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Val109Asp polymorphism in Intelectin 1 gene is associated with coronary artery disease severity in women

İntelektin 1 genindeki Val109Asp polimorfizminin kadınlarda koroner arter hastalığı şiddeti ile ilişkisi

ORIGINAL ARTICLE
KLİNİK ÇALIŞMA

ABSTRACT

Objective: Intelectin-1 is an anti-inflammatory adipokine encoded by the Intelectin 1 (*ITLN1*) gene. Genetic variations in the *ITLN1* gene affect the risk of coronary artery disease (CAD) and related CAD risk factors. In this study, we aimed to investigate whether the *ITLN1* gene Val109Asp polymorphism has an effect on the severity of CAD and serum lipid levels in both men and women.

Methods: A total of 493 subjects who underwent coronary angiography (43.5% women, mean age 63.1±9.5 years) were grouped as individuals with critical CAD (≥70% stenosis, n=202), non-critical CAD (31%-69% stenosis, n=90), and non-CAD (control group) (1%-30% stenosis, n=201). Genotyping was performed using LightSNiP assay in Real-Time PCR.

Results: The frequency of the Val allele was significantly different among all the patients with critical CAD (n=41) and non-CAD control (n=51) groups in women (p=0.033) but not in men (n=77 and n=38). Women with the Val allele had a 1.69-fold increased risk for critical CAD (p=0.033). In addition, the presence of Val allele was associated with higher coronary stenosis after adjustment for several confounders only in women with critical CAD (p=0.025). Furthermore, carriers of the Val allele exhibited an increased low-density lipoprotein cholesterol (LDL-C) in men with critical CAD than in those with non-CAD (p<0.05).

Conclusion: These results suggest that the Val allele of the *ITLN1* Val109Asp polymorphism is associated with critical CAD and high LDL-C levels in our study population. Further studies are required to elucidate the effect of Val109Asp polymorphism on CAD pathogenesis.

Keywords: Intelectin-1, *ITLN1* gene, coronary artery disease, Val109Asp

ÖZET

Amaç: Intelektin-1, anti-enflamatuvar bir adipokindir ve Intelektin 1 (*ITLN1*) geni tarafından kodlanır. *ITLN1* genindeki genetik değişimlerin, koroner arter hastalığı (KAH) ve KAH gelişiminde rol oynayan risk faktörleri üzerine etkilerinin olduğu öngörülmektedir. Bu çalışmada, *ITLN1* Val109Asp polimorfizminin hem erkeklerde hem de kadınlarda KAH'ın şiddeti ve serum lipit düzeyleri üzerine etkilerinin olup olmadığının araştırılması amaçlanmaktadır.

Yöntemler: Koroner anjiyografi (%43.5 kadın, ortalama yaş; 63.1±9.5 yaş) uygulanan 493 birey, belirlenen darlık düzeylerine göre kritik KAH olan (≥%70 darlık, n=202), kritik KAH olmayan (%31-69 darlık, n=90) ve KAH olmayan (kontrol grubu olarak) (%1-30 darlık, n=201) olarak gruplandırıldı. Anjiyografik ciddiyet ve aterosklerotik KAH yaygınlığı Gensini ve SYNTAX skorları kullanılarak değerlendirildi. Genotiplerinin belirlenmesi, Real-Time PCR LightCycler 480 cihazında LightSNiP assay kullanılarak yapıldı.

Bulgular: Seçilen Val109Asp polimorfizmi Val allel sıklığı, kritik KAH olan (n=34) ve KAH olmayan (n=46) kontrol gruplarında kadınlarda anlamlı derecede farklı bulunurken (p=0.033), erkek grupları (n=77 ve n=38) arasında anlamlı bir fark görülmedi. Val alleli taşıyıcısı olan kadınların, kritik KAH için 1.69 kat artmış riske sahip olduğu bulundu (p=0.033). Ek olarak, kritik KAH olan

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kadınlarda yaş, sigara tüketimi ve lipit düşürücü ilaç kullanımı için ayarlama yapıldığında, Val allel taşıyıcılığı daha yüksek darlık derecesi ile ilişkili bulundu (p=0.025). Ayrıca, bu polimorfizmin Val allelini taşıyıcısı olan kritik KAH sahip erkeklerde LDL-kolesterol (LDL-K) düzeyinde artış görüldü (p<0.05).

Sonuç: Bu sonuçlar, *ITLN1* Val109Asp polimorfizmin Val allelinin, kritik KAH ve LDL-K seviyeleri ile ilişkisini göstermektedir. *ITLN1* Val109Asp polimorfizminin KAH patogenezi üzerindeki etkisinin aydınlatılması için daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Intelektin-1, *ITLN1* geni, koroner arter hastalığı, Val109Asp

Coronary artery disease (CAD) is a complex multifactorial disease caused by interactions between genetic and environmental effects. It is associated with excessive inflammatory response in the vascular wall and is primarily because of atherosclerosis, an inflammatory process that leads to atheroma development and stenosis of the coronary arteries.[1]

Adipose tissue is a key endocrine organ, which produces several bioactive molecules (adipocytokines or adipokines), such as tumor necrosis factor-alpha, interleukin-6, and adiponectin with pro- or anti-inflammatory activities.[2] Adipokines play a crucial role in modulating atherogenesis in the development of cardiovascular diseases through insulin resistance and chronic inflammation.[3-7]

Intelectin-1 (ITLN-1, also known as omentin) encoded by the *ITLN1* gene, is a newly identified anti-inflammatory adipokine that is mainly expressed in the visceral adipose tissue and reduces inflammatory response.[8] Several lines of experimental and clinical evidence have shown that serum levels of ITLN-1 are negatively correlated with CAD,[9-11] peripheral artery disease,[12] established carotid atherosclerosis,[13] carotid intima-media thickness,[9,14] arterial stiffness,[14] and carotid plaque instability.[15]

The *ITLN1* Val109Asp missense polymorphism *(NM_017625.3, c.326T>A, p.Val109Asp, rs2274907)* in exon-4 leads to an amino acid alteration from valine to aspartic acid at codon 109. The Val109Asp polymorphism of the *ITLN1* gene has been associated with obesity, dyslipidemia, type 2 diabetes mellitus (T2DM), and CAD.[16-18] Moreover, the association of this polymorphism with anthropometric variables and CAD/T2DM-related complications, including lipid profile variations and insulin has been reported.[19] Several studies have shown inconsistent associations of *ITLN1* Val109Asp polymorphism with clinical CAD.[17,20-22] Nazar et al.[17] found that Val/Asp carriers were more frequent in Pakistani patients with CAD. In another study, Jha et al.[21] showed that the Val109Asp polymorphism was associated with CAD in the south Indian population, but no significant difference was found between men and women. In addition, Jamshidi et al.[22] reported that the male carriers of Asp had an increased risk of CAD.

In a previous study conducted in the Turkish population, the authors found no association between CAD and the *ITLN1* Val109Asp polymorphism, despite the higher frequency of Val/Val carriers in patients with CAD patients.[20] Studies investigating the severity of CAD with this polymorphism in Turkish patients have not been found in the literature till date.

Hence, in this study, we aimed to evaluate the effect of *ITLN1* Val109Asp polymorphism on serum lipid levels and the presence and severity of CAD in both sexes.

ABBREVIATIONS

BMI	Body mass index
CAD	Coronary artery disease
eQTL	Expression quantitative trait loci
HDL-C	High-density lipoprotein cholesterol
ITLN-1	Intelectin-1
LDL-C	Low-density lipoprotein cholesterol
MAF	Minor allele frequency
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
total-C	Total cholesterol

METHODS

Study population

The study was designed as a case-control study. The study population consisted of 493 consecutive individuals who underwent invasive coronary angiography at the Department of Cardiology, Ufuk University School of Medicine from 2014 to 2017 and were diagnosed as stable angina pectoris, myocardial ischemia, and acute coronary syndrome.

According to angiographic evaluation, the study subjects were classified as having critical CAD (≥70% stenosis, n=202), non-critical CAD (31%-69% stenosis, n=90), and non-CAD (control) (1%-30% stenosis,

n=201) groups. Two independent operators evaluated the coronary luminal narrowing following the guidelines of the American College of Cardiology/American Heart Association (ACC/AHA) for the classification of coronary lesions. The synergy between percutaneous coronary intervention with Taxus and Cardiac Surgery (SYNTAX) and Gensini scores were calculated for each sample following angiography to evaluate the complexity and severity of CAD.[23,24] The severity of the CAD was evaluated by Gensini score, an assessment based on coronary angiography. The Gensini score takes into consideration the degree of stenosis, multiplying factor that depends on the region of the stenosis, and collateral adjustment factor.[23] The SYNTAX score, used for the assessment of the complexity of CAD, was calculated using an online score calculator (version 2.28, www.syntaxscore.com). For selecting the non-CAD control group, in addition to coronary stenosis of 1%-30%, lack of cerebrovascular/peripheral artery disease and advanced age (≥55 years) were taken into consideration. The participants with a history of previous coronary bypass graft surgery, ongoing decompensated (New York Heart Association Functional Class IV) heart failure, advanced hepatic or renal failure, a life expectancy of less than a year, and known malignancy (except basal cell carcinoma and squamous cell carcinoma of the skin in full remission), and those with an active infectious or inflammatory disease or a rheumatologic disease were excluded from the study. Patients with a previous history of coronary brachytherapy were excluded from the study to eliminate the bias of the evaluation of coronary anatomy and severity of lesion resulted from vascular remodeling, which is a significant result of coronary brachytherapy. For eliminating CAD arising from familial hypercholesterolemia, participants with prior exposure to CETP or PCSK9 inhibitors were excluded from the study.

The presence of CAD risk factors was determined using the criteria of the European Society of Cardiology. A hypertensive condition was attributed when systolic blood pressure values were ≥140 mm Hg and/or diastolic blood pressure values were ≥90 mm Hg on at least two separate measurements. The subjects were considered smokers if they consumed cigarettes regardless of how many per day and non-smokers if they never smoked or had stopped smoking at least one year before sample collection. The weight of individuals was measured in their underwear without shoes using a scale. Body mass index (BMI) was calculated by dividing the body weight by their height in meter squared (kg/m^2). In the classification of obesity, individuals with a BMI ≥30 kg/m^2 were considered obese. Blood serum samples were collected before coronary angiography and stored at -80°C until analysis. Any lipemic, icteric, and hemolytic specimens were excluded. Analyses of the biochemical parameters were performed in two central laboratories. Concentrations of total cholesterol (total-C), fasting triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by UniCel DxC 800 (Beckman Coulter, USA).

The study was approved by the Ethics Committee of İstanbul University (Approval Date: February 28, 2014; Approval Number: 444), and written informed consent was obtained from all the participants.

Selection and functional annotation of *ITLN1* Val109Asp polymorphism

Single nucleotide polymorphism (SNP) selection was performed by literature search and bioinformatic analysis. We primarily focused on SNPs, which were associated with cardiovascular disease and its risk factors, especially those associated with CAD. On the basis of these criteria, Val109Asp (NM_017625.3:c.326T>A; rs2274907 A>T) polymorphism was selected.

The potential functional effect of this SNP was examined using the RegulomeDB database.[25] RegulomeDB uses a scoring system ranging from 1 to 7, where a score of 1 is the highest score indicating that SNP affects binding to transcription factors, and a score of 7 shows the least evidence of SNP being functional. RegulomeDB assigned a score of 4 to the Val109Asp polymorphism. This score indicates that Val109Asp polymorphism is related to transcription factor binding, regulatory motifs, and DNase peaks for DNase sensitivity. Although the results of these analyses point to Val109Asp polymorphism having functional effects, its expression quantitative trait loci (eQTL) data were not available.

Determination of the *ITLN1* genotyping

Genomic DNA was extracted from peripheral blood leukocytes using inorganic salting-out method.[26] Four hundred and ninety three participants (202 with critical CAD, 90 with non-critical CAD, and 201 non-CAD controls) were examined for *ITLN1* Val109Asp genotypes.

Genotyping was performed using LightSNiP assay (HybProbe hybridization probes) (TIB MOLBIOL GmbH, Germany) by Real-Time PCR LightCycler 480 instrument (Roche Life Science, USA). DNA amplification was set up in 96 well plates (ABGENE, UK). Typical 10 µL PCR reaction consists of 2 µL LightCycler® FastStart DNA

Table 1. Characteristics of study population (n=493)

Characteristics	Non-CAD control (n=201) Mean±SD; % (n)	Non-critical CAD patient (n=90) Mean±SD; % (n)	Critical CAD patient (n=202) Mean±SD; % (n)	p
Age (years)	57.4±11.8	64.3±9.2	63.6±10.9	<0 .001
BMI (kg/m^2)	28.4±3.9	28.8±3.8	29.3±4.3	0.075
Stenosis (%)	9.01±10.0	49.2±7.8	87.1±10.4	<0.001
Female	8.34±9.6	48.6±7.3	88.0±8.9	0.044
Male	9.88±10.3	49.6±8.1	86.7±11.0	
Gensini Score	1.95±3.02	16.4±12.3	67.4±50.1	<0 .001
SYNTAX Score	0.00±0.00	3.88±4.38	19.46±11.52	<0 .001
Total-Cholesterol (mg/dL)	203.5±49.1	185.0±44.1	196.0±47.3	0.009
HDL-Cholesterol (mg/dL)	41.9±10.0	39.3±10.3	39.8±11.3	0.061
LDL-Cholesterol (mg/dL)	119.4±37.1	108.5±34.9	114.0±37.0	0.054
Fasting Triglyceride (mg/dL)	143.1±1.66	132.9±1.69	146.5±1.70	0.333
HbA1c (%)¶	5.8±1.19	6.1±1.23	6.4±1.23	<0 .001
Fasting glucose (mg/dL)	111.8±40.2	125.2±51.8	125.0±45.4	0.006
Sex, Female*	57.2 (115)	38.9 (35)	31.2 (63)	<0 .001
Smoking status*	44.3 (89)	36.7 (33)	36.1 (73)	0.204
Diabetes mellitus*	29.9 (60)	45.6 (41)	46.8 (94)	0.001
Hypertension*	42.8 (86)	50.0 (45)	52.2 (105)	0.164
Obesity*	35.8 (72)	37.8 (34)	43.1 (87)	0.315
Positive family history of CAD*	36.3 (73)	42.2 (38)	49.5 (100)	0.028
Antidiabetic drug usage*	24.9 (50)	41.1 (37)	38.3 (77)	0.004
Lipid-lowering drug usage*	30.3 (61)	42.2 (38)	47.3 (95)	0.002

¶log-transformed variables expressed in geometric values.
*Categorical variables are presented in percentages (n).
BMI: body mass index; CAD: coronary artery disease; HbA1c: hemoglobin A1c; HDL: high-density lipoprotein; LDL: low-density lipoprotein; n: number of individuals; SD: standard deviation.

Master HybProbe Kit (Roche Life Science, USA) ready mix, 0.2 µL probes, 0.2 µL primers, and 6.6 µL distilled water. Genomic DNA 1 µL (15 ng) was added to the PCR mixture. PCR was carried out on LightCycler 480 under the following conditions: 95°C for 10 min, 95°C for 10 s, 60°C for 10 s, and 72°C 15 s (45 cycles). Melting curve analysis was assessed using the LightCycler 480 genotyping software (Supplementary Figure 1). The quality of genotyping for SNP was controlled using blind DNA duplicates. The accuracy of genotyping was 100% for each duplicate.

Statistical analysis

Genotype and allele distributions were compared using Pearson's chi-squared test. The Hardy-Weinberg equilibrium was computed to the expected genotype distribution. Genotype-phenotype associations were estimated using a dominant model (defined as Asp/Asp vs. Asp/Val+Val/Val). Because the number of individuals with the Val/Val genotype was low, homozygotes for the Val allele and heterozygotes were grouped as *ITLN1* Val allele carriers for statistical comparisons. All the variables were tested for normal distribution and found to be normally distributed, except TG. Owing to the skewed distribution, TG was logarithmically transformed for analyses and expressed as geometric means. Two-tailed and analysis of variance tests were used to compare continuous variables and expressed as means and standard deviation (SD), whereas categorical variables were compared using the Pearson's chi-squared test. Odds-ratio (OR) and 95% confidence interval (95% CI) were also assessed. Analyses were performed for men and women separately. Analyses of covariance were performed for values multi-adjusted for age, smoking, and usage of lipid-lowering drugs. A p value <0.05 was considered significant. All statistical analyses were performed using the SPSS for Windows version 23.0 software (IBM Corp., Armonk, NY, USA).

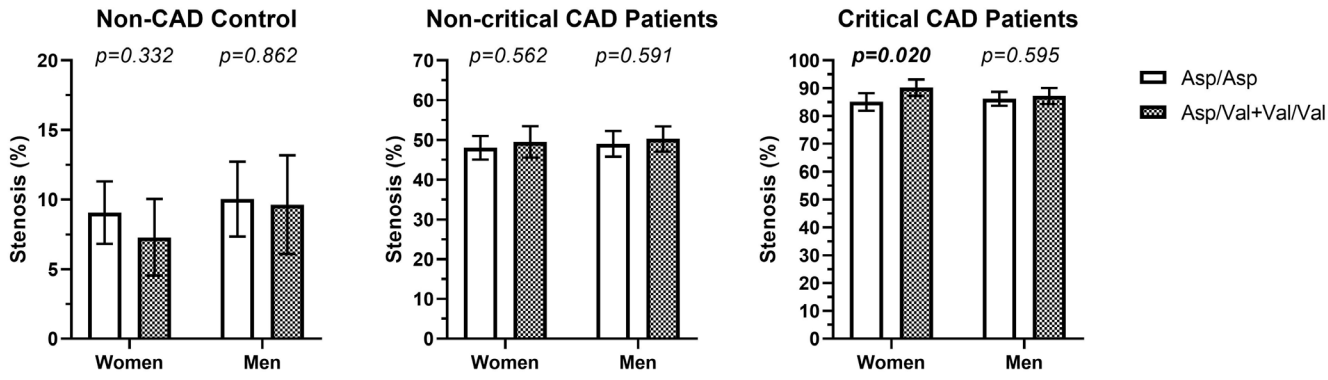

Figure 1. Estimated mean and 95% CI values for percentage of coronary stenosis across the genotypes of Val109Asp stratified by coronary stenosis and in women and men, after adjustment for age, smoking status, presence of diabetes, and usage of the lipid-lowering drug. Note the significant association among carriers of the Val109Asp Asp/Val+Val/Val and coronary stenosis in only women in critical CAD patient groups.

CI: confidence interval; CAD: coronary artery disease.

Table 2. Genotype and allele distribution of the *ITLN1* Val109Asp polymorphism in CAD groups, stratified to sex

	Non-CAD control	Non-critical CAD patient	Critical CAD patient		Non-CAD vs. Non-critical CAD		Non-CAD vs. Critical CAD		Non-critical CAD vs. Critical CAD	
	n (%)	n (%)	n (%)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Genotype distribution										
Women										
Asp/Asp	69 (60.0)	22 (62.9)	29 (46.0)	0.139	0.88 (0.40-1.93)	0.762	1.75 (0.94-3.26)	0.074	1.98 (0.85-4.62)	0.112
Asp/Val+Val/Val	46 (40.0)	13 (37.1)	34 (54.0)							
Men										
Asp/Asp	54 (62.8)	27 (49.1)	79 (56.8)	0.275	1.75 (0.88-3.47)	0.109	1.28 (0.73-2.22)	0.377	0.73 (0.39-1.36)	0.329
Asp/Val+Val/Val	32 (37.2)	28 (50.9)	60 (43.2)							
Allele Distribution										
Women										
Asp	179 (77.8)	54 (70.1)	85 (67.4)	0.081	1.49 (0.83-2.66)	0.173	1.69 (1.04-2.75)	0.033	1.13 (0.61-2.09)	0.691
Val	5 (22.2)	23 (29.9)	41 (32.6)							
Men										
Asp	134 (77.9)	78 (70.9)	201 (72.3)	0.317	1.44 (0.83-2.49)	0.185	1.35 (0.86-2.10)	0.186	0.93 (0.57-1.52)	0.783
Val	38 (22.1)	32 (29.1)	77 (27.7)							

Values are in % (n) and unadjusted. n: Number of individuals.
Odds ratios were calculated regarding the presence of the minor allele.
CAD: coronary artery disease; CI: confidence interval; OR: odds ratio.

RESULTS

Patient characteristics

General characteristics of critical CAD (mean age 63.6±10.9 years), non-critical CAD (mean age 64.3±9.2 years), and non-CAD control (mean age 57.4±11.8 years) groups are shown in Table 1. The percentage of patients with T2DM and a positive family history of CAD were significantly higher among critical CAD and non-critical groups than in the non-CAD group (Table 1). In addition, lipid-lowering and antidiabetic drug usage were higher in critical CAD and non-critical groups as shown in Table 1.

In the study participants, the genotype distribution of *ITLN1* Val109Asp polymorphism was 56.8% (n=280), 34.7% (n=171), and 8.5% (n=42) for AA, AT, and TT genotypes, respectively. The frequency of the rare allele, T, was found to be 0.26 in these participants.

Effects of the *ITLN1* Val109Asp polymorphism on severity of CAD

In the CAD groups, the genotype distributions of *ITLN1* Val109Asp for the Asp/Asp, Asp/Val, and Val/Val genotypes were 61.2% (n=123), 33.3% (n=67), and 5.5% (n=11) in non-CAD control; 54.4% (n=49), 37.8% (n=34), and 7.8% (n=7) in non-critical CAD; and 53.8% (n=108), 34.7% (n=70), and 11.9% (n=24) in critical CAD, respectively. The minor allele frequency (MAF) of the Val allele was 0.22, 0.26, and 0.29 for non-CAD control, non-critical CAD, and critical CAD respectively. The Hardy-Weinberg equilibrium test showed no deviation of the *ITLN1* Val109Asp polymorphism genotypes in 201 non-CAD control subjects. Because the number of individuals who were homozygous for the Val109 allele was very low, we chose the dominant model (Asp/Asp versus Asp/Val+Val/Val) for further statistical analyses.

Table 2 presents the distribution of *ITLN1* genotypes and allele stratified by CAD and sex in dominant model. Comparing the three CAD groups, no significant difference was observed in genotype distributions and allele frequencies in both sexes (p>0.05). The comparison of genotype distributions in pairwise groups showed no significant association existed in both men and women. A significant difference was found between critical CAD and non-CAD control groups when comparing the pairwise allelic frequencies in only women (p=0.033). ORs and their 95% CI based on allele distributions were calculated for critical CAD and non-CAD control groups (Table 2). In women, the Val allele frequency was 32.6% and 22.2% in critical CAD and non-CAD control groups, respectively. Val allele carriers had a 1.69-fold increased risk for critical CAD (p=0.033). However, no significant difference was found in pairwise comparisons of the non-critical CAD patient group (p>0.05) in both sexes.

Effects of the *ITLN1* Val109Asp polymorphism on cardiometabolic risk factors

The relationship between *ITLN1* Val109Asp genotypes and anthropometric and metabolic variables were analyzed in non-CAD, non-critical CAD, and critical CAD groups stratified to sex in the dominant model.

Crude analysis in women with critical CAD showed that coronary stenosis was significantly higher in Val allele carriers (mean±SD, 90.2±8.8) than in non-carriers (mean±SD, 85.3±8.3) (p=0.021). The univariate analysis adjusted for age, smoking status, presence of diabetes, and usage of lipid-lowering drugs showed that the presence of Val allele was associated with higher coronary stenosis only in women with critical CAD (p=0.020) (Figure 1).

In the crude analysis, significant effects of Asp/Val+Val/Val genotype existed on fasting higher TG (p=0.002) and lower HDL-C (p=0.044) levels in women with non-CAD and higher LDL-C (p=0.047) in men with critical CAD. The univariate analysis adjusted for age, smoking status, presence of diabetes, and lipid-lowering drug usage indicated that fasting TG, HDL-C, and LDL-C levels are associated with Val109Asp genotypes (Table 3). Higher TG and lower HDL-C in women with non-CAD were not observed in carriers of Val allele after age, smoking status, presence of diabetes, and lipid-lowering drug usage adjustments. Women with critical CAD who were carriers of the minor allele had higher LDL-C levels (p=0.070) after age, smoking status, presence of diabetes, and lipid-lowering drug usage adjustments; although this association was not found to be statistically significant. There were no significant associations between genotypes and fasting TG and HDL-C levels in women with non-critical CAD and critical CAD. In men with critical CAD, minor allele carriers were found to have significantly higher LDL-C levels than those with Asp/Asp genotype in an analysis adjusted for age, smoking status, presence of diabetes, and lipid-lowering drug usage (p=0.020). No significant associations were found between genotypes and fasting TG and HDL-C levels in men with non-CAD and non-critical CAD (p>0.05).

Table 3. Adjusted fasting TG, HDL-C, and LDL-C values according to the *ITLN1* Val109Asp genotype in CAD groups, stratified to sex

	Non-CAD control			Non-critical CAD patient			Critical CAD patient		
	Asp/Asp	Asp/ Val+Val/ Val	p	Asp/Asp	Asp/ Val+Val/ Val	p	Asp/Asp	Asp/ Val+Val/ Val	p
Women, n	69	46		22	13		29	33	
Fasting TG (mg/dL)¶	131.2±0.2	148.5±0.3	0.178	126.7±0.5	178.2±0.6	0.201	149.2±0.3	139.6±0.3	0.468
HDL-C (mg/dL)	46.1±1.1	43.0±1.4	0.112	45.2±1.9	41.0±2.5	0.218	42.6±2 .1	42.8±1.9	0.935
LDL-C (mg/dL)	120.7±4.8	123.2±5.9	0.746	115.7±7.1	111.6±9.4	0.738	116.6±5.8	131.5±5.4	0.070
Men, n	54	32		27	28		78	60	
Fasting TG (mg/dL)¶	140.6±0.3	168.2±0.4	0.237	118.8±0.4	134.2±0.4	0.406	146.9±0.3	151.3±0.3	0.668
HDL-C (mg/dL)	39.0±1.2	36.0±1.6	0.153	38.0±1.8	35.1±1.8	0.266	38.5±1.2	38.2±1.4	0.875
LDL-C (mg/dL)	114.5±4.5	119.3±6.0	0.534	106.7±6.4	103.2±6.3	0.699	103.1±3.9	117.6±4.5	0.020

Data are expressed as mean ± SE after adjustment for multiple covariates and p values are adjusted for multiple comparisons.
¶log-transformed variables expressed in geometric values, adjusted for age, smoking status, presence of diabetes, and usage of lipid-lowering drugs.
Associations are considered significant when p<0.05 and are indicated in bold.
CAD: coronary artery disease; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol TG: triglyceride.

Table 4. Data on *ITLN1* gene Val109Asp polymorphism affecting CAD in different study populations

Populations	Sample size (Case/Control)	Effect on CAD	Associated sex	Associated genotype	Associated Allele	Minor Allele	Minor Allele CAD	Frequency Non-CAD	Reference number
Indians	100/100	Increased risk of CAD	NA	Asp/Val	Val	Val	0.39	0.26	21
Pakistani	250/100	Increased risk of CAD	Not separated by sex	Asp/Val	NA	Val	0.38	0. 22	17
Iranian	200/200	Increased risk of CAD	Male	NA	Asp	Val	0.20	0.30	22
Turks	75/82	No effect	Not separated by sex	Val/Val	NA	Val	0.31	0.23	20
Turks	202/201*	Effect on Severity of CAD	Female	NA	Val	Val	0.29*	0.22	Present study

*critical CAD.
CAD: coronary artery disease; NA: no association.

Furthermore, there were no significant associations among genotypes, BMI, fasting glucose, HbA1c, Gensini Score, and Syntax Score in non-CAD, non-critical CAD, and critical CAD groups in both sexes in the crude analysis. No significant relationship was found between the *ITLN1* Val109Asp polymorphism and diabetes, obesity (BMI ≥30 kg/m^2), and hypertension in non-CAD, non-critical CAD, and critical CAD groups in both sexes using the chi-squared test (data not shown).

DISCUSSION

In this study, we investigated the role of the Val109Asp polymorphism of the *ITLN1* gene in Turkish patients with CAD and also investigated the probable differences between genotypes/alleles and the severity of CAD. This study also showed that the Val allele frequency in women was higher in patients with critical CAD than in non-CAD control, and female Val allele carriers had a 1.69 fold increased risk for critical CAD compared with non-CAD. Moreover, Asp/Val+Val/Val carriers were associated with higher stenosis in women with critical CAD independent of age, smoking, presence of diabetes, and drug usage. Male patients with critical CAD who were carriers of Asp/Val+Val/Val had significantly higher levels of LDL-C independent of age, smoking, presence of diabetes, and drug usage.

Associations of Val109Asp polymorphism with presence and severity of CAD

Several studies have reported the significant relationship between *ITLN1* Val109Asp polymorphism and the risk of CAD in different study populations such as Asian, Iranian, and Turkish. This relationship has been controversial in some[17,21,22] but not all[20] studies (Table 4).

In non-CAD subjects, the minor allele frequency was nearly 20% among Pakistanis and Turks, somewhat higher in Indians but as high as 30% in Iranians (Table 4). The frequency of minor allele in patients with CAD was 31%-39% among Pakistanis, Indians, and Turks and 20% among Iranians. The minor allele frequency of non-CAD (22%) and critical-CAD (29%) patients in our study is compatible with other studies.

The sex-specific effect of *ITLN1* Val109Asp polymorphism in CAD was reported in a study by Jamshidi et al.[22] In that study, they suggested that the common asp allele is a possible risk factor for CAD only in men.[22] To the best of our knowledge, to date, there is only one study performed in Turkish patients with CAD of this polymorphism.[20] In their study, Yörük et al.[20] observed a higher Val/Val genotype frequency in patients with CAD than in controls but did not find a significant difference between patients and controls owing to the small sample size. Compared with the study of Yörük et al.,[20] our work has a relatively larger sample size and unlike previous studies, the effect of both genotype and allele frequencies of *ITLN1* Val109Asp polymorphism on the severity of CAD was investigated in both the sexes. Our results indicate that the Val allele frequency was higher in patients with critical CAD than in the non-CAD control only in women. Val109Asp polymorphism in the *ITLN1* gene with a controversial relationship to CAD may be because of the small sample size and the differences in environmental factors affecting CAD. However, the fact that sex-specific effects may be owing to the differences in hormones, dietary habits, lifestyle, stress factors, or attitudes toward treatments and prevention between sexes should not be ruled out. CAD is the leading cause of death in both men and women; however, there are many sex-related differences in the clinical presentation and diagnosis of CAD.[27] Although women and men share similar risk factors for CAD including age, family history of CAD, hypertension, diabetes, dyslipidemia, smoking, and physical inactivity; the significance of these factors is different among sexes.[28] Furthermore, specific risk factors such as hypertensive diseases in pregnancy, menopause, and the use of hormonal therapies for contraception and menopausal symptoms are unique to women.[29] Sex-specific effects of several polymorphisms in various genes have been previously reported in CAD.[22,30,31] For example, *ESR1* PvuII polymorphisms affect CAD susceptibility only in men,[30] and APOE rs4420638 polymorphism was also significantly associated with increased coronary heart disease risk in men only.[31] Consequently, the sex-specific effect of the Val109Asp polymorphism may have resulted from the presence of the Val allele in addition to other factors mentioned above (such as sex).

The effect of the Val allele on the severity of CAD in women is likewise supported by high-grade stenosis in our study. Previous studies have reported the association of ITLN-1 levels with CAD severity and stenosis. Shang et al.[10] indicated that serum ITLN-1 levels were independently and negatively associated with the presence and angiographic severity of CAD in patients with metabolic syndrome. In addition, *ITLN1* expression in patients with CAD was lower in epicardial adipose tissue adjacent to coronary stenotic segments than in non-stenotic segments.[32] To the best of our knowledge, our study is the first one to report the association between Val109Asp polymorphism and stenosis. Carrying the Val allele in women is a risk factor for critical CAD, independent of age, smoking, presence of diabetes, and drug usage by increasing stenosis; however, such an effect of this allele was not observed in men. Our results suggest that the Val109Asp polymorphism effects CAD severity by increasing stenosis, independent of age, diabetes, cigarette, and statin use. The high risk of critical CAD in women carrying the Val allele might be owing to the fact that ITLN-1 interacts with cytokines such as adiponectin[33] and increases stenosis

via the mechanism of inflammation, apart from mechanisms such as obesity, impaired glucose tolerance, or dyslipidemia as no association was observed among any of these parameters.

It has been known that changes in functionally important regions of genes affect their activities and levels of their protein products.[34,35] Therefore, polymorphisms in the *ITLN1* gene can be expected to affect levels of ITLN-1. Previous studies did not observe any significant association between Val109Asp polymorphisms and circulating levels of ITLN-1 in different diseases.[19,36,37] In their study, Splichal et al.[19] observed 16% higher plasma ITLN-1 levels in Asp/Asp carriers than in Val/Val carriers, but this difference did not reach statistical significance, which might have been caused by the relatively small sample size used in the study. Later on, in another study, Splichal et al.[38] did not find any association between this polymorphism and circulating maternal serum ITLN-1 levels in women with spontaneous preterm birth and spontaneous term birth. In their study, Turan et al.[36] determined significantly lower levels of serum ITLN-1 in patients with psoriasis; however, the serum levels did not differ significantly between the Val109Asp genotypes. Therefore, they suggested that the low serum ITLN-1 levels were not associated with Val109Asp polymorphisms, and these polymorphisms had no effect on serum ITLN-1 levels. Rathwa et al.[37] observed significantly high levels of *ITLN1* mRNA in patients with T2DM; however, the mRNA levels did not show significant difference between AT and TT genotype of Val109Asp polymorphisms, although levels were lower in AT carriers. In the same study, plasma ITLN-1 levels showed significant decrease in patients with both T2DM and obesity than the controls; however, there was no difference in the plasma levels between TT and AT carriers. According to Regulome data, there is a possibility of transcription factor binding site in the region of this polymorphism. However, so far, there are no functional studies investigating the functional effect of this polymorphism. Further studies are needed to define the exact role of this polymorphism on circulating ITLN-1 levels.

Using the predication tool, the Val109Asp was classified as benign as the residue was not strongly conserved, and the allele frequency in population databases was greater than 0.05. However, this variant might influence the function of ITLN-1 protein because its physicochemical property changed from hydrophobic valine to negatively charged aspartic acid. The Val109 residue is located at the fibrinogen C terminal domain of the protein that can function as a molecular recognition unit that interacts with either proteins or carbohydrates. Therefore, this change can affect the interaction of ITLN-1 protein with other proteins or carbohydrates. Tsuji et al.[39] reported that ITLN-1 protein contains two potential N-glycosylation sites at Asn154 and Asn163, and these regions have a functional effect. However, the functional effect of Val109Asp has not been investigated yet, and further studies are needed to define the role of this polymorphism on protein function.

Linkage disequilibrium analysis revealed that the Val109Asp polymorphism was in moderate association with another polymorphism (rs1333062) in the *ITLN1* gene, and the haplotypes for these two polymorphisms were not found to be associated with T2DM.[32] Another study by Khoski et al.[40] showed that the Val109Asp and FTO rs9939609 polymorphisms were associated with insulin resistance and familial history of diabetes in the patients with newly diagnosed T2DM. The same study also showed that when the combinatorial effect of genotypes was examined, one polymorphic allele of these two genes coexisted more frequently in diabetic patients than in healthy controls.[40] However, the effect of other variants in the same gene or variants in other genes cannot be excluded.

Associations of Val109Asp polymorphism with other metabolic conditions

T2DM is a strong risk factor for CAD, and experts consider DM an equivalent to established CAD risk. Patients with diabetes have two to four-fold greater risk of developing CAD than non-diabetic patients.[41,42] In our study population, diabetes was frequently observed in the critical CAD group. No association was found among CAD groups regarding the presence or absence of diabetes. An association between Val109Asp polymorphism and T2DM was reported in Asians[40] but not in Indians[37] and Europeans.[18] A study by Rathwa et al.[37] did not find an association between Val109Asp genotypes and T2DM; however, high *ITLN1* mRNA expression and low protein level in plasma were found to be associated. This suggests that epigenetic and translational mechanisms rather than polymorphisms might be effective in the relationship of this gene with diabetes.

Associations of Val109Asp polymorphism with lipids

We also examined the effect of this polymorphism on serum lipid profile in non-CAD, non-critical CAD, and critical CAD groups in both sexes. Previous studies investigating the effect of Val109Asp polymorphism with CAD have reported no association of this polymorphism with total-C, LDL-C, HDL-C, and triglyceride.[17,20,37]

However, in a recent study, it has been reported that the Asp/Val genotype of Val109Asp polymorphism was associated with lower levels of LDL-C in CAD patients. [21] In our study, the Asp/Val+Val/Val genotype was associated with higher LDL-C in men with critical CAD, independent of age, smoking status, presence of diabetes, and lipid-lowering drug usage. Although LDL-C levels were found to be elevated in female carrier of the Val allele, this was not statistically significant. Previous studies have indicated that the effect of LDL-C on the development of cardiovascular disease was higher in men than in women.[43] However, LDL-C levels were found higher during menopause, and it was stated that women over the age of 65 had a higher average of LDL-C than men. [44] The difference in the effect of Val allele carriage on LDL-C levels observed in our study may be owing to the effect of hormones in women on LDL-C metabolism.

Limitations

Minor limitations of this study include focusing on Val109Asp polymorphism without assessing the combined effect of functional SNP(s) located in other regions of the gene and other interacting genetic factors on analyses. Potential confounding factors such as diet, physical activity, alcohol consumption, impaired glucose tolerance, and menopausal status could affect the pathogenesis of CAD. Therefore, another minor limitation might be the inability to adjust for these factors because of limited data. Considering this, serum ITLN-1 level was inversely associated with the presence and severity of CAD in patients with metabolic syndrome,[10] and lack of assessment of serum ITLN-1 levels may be considered as another limitation of this study. To determine the effect of this polymorphism on both serum ITLN-1 levels and CAD severity, further investigations are required.

CONCLUSION

Our results suggest that the Val allele of the *ITLN1* Val109Asp polymorphism is associated with CAD severity in women and higher coronary stenosis in women with critical CAD. In addition, the presence of the Val allele was associated with increased LDL-C levels in men. The results of this study showed that *ITLN1* Val109Asp polymorphism warrants further attention, and functional studies are required for the determination of its precise role in the mechanisms that lead to CAD. Although Val109Asp polymorphism was classified with a score of 4 on RegulomeDB, evidence that this variant interrupts the binding sites of the transcription factor needs to be elucidated. For the validation of the results of this study, more studies with larger sample sizes in different populations should be done.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of İstanbul University (Approval Date: February 28, 2014; Approval Number: 444).

Informed Consent: Written informed consent was obtained from the participants of this study.

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