010; Web of Science
2. **Rashev TR**, Marinova DM, Dobrev M, Trifonov SV. De novo design of a PCR-RFLP assay for detecting the leptin receptors single nucleotide polymorphism in hypothalamic nuclei. Journal of IMAB, 2022, Supplement 12 SEEC & 32 IMAB, vol. 28, pp.59-62; ISSN: 1312-773X; Web of Science
3. Marinova DM, Dobrev ML, **Rashev TR**, Gerasimov IK, Nankov V, Trifonov SV. Acetylcholine and its synthesizing enzyme choline acetyltranspherase in the enteric nervous system. Journal of IMAB, 2022, Oct-Dec, 28(4): 4671-4675; doi: 10.5272/jimab.2022284.4671; ISSN: 1312-773X; Web of Science

Conference Presentations

1. Desislava M. Marinova, **Tihomir R. Rashev**, Stefan V. Trifonov, Testing of primers and optimization of PCR for the detection of alternatively spliced variants of mouse ChAT mRNA, XXVI National Congress of the Bulgarian Anatomical Society, 29.09-01.10, 2023, Sofia.
2. **T. Rashev**, D. Marinova, M. Dobrev, S. Trifonov, De novo design of a PCR-RFLP assay for detecting the leptin single nucleotide polymorphism G – 2548A in the human genome, 20th Congress of the International Federation of Associations of Anatomists – IFAA 2022, 5-7.08.2022.
3. **T. Rashev**, D. Marinova, M. Dobrev, S. Trifonov, De novo design of a PCR-RFLP assay for detecting leptin receptor single nucleotide polymorphism in hypothalamic nuclei, 12th South-East European Conference of chemotherapy, infections and cancer and 32st Annual Assembly of International Medical Association Bulgaria, 20-23.10.2022, Trakia University, St. Zagora.

Research Project

Project D1/2021 – Medical University–Pleven

Title: Investigation of the G−2548A polymorphism in the leptin (LEP) gene and the Q223R polymorphism in the leptin receptor (LEPR) gene in patients with obesity and metabolic syndrome.

Appendix 1

Overview of Studies Describing Cross-talk and Cross-activation of the Leptin Receptor (Ob-R/LEPR)

Receptor System	Model Type	Experimental Model	Key Effects of Leptin	Reference
EGFR	In vitro	Gastric cancer cells (MKN28, MKN74)	↑ EGFR phosphorylation; EGFR inhibitor (AG1478) блокира JAK2 и ERK1/2	(237)
	In vitro	Breast cancer cells (MCF7, MDA-MB-231)	↑ пролиферация, миграция, survivin, Notch-I, EGFR phosphorylation	(238)
	In vitro	Esophageal cancer cells	↑ пролиферация, ↓ апоптоза, ↑ EGFR/ERK1/2; инхибитори блокират ефекта	(239, 240)

	In vitro	Rat vascular smooth muscle	↑ EGFR, ERK1/2, endothelin-1	(241)
	In vitro	Gastric mucosal cells	↑ EGFR, cPLA2; защита от цитотоксичност	(242)
	In vitro	Salivary gland cells	↑ EGFR, cPLA2; защитен ефект	(243)
	In vitro	HEK293T (transfected)	Long & short ObR → EGFR activation	(244)
	In vivo	Rat kidney	↑ Na^+/K^+-ATPase, H_2O_2, ERK1/2	(245)
ERα	In vitro	Breast cancer cells	ERα необходим за STAT3 activation; ↑ клетъчна жизнеспособност	(246)
	In vitro	MCF7	↑ aromatase чрез STAT3/ERK	(247)
	In vitro	MCF7, HeLa	↑ nuclear ERα; ↑ expression	(248)
	In vitro	MCF7	↑ ERα levels	(249)
	Ex vivo	Breast cancer patients	Корелация ObR ↔ ERα	—
	In vivo	Mouse xenograft	↑ ERα, ↓ ERβ	(250)
IGF-IR	In vitro	Breast cancer cells	Co-IP ObR + IGF-IR; IGF-I → ObR phosphorylation	(251)
	In vitro	Breast cancer cells	Bidirectional activation; синергия с EGFR; ↑ метастаза	(252)
LRP1	In vivo	Knockout mice	↓ leptin signaling; obesity	(253)
			LRP1 необходим за STAT3 activation	
LRP2 (Megalin)	In vivo/in vitro	Multiple species	↑ leptin uptake (kidney, cells)	(254)
	In vivo	Rats	Transport през choroid plexus	(255)
	In vivo/in vitro	Rats, cells	Binding (Ca^{2+}-dependent), internalization, degradation	(256)
VEGFR	In vitro	HUVEC cells	↑ proliferation, COX-2, VEGFR2 phosphorylation	(257)

	In vitro	Endothelial cells	Trans-phosphorylation VEGFR1/2; ↑ Notch, angiogenesis	(258)

Appendix 2A.

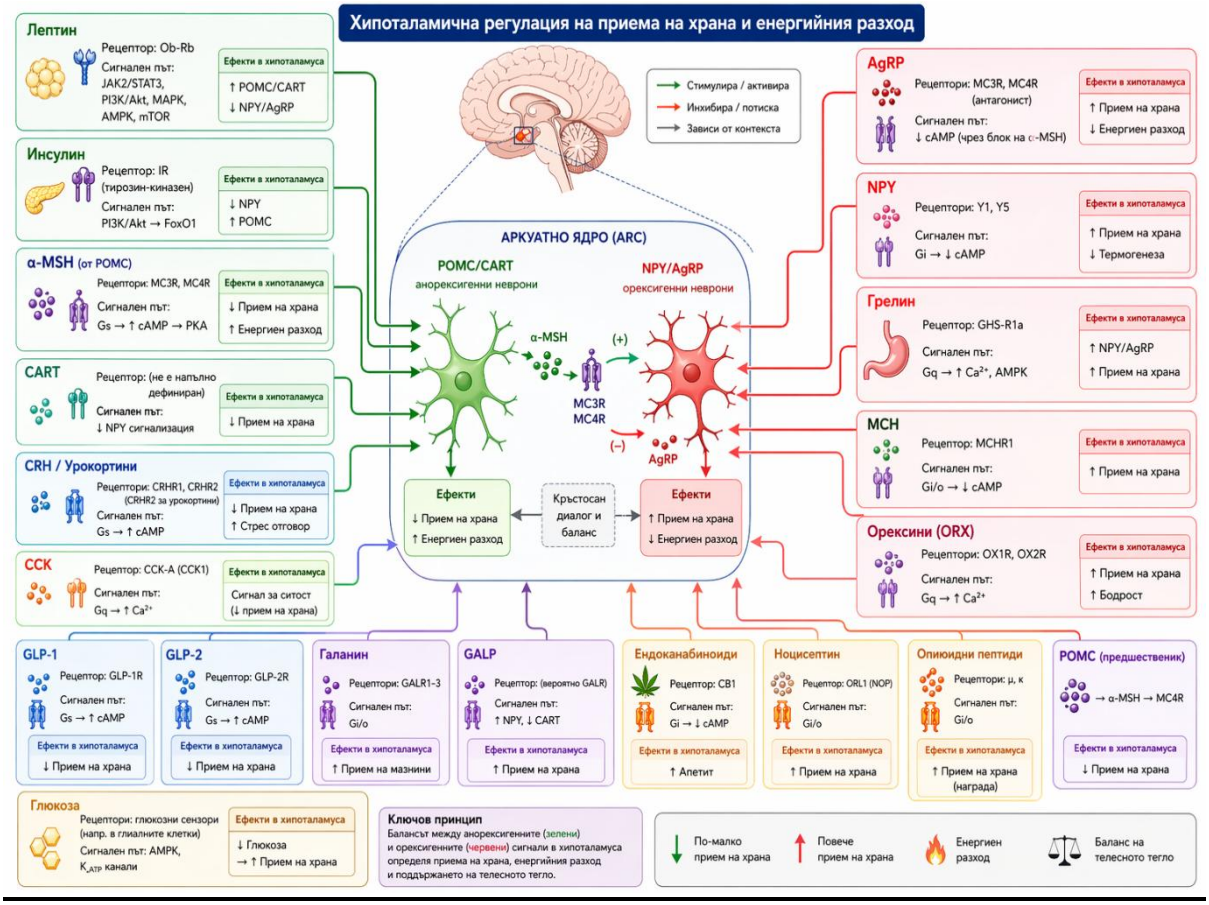
Functionally Oriented Table of Receptors and Major Signaling Pathways in Hypothalamic Nuclei

Molecule / Neuropeptide	**Effect**	**Receptor(s)**	**Major Signaling Pathways**	**Primary Effects in the Hypothalamus**
AgRP	Orexigenic	MC3R, MC4R (antagonist)	↓ cAMP (via inhibition of α-MSH signaling)	↑ Food intake, ↓ energy expenditure
α-MSH (POMC-derived)	Anorexigenic	MC3R, MC4R	Gs → ↑ cAMP → PKA	↓ Food intake, ↑ energy expenditure
NPY	Strongly orexigenic	Y1, Y5	Gi → ↓ cAMP	↑ Food intake, ↓ thermogenesis
CART	Anorexigenic	(Not fully characterized)	Inhibition of NPY signaling	↓ Food intake
Leptin	Anorexigenic	Ob-Rb (LEPR)	JAK2/STAT3, PI3K/Akt, MAPK/ERK, AMPK, mTOR	↑ POMC/CART, ↓ NPY/AgRP
Insulin	Anorexigenic	Insulin receptor (IR)	PI3K/Akt → FoxO1	↓ NPY, ↑ POMC
Ghrelin	Orexigenic	GHS-R1a	Gq → ↑ Ca^{2+}, AMPK	↑ NPY/AgRP activity
Melanin-Concentrating Hormone (MCH)	Orexigenic	MCHR1	Gi/o → ↓ cAMP	↑ Food intake

Orexins (Hypocretins)	Orexigenic	OX1R, OX2R	Gq → ↑ Ca^{2+}	↑ Food intake, ↑ arousal/wakefulness
Corticotropin-Releasing Hormone (CRH)	Anorexigenic	CRHR1, CRHR2	Gs → ↑ cAMP	↓ Food intake, ↑ stress response
Urocortins	Anorexigenic	CRHR2	Gs → ↑ cAMP	↓ Appetite
Cholecystokinin (CCK)	Anorexigenic	CCK-A (CCK1R)	Gq → ↑ Ca^{2+}	Satiety signaling
GLP-1	Anorexigenic	GLP-1R	Gs → ↑ cAMP	↓ Food intake
GLP-2	Anorexigenic	GLP-2R	Gs	↓ Food intake
Galanin	Orexigenic	GALR1–3	Gi/o	↑ Fat intake
GALP (Galanin-like peptide)	Orexigenic	(Likely GALR)	↑ NPY, ↓ CART	↑ Food intake
Endocannabinoids	Orexigenic	CB1	Gi → ↓ cAMP	↑ Appetite
Nociceptin (Orphanin FQ)	Orexigenic	NOP (ORL1)	Gi/o	↑ Food intake
Opioid peptides	Orexigenic	μ-, κ-opioid receptors	Gi/o	↑ Food intake (reward-driven feeding)
POMC	Anorexigenic	(Precursor peptide)	Processed to α-MSH → MC4R activation	↓ Food intake
Glucose	Metabolic regulator	Glucose sensors	AMPK, KATP channels	↓ Blood glucose → ↑ Food intake

Appendix 2B

Functionally Oriented Diagram of Major Receptors and Signaling Pathways in the Hypothalamic Nuclei



Appendix 3

Patient Information Sheet

Project Title

"Investigation of the Carrier Status of Leptin G-2548A, Leptin Receptor Q223R, and Adiponectin rs266729 Polymorphisms in Patients with Obesity and Metabolic Syndrome"

Information for the Patient Regarding the Nature of the Study

Dear Sir/Madam,

We would like to invite you to participate in the above-mentioned research project. Your participation is entirely voluntary, and you are under no obligation to take part if you do not wish to do so.

It is important that you read this information carefully before deciding whether to participate. After reviewing the information, you have the right to ask any questions you may have. If you receive satisfactory answers and decide to participate, please complete the consent form, thereby confirming your voluntary agreement to participate in the study.

If you decide not to participate or wish to withdraw from the study at any time, you may do so without providing any explanation. Your decision will not affect your medical treatment, the

quality of care you receive, or your relationship with the medical staff. If you decide to withdraw from the study, we kindly ask you to inform the research team.

The study will be conducted by physicians from the **Medical University – Pleven**.

If you have any questions regarding the study, you may contact the Principal Investigator:

Assoc. Prof. Stefan Venelinov Trifonov, MD, PhD
Department of Anatomy, Histology, Cytology and Biology
Medical University – Pleven

We invite you to participate in this study in order to determine whether your condition has a genetic basis and whether elevated levels of specific adipokines, increased fasting plasma glucose, arterial hypertension, and obesity are associated with genetic factors. The results of this project will contribute to a better understanding of the mechanisms underlying these conditions. Such knowledge may improve the identification of individuals at increased risk and facilitate the selection of the most appropriate therapeutic approach for each patient.

The duration of the study is **one year**.

At the beginning of the study, a physician from the research team will ask you questions regarding your current and previous medical conditions, as well as collect certain personal information, including your date of birth, address, telephone number, and contact information for a close relative, in order to maintain contact with you after your discharge from the hospital. This information will be recorded in a Patient Record Form, which will be accessible only to members of the research team.

In addition to the routine laboratory investigations required for patients with obesity and metabolic syndrome, approximately **10 mL of venous blood** will be collected for DNA analysis. The risks associated with this blood sampling do not exceed those associated with routine venipuncture performed for standard laboratory tests. These risks will be minimized because the blood sample will be collected by trained healthcare personnel following established procedures for safe venous blood collection.

The project does not provide reimbursement for transportation expenses related to follow-up visits.

If you decide to participate, all information collected about you will remain strictly confidential. Authorized members of the research team will have access to your medical records only for the purposes of this study and under strict confidentiality.

No financial compensation is предусмотрена for participation or for any potential harm associated with the study, as participation does not involve additional risks beyond those

associated with routine venous blood collection, provided that all safety procedures are followed and blood samples are collected by qualified personnel.

Informed Consent Form

Project Title

"Investigation of the Carrier Status of Leptin G-2548A, Leptin Receptor Q223R, and Adiponectin rs266729 Polymorphisms in Patients with Obesity and Metabolic Syndrome"

Please indicate **Yes** or **No** for each of the following statements by circling or underlining your answer.

I was asked to provide my own informed consent.
Yes / No

I have read the Patient Information Sheet.
Yes / No

I was given the opportunity to ask all questions that were important to me and to discuss this research project.
Yes / No

I received satisfactory answers to all of my questions.
Yes / No

I received sufficient information about the study.
Yes / No

The study was explained to me by:

(Name of the Investigator)

Signature of the Investigator:

I understand that my participation in this research project is entirely voluntary and that I am free to withdraw my consent and discontinue participation at any time, without providing any reason, and without this affecting my current or future medical care in any way.

Date: _____

Patient's Full Name (First Name, Middle Name, Last Name):

Patient's Signature: _____

Appendix 4

Salting-Out DNA Extraction Method

Peripheral venous blood (3–10 mL) collected into **EDTA anticoagulant tubes** (EDTA K2, VENOJECT II) was used for DNA extraction. Hemolyzed, clotted, or insufficient blood samples do not guarantee adequate quantity and quality of the isolated DNA. If DNA extraction cannot be performed immediately, blood samples may be stored for several hours at room temperature, up to **48 hours at 4°C**, or for several months at **−20°C**. The use of specialized **Becton Dickinson collection tubes** allows storage and transportation of samples at room temperature for up to **72 hours**.

I. Preparation of the Nuclear Cell Lysate

Three to ten milliliters of EDTA-anticoagulated venous blood were transferred into a **50-mL conical centrifuge tube**, and **1× erythrocyte lysis buffer** was added to a final volume of **40 mL**. The tube was gently inverted several times to ensure thorough mixing and incubated on an ice-water bath for **30 minutes**.

Samples were centrifuged at **3,000 rpm for 10 minutes** at **4°C** (or alternatively at **18°C**). Leukocyte nuclei formed a pellet, while erythrocyte debris and other cellular components remained in the supernatant. The supernatant was carefully discarded, and the pellet was resuspended in **20 mL of cold erythrocyte lysis buffer**. After a second centrifugation under identical conditions, the supernatant was removed and the remaining cell pellet was resuspended in **5 mL of SE (nuclear lysis) buffer**.

II. Digestion of Proteins and RNA

To the suspension containing the nucleated cells, **25 µL Proteinase K (10 mg/mL)** and **400 µL of 10% sodium dodecyl sulfate (SDS)** were added. The mixture was gently mixed and incubated either **overnight (approximately 12 hours) at 37°C** or for **5 hours at 55°C**.

Cell lysis was monitored visually. Complete lysis was indicated by a clear, non-opalescent solution.

III. DNA Purification by Salting-Out

Following complete lysis, **2 mL of 6 M sodium chloride (NaCl)** was added to the lysate, followed by vigorous vortex mixing for **15 seconds**.

The samples were centrifuged at **3,000 rpm for 15 minutes**. Proteins and other contaminants formed a pellet, while genomic DNA remained in the supernatant.

IV. DNA Precipitation

The DNA-containing supernatant was carefully transferred into a new tube, and **two volumes of cold absolute ethanol** were added. Genomic DNA became visible as white fibrous strands that could be spooled using a sterile Pasteur pipette.

For long-term storage in a DNA biobank, the DNA was transferred into tubes containing **96% ethanol**.

The precipitated DNA was washed with **1 mL of 80% ethanol**, air-dried, and dissolved in **TE buffer** using a final volume between **300 µL and 1.5 mL**, depending on DNA yield. Tubes were sealed with parafilm, appropriately labeled, and placed on a laboratory shaker at room temperature overnight to ensure complete dissolution.

The expected DNA yield from **10 mL of peripheral blood** was approximately **300–800 µg** of high-molecular-weight genomic DNA.

DNA Polymorphisms (Indirect DNA Analysis)

Individuals differ genetically because they carry different alleles at the same genetic locus, particularly within **non-coding regions of DNA**. These variations contribute to the extensive genetic diversity observed within populations while simultaneously providing genetic uniqueness to each individual.

Differences in nucleotide sequences located within non-coding DNA regions are referred to as **DNA polymorphisms**.

Definition

A genetic locus is considered **polymorphic** when the frequency of its least common allele is **≥1%**, or when the frequency of its most common allele is **≤99%** within a given population.

Polymorphic loci suitable for laboratory analysis may serve as **genetic markers** for the diagnosis and investigation of hereditary diseases. Particularly valuable markers are those exhibiting a high degree of polymorphism, possessing numerous alleles with frequencies of **≥1%** within the studied population.

Localization of DNA Polymorphisms

DNA polymorphisms may occur:

- **Outside coding regions**, within non-coding DNA sequences, where they are generally regarded as neutral genetic variants.
- **Within intronic regions**, where they usually have no functional consequences and therefore are not associated with phenotypic effects.
- **Within coding sequences**, although such variants are relatively uncommon and may also occur without producing detectable phenotypic changes.

Inheritance

DNA polymorphisms are inherited as **codominant genetic markers**, meaning that offspring inherit one allele from each parent.

Significance of DNA Polymorphisms

DNA polymorphisms:

- contribute substantially to genetic diversity within populations;
- provide genetic individuality for each person;
- are generally not associated with clinical phenotypes;
- can serve as highly informative **genetic markers**, provided they exhibit:
    - a high degree of polymorphism; and
    - localization within or in close proximity to the gene of interest.

Types of DNA Polymorphisms

One of the most common forms of genetic variation is the **single nucleotide polymorphism (SNP)**, which results from the substitution of one nucleotide by another.

Characteristics of SNPs

- **Biallelic markers**, with each individual carrying two alleles at a given locus.
- **Maximum heterozygosity of approximately 50%.**
- Possible genotypes:
    - Homozygous wild-type (+/+)
    - Heterozygous (+/−)
    - Homozygous variant (−/−)
- **Relatively low individual information content**, although their abundance throughout the genome makes them highly valuable for genetic association studies and disease susceptibility analyses.

Single Nucleotide Polymorphisms (SNPs)

- Do **not** alter restriction enzyme recognition sites.
- Are most commonly analyzed by **direct DNA sequencing** or allele-specific genotyping methods.

Restriction Fragment Length Polymorphisms (RFLPs)

- Result from sequence variations that **create or abolish restriction enzyme recognition sites**.
- Are most commonly analyzed by **restriction enzyme digestion (restriction analysis)** followed by fragment size separation.

Tandem Repeat Length Polymorphisms

Synonyms:

- Variable Number Tandem Repeats (**VNTRs**)
- Short Tandem Repeats (**STRs**) or Microsatellites (depending on repeat length)

Characteristics:

- **Multiallelic** genetic markers.
- **Maximum heterozygosity:** typically **70–99%**.
- **Information content:** highly polymorphic, providing excellent discriminatory power for genetic linkage studies, population genetics, and forensic identification.