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ORIGINAL RESEARCH

Involvement of Single Nucleotide Variants in the *Klotho* Gene Among Obesity Individuals with and without Type 2 Diabetes Mellitus in the Saudi Population

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Purpose: Aging is characterized by the gradual physiological changes and alterations that accumulate over time in the human body. The combination of obesity and ageing can lead to an increased risk of serious health issues or death. Single nucleotide variants (SNVs) in the *Klotho* gene were commonly studied, including that in type 2 diabetes mellitus (T2DM).

Aim: The aim of this study is to examine the possible effect of SNVs in *Klotho* on the obese population in Saudi Arabia using middle-aged participants with and without T2DM.

Methods: This study consists of 100 controls and 100 obesity patients, in which 50 had T2DM and the remaining 50 were obese without T2DM. Genotyping was performed with PCR, and Sanger sequencing analysis was used to validate the molecular association.

Results: In this study, rs1207568 (p = 0.001–0.003) and rs9527025 (p = 0.001–0.00004) SNVs were associated with obesity cases. However, none of the genotypes or allele frequencies showed a positive association with the rs564481 SNV (p = 0.344–0.881). The multiple linear regression model showed that waist and hip were associated (p = 0.01–0.02). ANOVA analysis showed age (p = 0.04), hip (p = 0.002), SBP, and TC (p = 0.02) were associated. Finally, SNV (rs1207568 and rs95270250) and obesity ($p < 0.001$) associations were confirmed through gene multifactor dimensionality reduction analysis with gene–gene interaction, dendrogram, and graphical depletion method.

Conclusion: This study concludes that rs1207568 and rs9527025 SNVs are associated with obesity in the Saudi population. Additional genetical statistics showed significant association between dependent and independent variables. SNVs in *Klotho* play a role in the Saudi population's susceptibility to obesity.

Keywords: *Klotho* gene, obesity, type 2 diabetes mellitus, rs1207568/G395A, rs564481 /C1818T, rs9527025/C370S

Introduction

Obesity is a chronic, multifaceted, and complex condition characterized by excess adiposity that can impact the human health.[1] It is a neuroendocrine[2] and multifactorial disease, influenced by an obesogenic environment and physio-social variables.[3] Presently, the World Obesity Federation estimates 800 million individuals globally are obese, and one billion individuals will be overweight or obese in the future.[4] The imbalance between metabolic substrate utilization; consumption of a high-carbohydrate and-fat diet combined, with a habitual sedentary lifestyle will lead to obesity or an increase in body mass index (BMI).[5,6] Obesity can lead to hypertension (HTN), hypercholesterolemia, type 2 diabetes mellitus (T2DM), gallbladder disease, osteoarthritis, sleep apnea, and cancer.[5,7] Obesity is prevalent in 650 million adults, 340 million adolescents, and 39 million children worldwide.[8] The highest prevalence of obesity was reported in Pacific Island states at over 50%, followed by 23–38% in the United States.[9] According to a previous World Health Organization (WHO) assessment, the prevalence of overweight in Saudi Arabia was 68%, with 68% of both women and men engaged, while obesity was found to be 33.7%,

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with 39.5% of women and 29.5% of men involved.[10] Bell confirmed almost 60% of the adult population in Saudi Arabia are obese. As per the Global Obesity Observatory, 20.2% were obese and 38.2% were overweight in Saudi Arabia. The Kingdom of Saudi Arabia was found to have the lowest prevalence of obesity among GCC nations, compared to that for Kuwait (80%), Bahrain (72.4%), Qatar (70.1%), UAE (67.9%), and Oman (66.2%).[11] Obesity is substantially linked to insulin resistance, which is a significant risk factor for developing diabetes and cardiovascular disease (CVD). Natural aging-related acceleration of cellular processes might lead to obesity-related diseases. Inflammation and oxidative stress seem to be important mediators of this relationship.[12]

Aging is an irreversible pathologic process that progresses gradually, characterized by decreases in tissue and cell functioning as well as large increases in the risks of a variety of aging-related diseases, including as CVD, neurodegenerative, metabolic disorders, musculoskeletal, and immune system diseases.[13] The scientific definition of aging, also known as senescence, refers to the biological process of growing older and experiencing a decline in physiological function over time.[14] Aging is a complex and multifactorial process influenced by a combination of genetic, environmental, and lifestyle factors. It affects all living organisms, from single-celled organisms to multicellular organisms, such as plants, animals, and humans.[15] Previous studies have showed the association between obesity and ageing through oxidative stress, mitochondria, telomere shortenings, gene expression, DNA mutations, immune system deficiencies, and changes in hormonal levels.[16] Excessive abdominal obesity is a hallmark of aging, and it plays an influential part in the development of insulin resistance and metabolic syndrome, lower immunity, exacerbated systemic inflammation, and fluctuations in tissue and body compositions. Obesity reduces life expectancy by 5.8 years in men and 7.1 years in women after the age of 40, due to ageing.[17]

Klotho is an anti-aging molecule, and *Klotho* gene was initially discovered as an aging gene by Kuro et al[18] during the development of transgenic mice models.[19] The humoral factor and its membrane-bound analog, Klotho, regulate phosphate homeostasis by increasing FGF23 activity and directly decreasing NaPi-2a transporter function. The α-Klotho is known to be transmembrane protein, which encodes in the *Klotho* gene. It is expressed in the kidney and brain and implicated in various biological processes, such as calcium homeostasis, phosphate control, and the insulin signaling pathway.[20] Lower α-Klotho levels have been noted in various aging-related diseases, including cancer, HTN, and kidney disease,[21] where high BMI levels have been identified as risk factors for these and other chronic diseases.[22] In vitro FGF receptor 1c (FGFR1c), β-Klotho, acts as an obligatory coreceptor for FGF21 via the formation of a binary complex. FGF21 is of great interest as a biologic to treat metabolic disorders because it promotes glucose absorption and lipolysis in adipose tissue and triggers thermogenesis.[23] The *Klotho* gene is connected to obesity through adipose tissue, which is controlled by adipogenesis and adipose function, which affects fat accumulation.[24] Additionally, activation of a hormone that suppresses blood glucose levels in rat fat cells by an ancestral form of the anti-aging *Klotho* provides an exciting target for the development of medications to treat human obesity and diabetes.[24] *Klotho* in humans is comprised of five exons that contain 1012 amino acids. It is found in the 13q12 region and spans more than 50 kb. The human *Klotho* gene has 10 known single nucleotide variants (SNVs). The rs1207568 SNV is mostly found in the promoter region, and guanine is replaced with adenine at position 395. The rs564481 synonymous SNV is found on exon-4, and cytosine is replaced with thymidine at codon 1767, resulting in no alteration at histidine position.[25] The rs9527025 is a missense SNV found to be a functional *Klotho* variant (KL-VS) in which six SNVs were involved in linkage disequilibrium and C370S SNV was found to have an amino acid substitution at position 398, where a homozygous GG genotype encodes methionine (Met) and homozygous AA genotype encodes valine (Val).[26]

Obesity is a central problem towards developing future risk of CVD, T2DM, cancer, and other chronic diseases, and previous studies revealed inconsistent results in sedentary middle-aged obese participants who had higher blood Klotho levels when compared to normal BMI participants.[20] According to the CDC, individuals with obesity are categorized into: (i) young adults: 20–39 years, (ii) middle-aged adults: 40–59 years; and (iii) elder individuals above 60 years of age.[27] In this study, middle-aged case–control participants were recruited due to the limitation of elder individuals in Saudi Arabia. In accordance with Worldometer, the average age of the Saudi population in 2023 was 30.6 years, with a total population of 36.99 million.[28] Individuals with T2DM were either overweight or obese in 88% of the cases. AlShahrani[29] discussed earlier findings conducted in Saudi Arabia which found T2DM in 71–86% of high BMI cases (25.1 to >30kg/m^2).[29] Based on previously available reports,[20,28,29] this study was designed to select the middle-aged

participants to perform this case–control study. The aim of this manuscript is to investigate the molecular role of rs1207568 (G395A), rs564481 (C1818T), and rs9527025 (C370S) SNVs studied in the *Klotho* gene among individuals with obesity in the Saudi population.

Materials and Methods

Obtained Study Participants

This study was designed at G-141 Laboratory, Department of Clinical Laboratory Sciences, King Saud University (KSU) as a case–control study between middle-aged Saudi participants ranging from 41–59 years of age. In this hospital-based case–control study, 100 obesity samples were selected in which 50 patients were diagnosed with T2DM and 50 did not have T2DM. Healthy controls (n = 100) were confirmed as BMI levels were <25kg/m^2 (non-obese participants). Saudi participants were all recruited on the KSU premises, including different outpatient clinics. The age range of individuals was between 46 and 59 years and 50 Saudi individuals were diagnosed with T2DM. All the individuals with obesity were confirmed through clinicians. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines were used throughout the design, review, interpretation, planning, and amendments of this study. STROBE was established to enhance the transparency and accuracy of observational study reporting across cohort, case–control, and cross-sectional studies. The current study was designed as a case–control study. Saudi individuals whose BMI was lower than were included as healthy controls or individuals without obesity. Elevated BMI levels were considered to be exclusion criteria for controls. The inclusion criteria for individuals with obesity[30] was BMI >30kg/m^2, in which additionally elevated fasting plasma glucose (FBG) levels were also found. Finally, individuals with obesity had elevated BMI levels, while those with T2DM had elevated BMI and FPG levels. All non-Saudi individuals and those diagnosed with other chronic diseases were excluded from this study. The study was approved by the Institutional Review Board of the Medical College at KSU (E-2023-07835). All the individuals signed the patient consent form and those who denied the consent form were excluded from this study. This study was conducted in accordance with the Helsinki Declaration.

Patient Details and Sample Collection

A questionnaire was prepared along with the consent form, and approval was obtained from 200 Saudi individuals. To evaluate the anthropometric and demographic information, the data was collected from eSIHI within the hospital premises. Age, sex, weight, height, BMI, waist, and hip details were recorded. BMI was computed using the combined weight and height information as kilograms (kg), centimeters (cm), and square meter (m^2) as per WHO guidelines, that is, 30 kg/m^2 for obesity. Waist circumference was measured at the lower border of the last rib and the top rim of the antero-superior iliac crest. The normal values of waist in men and women were 94–102 cm and 80–88 cm, respectively. The hip circumference was measured in a standing position, with the arms at the sides and the feet together, around the largest portion of the buttocks. In men, the normal range of hip was 94–105 cm and 97–108 cm in women. HTN or blood pressure was taken from the left arm in the sitting posture following a 10-min rest period. A minimum of two to three measurements were collected at 5-min intervals, with an average of two recorded. Finally, HTN was calculated using SBP and DBP. Each patient has been given 4 mL of peripheral into 2 mL of plain serum tube after an overnight fasting for a minimum of one-third of a day and the remaining 2 mL in EDTA tube. The plain sample was collected in an anticoagulant tube, mixed well and centrifuged for 10 min at 3500 rpm, and then serum was separated and stored at −80°C. Using a Roche kit with Cobas e411 immunoanalyzer equipment, FPG levels were measured. HbA1c was measured using the Helena Glyco-Tek Affinity column method.[31]

DNA and Sequencing Analysis

Using the remaining EDTA blood, genomic DNA was isolated using a Qiagen purification kit protocol along with the recommended protocol. The genomic DNA was quantified using a NanoDrop Spectrophotometer, and 200 genomic DNA was converted into 20 µg/mL. The extracted genomic DNA was stored in −80°C for further analysis. Based on previous studies[32,33] involving aging and different chronic diseases, rs1207568, rs564481, and rs9527025 SNVs were selected from HapMap and

NCBI dbSNP databases. Furthermore, using the SNPinfo website, these three SNVs were predicted with functional consequences and described with 10% of the aforementioned minor allele frequencies in the 1000 genomes research project. Genotyping for rs1207568, rs564481, and rs9527025 SNVs was carried out using polymerase chain reaction (PCR) analysis. Oligonucleotides used for rs1207568 (66°C) SNV were 5′-F: TGGACGCTCAGGTTCATTCT-3′; R: 5′-CCTCTAG GATTTCGGCCAGT-3′, for SNV rs564481 (60°C) were F: 5′-TGTCTCAGTTTACCGACCTGAATGT-3′; R: 5′-ATTCAT CGTTATCCAAAGCTTGACG-3′, and for the final SNV rs9527025 (62°C) were F: 5′-AGGCTCATGCCAAAGTCTGG-3′; ′R: 5′-GTTTCCATGATGAACTTTTTGAGG-3′. The protocol for amplification of PCR was as follows: 95°C for 5 min for initial denaturation, 95°C for 30 s for denaturation, 95°C and 60–66°C (the rs1207568 [60°C] SNVs, rs564481 [66°C], and rs9527025 [64°C]) as annealing temperatures, 72°C for 45 s as extension, and final extension was considered to be 72°C for 5 min along with 35 cycles and holds at 4°C. Using a 50-µL reaction with the Qiagen PCR master mix, which includes the combination of 10× Buffer, $MgCl_2$, dNTPs, and Taq DNA polymerase and using double purified water, the amplification was conducted with 20 ng of 200 different genomic DNA. Amplification was carried out for approximately 1.35 min. Unpurified PCR products were loaded on a 2% agarose gel run to confirm the precise bands of 243, 452, and 505 bp for rs1207568, rs564481, and rs9527025 SNVs, respectively, stained with ethidium bromide and visualized using UVI gel documentation system. All 200 PCR products for three SNVs were purified and used for Sanger sequencing analysis with Big Dye and primers. Multicapillary sequencing analysis was carried out for the studied rs1207568, rs564481, and rs9527025 SNVs. Genomic DNA isolation and amplification for rs1207568, rs564481, and rs9527025 SNVs were conducted at G-141 laboratory and Sanger sequencing (Figure 1) was conducted outside the G-141 laboratory of CLS-KSU premises.

Statistical Investigation

All the collected data was inserted into Excel, and both qualitative (total number and percentages) and qualitative variables (mean ± standard deviations) were studied between cases and control groups using Mann–Whitney *U*-test for analyzing *t*-tests. Using Pearson χ^2 tests, Hardy–Weinberg equilibrium (HWE) analysis was studied between expected as well as observed genotype distributions in rs1207568, rs564481, and rs9527025 SNVs. Utilizing SNPstats software, the inheritance hypothesis was evaluated between cases and controls utilizing genotypes, alleles, and genetic models, such as dominant, recessive, and co-dominant models with odds ratios (OR), p values, and 95% confidence intervals (95% CIs). A linear logistic regression model was tested to estimate the association between rs1207568, rs564481, and rs9527025 SNVs and obesity parameters using the SPSS software. One-way ANOVA analysis was studied between rs1207568, rs564481, and rs9527025 SNVs and obesity parameters as well as categorization of BMI into: (i) Obesity, (ii) Morbid Obese-I, and (iii) Morbid Obese-II vs 13 dependent variables using Jamovi Software. Using three SNPs in the *Klotho* gene, the generalized multifactor dimensionality reduction (GMDR) model[34] was used to evaluate gene–gene interaction, dendrogram, and graphical depletion models. $P \leq 0.05$ was considered statistically significant.

Results

Characteristics of Saudi Participants with and without Obesity

A total of 200 individuals were recruited based on the study's inclusion and exclusion criteria and after obtaining their signed consent forms. One hundred participants were non-obese (BMI≤25kg/m^2) and used as controls of this study; and the remaining 100 participants were categorized obese, which 50 had T2DM. Table 1 shows baseline characteristic details. The mean age of cases and controls was 56.67 ± 2.65 and 54.01 ± 2.46, respectively, which was high, but not significant when compared with controls (p = 0.462). A total of 63.5% females and 36.5% males were involved in this study (n = 200), in which 59% and 41% were males and females with obesity, respectively; and 68% and 32% were females and males among control population, respectively. Sex (p = 0.085) and height (p = 0.756) were non-significantly higher in controls than in cases. Other parameters, such as weight, BMI, waist, hip, SBP, DBP, FPG, HbA1c, and lipid profile parameters, such as TG, TC, HDLc, and LDLc levels were significantly higher with obesity ($p < 0.05$), compared to those of controls. The similar parameters were shown to be associated when compared between obesity with T2DM (n = 50) and controls ($p < 0.05$). Among individuals with obesity without T2DM (n = 50), a similar association ($p < 0.05$) was confirmed apart from FPG (p = 0.521) and HbA1c (p = 0.581), when compared with control participants.

Figure 1 Chromatograms represent the SNVs present in the *Klotho* gene.

Genotype and Allelic Associations

Table 2 describes the observed frequencies of SNVs present in *Klotho* between obesity and controls. Genotype distribution for rs1207568 SNV had 67%, 19%, and 14% of GG, GA, and AA genotypes for obesity, and 86%, 11%, and 4% for the control population, respectively. Only homozygous codominant (AA vs GG: OR 5.99 [95% CI: 1.65–21.70] p = 0.002) and dominant models (GA+AA vs GG: OR 3.02 [95% CI: 1.51–6.11] p = 0.001) showed significant association with obesity. However, heterozygous codominant (AG vs GG: OR 2.21 [95% CI: 0.98–4.97] p = 0.042), recessive (AA vs GG: OR 0.53 [95% CI: 0.23–1.17] p = 0.112), and codominant models (AA vs GG: OR 0.19 [95% CI: 0.05–0.68] p = 0.005) showed negative associations with obesity. The A allele was associated with 23.5% and

Table 1 Characteristics of Controls and Cases as Well as Serum Analysis Studies

Demographic Details	Total Cases (n=100)	Controls (n=100)	P Value	Obesity Without T2DM (n=50)	P Value	T2DM Cases (n=50)	P Value
Age	56.57 ± 2.65	54.01 ± 2.46	0.462	56.38 ± 2.39	0.831	56.76 ± 2.91	0.151
Sex (Female: Male)	59 (59%):41 (41%)	68 (68%):32 (32%)	0.085	28 (56%): 22 (44%)	0.007	31 (62%): 19 (38%)	0.310
Weight	86.02 ± 10.81	69.06 ± 8.98	<0.0001	85.63 ± 9.32	<0.0001	86.41 ± 12.21	<0.0001
Height	158.03 ± 6.92	160.66 ± 7.14	0.756	157.98 ± 6.59	0.541	158.08 ± 7.30	0.831
BMI	34.44 ± 3.53	26.69 ± 2.43	<0.0001	34.33 ± 3.30	<0.0001	34.56 ± 3.77	<0.0001
Waist	100.40 ± 12.99	89.27 ± 13.11	<0.0001	102.10 ± 6.17	<0.0001	98.16 ± 18.42	0.0008
Hip	113.58 ± 14.41	94.12 ± 14.65	<0.0001	121.68 ± 10.02	<0.0001	103.13 ± 12.40	0.0002
SBP	132.10 ± 6.12	116.42 ± 6.36	<0.0001	131.90 ± 5.70	<0.0001	132.31 ± 6.56	<0.0001
DBP	81.96 ± 4.75	77.08 ± 5.53	<0.0001	81.68 ± 5.37	0.0001	82.23 ± 4.09	<0.0001
FPG	9.85 ± 5.50	5.43 ± 0.47	<0.0001	5.37 ± 0.66	0.521	14.33 ± 4.44	<0.0001
HbA1c	6.58 ± 5.50	5.36 ± 0.41	0.020	5.32 ± 0.46	0.581	7.84 ± 0.55	<0.0001
TG	2.11 ± 1.98	1.40 ± 0.46	0.0005	2.15 ± 2.46	0.0030	2.06 ± 1.39	0.0001
TC	5.76 ± 1.20	5.19 ± 0.91	0.0002	5.73 ± 1.29	0.0035	5.79 ± 1.12	0.0005
HDLc	1.37 ± 1.20	0.68 ± 0.30	<0.0001	1.26 ± 0.98	0.0001	1.48 ± 1.37	0.0001
LDLc	3.81 ± 0.98	3.01 ± 1.09	0.0001	3.87 ± 0.99	0.0006	3.73 ± 0.98	0.0001

Table 2 Genotype and Allele Frequency Studies Between G395A, C1818T, and C370S SNVs in Klotho Gene Between Cases and Controls

Gene (rs number)	Genotypes	Cases (n=100)	Controls (n=100)	ORs	95% CIs	P value
Klotho (rs1207568)	GG	67 (67%)	86 (86%)	Reference	Reference	Reference
	GA	19 (19%)	11 (11%)	2.21	0.98–4.97	0.042
	AA	14 (14%)	03 (03%)	5.99	1.65–21.70	0.002
	GA+AA vs GG	33 (33%)	14 (14%)	3.02	1.51–6.11	0.001
	AA+GG vs GA	81 (81%)	89 (89%)	0.53	0.23–1.17	0.112
	GG+GA vs AA	86 (86%)	97 (97%)	0.19	0.05–0.68	0.005
	G allele	153 (76.5%)	183 (91.5%)	Reference	Reference	Reference
	A allele	47 (23.5%)	17 (8.5%)	3.31	1.82–5.99	0.003
	HWE analysis	χ^2=22.23	χ^2=8.57			
Klotho (rs564481)	CC	46 (46%)	51 (51%)	Reference	Reference	Reference
	CT	34 (34%)	33 (33%)	1.14	0.61–2.13	0.675
	TT	20 (20%)	16 (16%)	1.38	0.64–2.98	0.404
	CT+TT vs CC	54 (54%)	49 (49%)	1.22	0.71–2.12	0.479
	TT+CC vs CT	66 (66%)	67 (67%)	0.95	0.53–1.72	0.881
	CC+CT vs TT	80 (80%)	84 (84%)	0.76	0.36–1.57	0.461
	C allele	126 (63%)	135 (67.5%)	Reference	Reference	Reference
	T allele	74 (37%)	65 (32.5%)	1.22	0.81–1.84	0.344
	HWE analysis	χ^2=7.32	χ^2=6.14			

(*Continued*)

Table 2 (Continued).

Gene (rs number)	Genotypes	Cases (n=100)	Controls (n=100)	ORs	95% CIs	P value
Klotho (rs9527025)	GG	59 (59%)	83 (83%)	Reference	Reference	Reference
	GC	28 (28%)	12 (12%)	3.28	1.54–6.97	0.001
	CC	13 (13%)	05 (05%)	3.65	1.23–10.81	0.013
	GC+CC vs GG	41 (41%)	17 (17%)	3.39	1.76–6.54	0.0001
	CC+GG vs GC	72 (72%)	88 (88%)	0.35	0.16–0.73	0.004
	GG+GC vs CC	87 (87%)	95 (95%)	0.35	0.12–1.02	0.048
	G allele	146 (73%)	178 (89%)	Reference	Reference	Reference
	C allele	54 (27%)	22 (11%)	2.99	1.74–5.14	0.00004
	HWE analysis	χ^2=8.39	χ^2=14.98			

8.5% of individuals with obesity and controls, respectively. Additionally, the G allele was associated with 76.5% and 91.5% of the individuals with obesity and controls, respectively. The allele frequency was associated with obesity compared with that of the controls (G vs A: OR 3.31 [95% CI: 1.82–5.99] p = 0.003). Regarding rs564481, CC, CT, and TT genotypes were associated with 46%, 34%, and 20% of the individuals obesity and 51%, 33%, and 16% of the control population, respectively. The T allele was present in 37% and 32.5% of the obesity and control groups, respectively. The C allele was present in 63% and 67.5% of the obesity and control groups, respectively. There was no statistical association with genotypes (CT vs CC: OR1.14 [95% CI: 0.61–2.13] p = 0.675; CT vs CC: OR 1.38 [95% CI: 0.64–2.98] p = 0.404), genetic models (CT+TT vs CC: OR 1.22 [95% CI: 0.71–2.12] p = 0.479; TT+CT vs CC: OR 0.95 [95% CI: 0.53–1.72] p = 0.881; CC+CT vs TT: OR 0.76 [95% CI: 0.36–1.57] p = 0.461), and in alleles (T vs C: OR 1.22 [95% CI: 0.81–1.84] p = 0.344). This study confirms that the rs564481 SNV has no role in the Saudi population with obesity with and without T2DM. The rs9527025 SNV showed a strong association between genotypes: (GC vs CC: OR-3.28 [95% CI: 1.54–6.97] p = 0.001; CC vs CC: OR 3.65 [95% CI: 1.23–10.81] p = 0.013), dominant model (GG +GC vs CC: OR 3.39 [95% CI: 1.76–6.54] p = 0.0001), and alleles (G vs C: OR 2.99 [95% CI: 1.74–5.14] p = 0.00004). The genotype and allele frequencies for obesity and controls were 59%, 28%, and 13% vs 83%, 12%, and 5%; whereas C and G alleles were found to be 27% and 73% vs 11% and 89%, respectively. However, the other genetic models, such as co-dominant (CC+GG vs CG: OR 0.35 [95% CI: 0.16–0.73] p=0.004), and recessive models (GG+GC vs CC: OR 0.35 [95% CI: 0.12–1.02] p = 0.048) were associated statistically.

Linear Multiple Regression Model Analysis

In this study, the multiple linear regression model was used to establish dependent and independent variables (Table 3). The 13 covariates, such as age, weight, BMI, Waist, Hip, SBP, DBP, FPG, HBA1c, TG, TC, HDL/LDLc levels were confirmed as

Table 3 Linear Regression Model Analysis Between SNVs in Klotho Gene and Covariates Present in Total Cases

Covariates	R-Value	Adjusted R Square Value	Standardized β-Coefficient for rs1207568	Standardized β-Coefficient for rs564481	Standardized β-Coefficient for rs9527025	F	p Value
Age	0.268	0.043	−0.160	0.166	−0.111	2.476	0.066
Gender	0.260	0.038	−0.107	−0.133	−0.209	2.320	0.080
Weight	0.174	0.000	−0.131	−0.088	−0.092	0.993	0.399
BMI	0.132	−0.013	−0.005	−0.072	−0.114	0.572	0.635
Waist	0.308	0.067	−0.244	−0.170	−0.130	3.351	0.022
Hip	0.386	0.123	−0.178	−0.087	−0.341	5.608	0.001

(*Continued*)

Table 3 (Continued).

Covariates	R-Value	Adjusted R Square Value	Standardized β-Coefficient for rs1207568	Standardized β-Coefficient for rs564481	Standardized β-Coefficient for rs9527025	F	p Value
SBP	0.203	0.011	0.015	−0.182	−0.093	1.376	0.255
DBP	0.184	0.004	0.121	0.105	−0.100	1.120	0.345
FPG	0.213	0.015	0.180	−0.011	0.112	1.518	0.215
Hb1Ac	0.261	0.039	0.183	0.022	0.189	2.333	0.079
TG	0.159	−0.005	0.073	−0.122	−0.059	0.825	0.483
TC	0.107	−0.020	0.098	−0.020	0.031	0.368	0.776
HDLc	0.038	−0.030	0.028	0.011	0.025	0.047	0.987
LDLc	0.112	−0.018	−0.022	0.016	0.109	0.409	0.747

dependent variables and rs1207568, rs564481, and rs9527025 SNVs were considered as independent variables. The three genotypes were documented as one, two, and three for the homozygous codominant genotype (GG/CC/GG), heterozygous codominant genotype (GA/CT/GC), and homozygous codominant variant genotype (AA/TT/CC). The statistical analysis calculated between 13 dependent and 3 independent variables with SPSS software confirmed Waist ($p = 0.02$) and Hip ($p = 0.001$) was associated with obesity and another 11 covariates was not ($p > 0.05$).

ANOVA Analysis in Klotho SNVs

One-way ANOVA analysis was studied between 13 dependent and 3 independent variables in *Klotho*-gene and obesity cases. In Table 4, the analysis showed age ($p = 0.04$), SBP ($p = 0.02$), and TC ($p = 0.02$) were positively associated with the rs1207568 SNV and hip ($p = 0.002$) was strongly associated with the rs9527025 SNV in *Klotho*. The rs564481 SNV has no role in any of the studied variables, but weight (87.49 ± 11.23) and BMI (35.05 ± 4.14) had elevated levels among CC genotypes in the studied SNVs. For the rs9527025 SNV, hip (118.02 ± 14.54) had elevated levels of GG and in CC genotypes, and HbA1c (7.06 ± 1.42) and LDLc (4.06 ± 0.80) levels were high. Finally, for the rs1205768 SNV, GG genotypes were elevated at the waist (102.40 ± 14.58). The GA genotypes were associated with in age (57.11 ± 2.4), SBP (135.42 ± 7.71), and FBG (11.88 ± 6.87). DBP (83.77 ± 4.62), TG (2.75 ± 2.24), TC (6.41 ± 1.02), and HDLc (1.59 ± 1.18) levels were highly associated with the AA genotype. A potential relationship was developed between age, SBP, TC, and Hip among SNVs in *Klotho* gene and obesity cases.

Correlation Between BMI Categorization Among Dependent Variables with Obesity

Table 5 defines the association between categorization of BMI in comparison with 13 dependent variables. BMI was categorized into (i) obesity (>30.0 kg/m^2), (ii) morbid obese-I (>35.0 kg/m^2), and (iii) morbid obese-II (>40.0 kg/m^2). The ANOVA analysis studied in Table 5 describes weight ($p = 0.0001$), BMI ($p = 0.0003$), and waist ($p = 0.0002$) were significantly associated with obesity, among three categories of BMI in the individuals with obesity. Age (58.00 ± 0.87), Weight (107.27 ± 13.89), BMI (43.01 ± 2.40), Waist (121.20 ± 32.35), FPG (11.37 ± 5.45), and Hb1Ac (7.13 ± 1.17) were found to have elevated levels in the third category of individuals with obesity. Hip (114.33 ± 14.81), SBP (132.23 ± 6.50), HDL-c (1.39 ± 1.33), and LDL-c (3.89 ± 0.96) were found to have high values in the first category of individuals with obesity. Finally, DBP (83.22 ± 5.37), TG (2.65 ± 3.58), and TC (5.83 ± 1.24) levels were found to be elevated in the morbid-obese I group. The overall ANOVA analysis confirms that weight, BMI, and waist were associated with obesity ($p < 0.05$).

Table 4 ANOVA Analysis Studied Between 3 SNVs with Its Covariances Appears in All the Cases

	Klotho (rs1207568)				*Klotho* (rs564481)				*Klotho* (rs9527025)			
	GG (n=67)	**GA (n=19)**	**AA (n=14)**	**p**	**CC (n=46)**	**CT (n=34)**	**TT (n=20)**	**p**	**GG (n=59)**	**GC (n=28)**	**CC (n=13)**	**p**
Age	56.75 ± 2.48	57.11 ± 2.40	55.01 ± 3.33	0.049	55.98 ± 2.92	57.06 ± 2.30	57.10 ± 2.40	0.122	56.73 ± 2.46	56.68 ± 2.72	55.62 ± 3.33	0.382
Weight	87.12 ± 11.90	83.23 ± 7.27	84.54 ± 8.87	0.332	87.49 ± 11.23	83.78 ± 10.11	86.45 ± 10.89	0.312	86.56 ± 9.73	86.16 ± 11.10	83.28 ± 14.83	0.451
BMI	34.49 ± 4.02	34.06 ± 2.21	34.74 ± 2.36	0.848	35.05 ± 4.14	33.33 ±2.58	34.94 ± 3.06	0.075	34.63 ± 3.59	34.64 ± 3.57	33.16 ± 3.13	0.371
Waist	102.40 ± 14.58	99.33 ± 8.17	94.25 ± 9.72	0.096	101.93 ± 10.70	98.18 ± 8.82	101.57 ± 22.07	0.428	101.02 ± 8.39	102.05 ± 20.83	94.50 ± 6.90	0.201
Hip	114.33 ± 13.65	116.56 ± 16.20	106.92 ± 13.73	0.133	112.52 ± 13.87	113.89 ± 14.66	115.14 ± 15.86	0.784	118.02 ± 14.54	108.58 ± 12.69	105.64 ± 10.97	0.002
SBP	131.52 ± 5.82	135.42 ± 7.71	130.36 ± 3.08	0.023	133.40 ±5.66	131.18 ± 6.04	130.75 ± 6.93	0.148	132.34 ± 6.34	132.41 ± 6.41	130.38 ± 4.31	0.556
DBP	81.82 ± 4.13	81.12 ± 6.68	83.77 ± 4.62	0.268	81.57 ± 5.39	81.61 ± 2.87	83.35 ± 5.70	0.337	82.18 ± 3.87	82.15 ± 4.43	80.62 ± 8.05	0.547
FPG	9.03 ± 4.78	11.88 ± 6.87	11.03 ± 6.13	0.093	10.23 ± 6.01	9.25 ± 4.81	10.01 ± 5.54	0.729	9.46 ± 5.71	9.96 ± 5.06	11.41 ± 5.54	0.512
Hb1Ac	6.40 ± 1.31	6.93 ± 1.33	6.97 ± 1.54	0.166	6.62 ± 1.38	6.47 ± 1.33	6.66 ± 1.43	0.851	6.38 ± 1.36	6.78 ± 1.30	7.06 ± 1.42	0.175
TG	2.05 ± 2.10	1.81 ± 1.25	2.75 ± 2.24	0.383	2.31 ± 2.73	2.11 ± 1.14	1.61 ± 0.61	0.426	2.25 ± 2.46	1.79 ± 0.92	2.25 ± 1.01	0.586
TC	5.76 ± 1.14	5.26 ± 1.36	6.41 ± 1.02	0.023	5.75 ± 1.15	5.86 ± 1.08	5.60 ± 1.52	0.747	5.76 ± 1.22	5.67 ± 1.23	5.95 ± 1.13	0.789
HDL-c	1.38 ± 1.30	1.16 ± 0.73	1.59 ± 1.18	0.622	1.32 ± 1.13	1.47 ± 1.48	1.30 ±0.79	0.828	1.34 ± 0.98	1.42 ± 1.45	1.39 ± 1.56	0.957
LDL-c	3.84 ± 0.91	3.67 ± 1.19	3.83 ± 1.07	0.801	3.80 ± 1.01	3.78 ± 0.79	3.85 ± 1.24	0.968	3.73 ± 0.95	3.84 ± 1.13	4.06 ± 0.80	0.539

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Table 5 Relation Between Different Forms of Obesity and Dependent Variables Used in Obesity Participants (n = 100) with and without T2DM

Dependent Variables	Obesity (n=65)	Morbid Obese-I (n=26)	Morbid Obese-II (n=09)	P value
Age	56.31 ± 2.92	56.73 ± 2.22	58.00 ± 0.87	0.191
Weight	82.50 ± 7.65	87.48 ± 7.39	107.27 ± 13.89	0.0001
BMI	32.44 ± 1.36	36.49 ± 1.21	43.01 ± 2.40	0.0003
Waist	98.83 ± 9.84	98.90 ± 7.31	121.20 ± 32.35	0.0002
Hip	114.33 ± 14.81	113.10 ± 12.52	108.60 ± 19.55	0.545
SBP	132.23 ± 6.50	132.12 ±5.32	131.11 ± 6.01	0.878
DBP	81.70 ± 4.76	83.22 ± 5.37	80.56 ± 1.67	0.252
FPG	10.35 ± 5.92	8.06 ± 3.96	11.37 ± 5.45	0.136
Hb1Ac	6.56 ± 1.39	6.43 ± 1.34	7.13 ± 1.17	0.409
TG	1.95 ± 0.92	2.65 ± 3.58	1.68 ± 0.82	0.253
TC	5.81 ± 1.23	5.83 ± 1.24	5.18 ± 0.78	0.321
HDL-c	1.39 ± 1.33	1.39 ± 0.98	1.10 ± 0.69	0.789
LDL-c	3.89 ± 0.96	3.76 ± 1.10	3.30 ± 0.61	0.232

GMDR Model Analysis

The genotype data of three SNVs for the individuals with obesity were denoted as one and zero for controls, to calculate the gene–gene interaction analysis (Table 6). The S1 model consists of the rs9527025 SNV; S2 model consists of the rs1207568 and rs9527025 SNVs; and S3 model has a combination of rs1207568, rs564481, and rs9527025 SNVs. The overall analysis of gene–gene interaction when combined with all the genotype data in GDMR software confirmed S3 (0.83) as the best studied model in this study, followed by, S2 (0.7617) and S1 (0.62). All S1-S3 models showed significant association ($p < 0.05$) with obesity and overall analysis towards gene–gene interaction confirmed that the S3 model was a risk element between studied cases and the three SNVs in *Klotho*. Moreover, dendrogram analysis (Figure 2) confirmed substantial association of rs1207568, rs564481, and rs9527025 SNVs with obesity. Additionally, Figure 2 shows the graphical depletion model which confirms the high risk via dark cells and low risk with light cells. However, the absence of genotypes indicates with the white or blank cells. The bar validates the left-hand hypothetical cases (obesity) and the right-hand control population. The analysis of the graphical depletion model showed the limited risk combination present between the SNVs in rs564481/rs1207568 and rs9527025/rs1207568.

Discussion

Notably, obesity was not considered as a disease due to the lack of specific symptoms. Poor diet control, physical inactivity, laziness, inherited alleles from the family, and many other factors contributed to the excessive accumulation of body fat. This led to underweight to normal BMI levels, and then overweight, obese, and morbid obesity. Therefore, the energy imbalance between the consumption and exertion of calories led to weight gain. In 1980, the prevalence of obesity was 6.4% and it increased to 12% in 2008.[35] After substantial debates, the American Medical Association declared obesity as a disease in 2003. By 2013, it was recognized as a complex disease with links to various health issues, including T2DM, CVD, and infertility in both men and women. In GCC countries, including Saudi Arabia, the cultural socioeconomic factor added towards the development of obesity and other chronic diseases. Unhealthy diet is one of the major reasons for developing weight gain in Saudi Arabia. High frequency of consuming snacks, missing of breakfast, and harmful dietary habits are the major contributors to increased BMI in the Saudi population.[36] Physical inactivity is

Table 6 Gene–Gene Interaction to Determine the Risk of T2DM Patients

Model No	Best Combination of Genes	Training Accuracy	Testing Accuracy	CVC	P-value	Total Sensitivity	Total Specificity	χ^2	OR (95% CI)	F -Measure	Kappa
1	rs9527025 (S1)	0.62	0.62	10/10	0.0004	0.41	0.83	13.99	3.39 (1.75–6.54)	0.519	0.24
2	rs1207568, rs9527025 (S2)	0.7617	0.74	10/10	<0.0001	0.60	0.92	60.24	17.25 (7.56–39.39)	0.7143	0.52
3	rs1207568, rs564481, rs9527025 (S3)	0.83	0.82	10/10	<0.0001	0.69	0.97	94.53	71.96 (21.15–244.91)	0.8023	0.66

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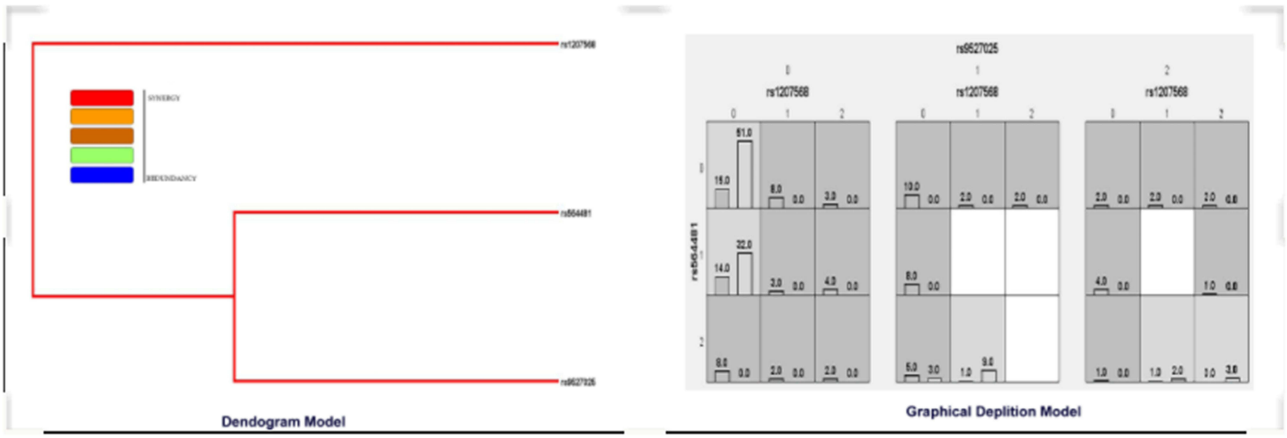

Figure 2 Representation of dendrogram and graphical depletion model in this study.

a global health issue that contributes to higher BMI and the development of noncommunicable diseases.[37] Cultural perspectives substantially influence lifestyle choices. Notably, cultural attitudes should be addressed with compassion and knowledge that people within a culture may have a wide range of opinions and actions. However, several cultural factors have been recognized as possible causes of obesity in Saudi Arabia.[38] Among the younger generations, snacking is the major issue in elevating the weight gain in the present scenario. The combination of chocolates, chips, and soft drinks are considered to be as snacks which further affected the negative health outcome. Both the snacking and poor diet contribute to overweight and obesity.[39] In Saudi Arabia, the high prevalence of inactivity, especially among females, is a serious public health burden and a forerunner to obesity prevalence. Furthermore, as compared to males, Saudi women have less access to exercise facilities and less opportunity to participate in physical activity.[40] Reduced physical activity in Saudi Arabia may be attributed to growing reliance on motorized vehicles, sedentary lifestyles or working in cubicles at office premises, and consumption of processed foods and sugar-laden beverages in excessive amounts. There may be cultural attitudes in the GCC that associate elevated BMI levels with obesity. However, the younger generation of Saudi Arabia is focusing on weight loss programs to avoid the future complications,[41] particularly among Saudi women.[42] In addition to obesity, the prevalence of T2DM and CVD is also increasing in Saudi Arabia along with aging. To perform a molecular study to confirm aging, obesity, and T2DM associations, this study involved the *Klotho* gene and three SNVs that had an amino acid amendment. *Klotho* was not studied in the Saudi population, therefore, this can be considered a novel study, with 100 control and 100 obesity participant screens in which 50% have developed T2DM.

The outline of this study confirmed strong association between cases and controls using Mann Whitney *U*-test in BMI, H/W, HTN, and serum levels ($p = 0.0001$ to $p < 0.0001$; Table 1). These *t*-test was performed between cases and controls to analyze the statistical association towards a specific variable. Genotype variants, dominant model, and allele frequencies were associated with rs1207568 and rs9527025 SNVs in *Klotho* ($p = 0.01$ to $p = 0.00004$; Table 2). Multiple linear regression analysis was used in population genetics and epidemiological studies to determine if individual SNVs are independently associated with the disease after adjusting for other relevant variables. This similarly applies with ANOVA analysis and had a minimum of three groups to determine whether any statistical association is present. The SNVs were examined to explore their potential association with diseases. The linear regression model (Table 3) showed that waist ($p = 0.02$) and hip ($p = 0.001$) were strongly associated with dependent and independent variables. The similar variables were used to study via ANOVA analysis, and study results confirmed age ($p = 0.04$), SBP ($p = 0.02$), TC ($p = 0.02$), and hip ($p = 0.002$) were associated (Table 4) with obesity. The GMDR statistical model is used in population-based molecular studies to determine and characterize gene–gene interactions. When a minimum of two or more SNVs data are combined and examined in gene–gene interaction, there was an effect of a single or multiple SNVs in that disease. This paradigm is important because it links biological pathways to disease susceptibility mechanisms. The dendrogram is a graphical depiction of the results of an interaction exploration involving two or more SNVs. It shows the combination of SNVs grouped together based on their conjoint effect with a specific color. The depletion model is used to

differentiate the high- and low-risk combination of genetic factors. Finally, GMDR analysis showed that the gene–gene interaction, dendrogram, and depletion model confirmed a relationship between SNVs in *Klotho* and obesity cases ($p < 0.05$; Table 6).

Klotho may play an inhibitory role in obesity in Saudi Arabia. Li et al[43] showed that the rs7670903 SNV was associated with obesity in the Chinese population, which is present in the *Klotho* β gene. In the Japanese population, both G395A and C1818T SNVs was studied in lipid and glucose metabolisms in men and showed a positive association.[44] Klotho serum levels were studied in the obese population in USA, which confirmed the significant association among adult women.[45] However, previous studies on serum levels in the obese population produced inconsistent findings.[46–50] A previous study in MetS involving the G395A SNV showed association with obesity.[51] Both G395 and C1818T SNVs was studied in T2DM among the Pakistani population and showed both positive and negative associations.[52] There are many studies, such as case–control, epidemiological, and meta-analysis, performed on many human diseases excluding obesity as well as T2DM. In our study, Tables 3 and 4 showed that waist/hip,[53] SBP,[54] TC,[44] and aging[55] were associated with obesity and the role of *Klotho* was established. Limitations of this study were the lack of serum-level measurements and no documentation of detailed clinical conditions. Additionally, we could not record the immune- and chronic-related diseases present in all the participants. Involving Saudi individuals with obesity with and without T2DM was one of the strengths of this study. Furthermore, Sanger sequencing analysis was also added for robust results.

Conclusion

The rs1207568 and rs9527025 SNVs were associated with individuals with obesity in the Saudi population. Future studies are recommended to be carried out using similar sample criteria, but with the large sample size because *Klotho* has a role in both obesity and T2DM. Moreover, we recommend measuring the Klotho serum levels.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of KING SAUD UNIVERSITY (E-23-7835 and 22/06/2023) for studies involving humans.

Data Sharing Statement

All the data are available in this study.

Informed Consent Statement

Informed consent was obtained from all participants involved in the study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Both the authors declare as no conflict of interest.

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