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Original research

Association of NFKB1 -94 ins/del variants with BMI in patients with myocardial infarction

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ABSTRACT

Background: Myocardial infarction (MI) is one of the major causes of death and disability in the world.
Aim: We studied the functional polymorphisms of *IL17A* (HGNC:5981) G197A (rs2275913; NC_000006.11:g.52051033G > A) and −94 *NFKB1* (HGNC:7794) ATTG ins/del (rs28362491, NC_000004.12:g.102500998_102501001ATTG) in patients with MI.
Methods: The selected polymorphisms were assessed in 201 MI patients and 201 healthy blood donors from Fars Province. Associations between allele frequencies, serum CXCL1 levels and clinicopathological criteria of MI were examined.
Results: A significant increase in del minor allele of *NFKB1* in individuals with a BMI lower than 25 (P = 0.048) and in patients who needed coronary artery bypass graft during the course of treatment (P = 0.044) was observed. Also we found a significant association between plasma content of CXCL1 and -94 *NFKB1* ATTG ins/del (rs28362491) polymorphism (P = 0.01). A trend of increase in the *IL17A* A-allele in patients with left ventricular hypertrophy and those with apical hypokinesia was observed as well.
Conclusion: The minor alleles of genes upstream and downstream of IL-17A pathway are associated with BMI and clinicopathological parameters of MI severity.

1. Introduction

Myocardial infarction (MI) is one of the major causes of death and disability in many countries around the world (Thygesen et al., 2007). Atherosclerosis causes myocardial infarction through coronary artery blockage or plaque rupture. Immunological and inflammatory responses have considerable roles in the progression of atherosclerosis which eventually lead to MI, speed up the myocardial disability or contribute to a worse outcome for the patients (Viles-Gonzalez et al., 2004; Hansson, 2009) Inflammatory cytokines secreted by T lymphocytes impact atherosclerosis development; of which IL-17A secreted by Th17 lymphocytes (Eid et al., 2009; de Boer et al., 2010; Taleb et al., 2009) and other cells such as dendritic cell, neutrophil, basophil, eosinophil, mast cell can be named (Gaffen, 2009; Cua and Tato, 2010). IL-17A induces the production of chemokines, inflammatory cytokines, and matrix metalloprotease and activates caspase 3 and caspase 9; that cause apoptosis in endothelial cells and cardiomyocytes thereby exerting a pro-atherogenic role (Liuzzo et al., 2013). Increased frequency of Th17 and its respective cytokines: IL-17, IL-6, IL-23 are seen in patient with acute MI (Liang et al., 2009; Ávalos et al., 2012). In addition, increased IL-17A expression in dead tissue of the left ventricle is seen in the rat MI model (Ávalos et al., 2012). It is shown that ischemic reperfusion injury in heart causes elevated expression of IL-17 and its receptor (IL-17RA subunits) (Barry et al., 2013). This is a notable observation because Percutaneous Coronary Intervention (PCI), which is a common therapeutic intervention to reduce the damage of ischemic myocardium, can cause Ischemic-Reperfusion injury (Piper et al., 1998; Yellon and Hausenloy, 2007). Enhanced IL-17A signaling via the activation of a number of MAPK leads to activation of several transcription factors including NF-κB which cause transcription of chemokines, cytokines and MMPs.

In vivo studies in rat MI model have shown that IL-17 neutralization results in reduced necrosis and reduced apoptosis of cardiomyocytes (Barry et al., 2013). Treatment by monoclonal antibodies and IL-17A genetic defects lead to the improvement of Ischemic-Reperfusion injury, decline of the damaged tissue size, reduced heart cardiac troponin T level and better heart function that are associated with reduced Neutrophil infiltration and cardiomyocytes apoptosis (Liao et al., 2012). Previous reports suggest that the rs2275913 (G197A) from IL-17A gene promoter is markedly associated with inflammatory diseases (Geng

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et al., 2014; Arisawa et al., 2008, 2012). Moreover, rs28362491 from *NFKB1* gene is associated with increased risk of coronary artery disease (Yang et al., 2014; Vogel et al., 2011). Since NF-κB1 works downstream of IL-17 signaling, we aimed to study functional polymorphisms in the *IL-17A* and *NFKB1* gene promoters in patients who were hospitalized after myocardial infarction and also in a group of healthy blood donors in the same geographic region. Also, the correlation of plasma CXCL1, one of the target genes of IL-17A signaling pathway, with the studied polymorphisms was assessed.

2. Subjects, materials and methods

2.1. Study population

All confirmed MI cases (n = 201) referring to the affiliated hospitals of Shiraz University of Medical Sciences in a one-year period were included in this study. MI diagnosis was approved by collaborating cardiologist on the basis of typical ECG changes and increased cardiac markers. The MI diagnosis was confirmed by coronary angiography. The patients' clinical and pathological criteria are shown in Table 1. Control individuals (n = 201), were selected from among healthy blood donors of the same age range and gender (Mean age ± SD = 57.80 ± 11.8 yrs, 175 Male/26 Female) who referred to Fars Blood Transfusion Center. First they were examined by a physician and were evaluated for underlying diseases, including: cardiovascular diseases, hyperthyroidism, hypertension, pulmonary diseases, stroke, diabetes, cancer and autoimmune diseases. Second, they were asked and examined for recent infectious diseases including common cold, influenza, lung infection, urinary tract infection, brucellosis, tuberculosis, and typhoid and excluded if positive. Thirdly, the recipients of recent surgery and dentistry and those who were on any medications such as antibiotics, aspirin, propranolol, or had recently received any vaccine or blood products were excluded from study. Travel to endemic malaria areas in the country, tattooing, acupuncture, high or low blood pressure and anemia, G6PD deficiency and, risky behaviors were also considered as exclusion criteria. Blood samples from these individuals were further tested for infectious diseases such as hepatitis C, hepatitis B, AIDS and Syphilis. All of these 201 peoples were negative for those criteria.

The lipid panel (HDL, LDL, TG, and Cholesterol) and blood glucose levels were not studied due to the fact that the control individuals were not fasting. Having said so, we only included individuals who had had their lipid profile and blood sugar levels checked up to 3 month before sampling and turned normal.

Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. Categorization of BMI was done based on previously defined criteria by WHO (World Health Organization (WHO), 2000). Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or the use of oral antihypertensive therapy. Type 2 Diabetes mellitus (T2DM) was diagnosed according to World Health Organization criteria. Hyperlipidemia was defined as elevated levels of any or all lipids and/or lipoproteins in the blood.

Table 1
The demographical and clinicopathologic criteria of patients with myocardial infraction.

Characteristic	MI patients no. (%)
No. of subjects	201
Age (means ± SD, yrs)	59.30 ± 12.4
Sex (male/female, no %)	175 (87.1)/26 (12.91)
Body Mass Index (112)	
< 18.4	1 (1.8)
18.5–24.9	52 (46.4)
25–30	45 (40.2)
> 30	13 (11.6)
Hyperlipidemia (163)	
Yes	42 (25.8)
No	121 (74.2)
Diabetes Mellitus (162)	
Yes	40 (24.7)
No	122 (75.3)
Family history (163)	
Yes	48 (29.4)
No	115 (70.6)
Hypertension (163)	
Yes	113 (69.3)
No	50 (30.7)
Smoking (163)	
Yes	78 (47.9)
No	85 (52.1)

2.2. Blood samples and DNA extraction

Six ml venous blood was collected from all subjects in tubes containing Ethylene diamine tetra acetic acid anticoagulant. At first, plasma was separated for ELISA test and then DNA extraction was performed by salting out method. DNA concentration and protein contamination were determined by means of spectrophotometer in 260 and 280 wave length. The concentration of DNA samples was standardized to 0.3 μg/μL.

2.3. Genotyping

We studied G197A (rs2275913; NC_000006.11:g.52051033G > A) polymorphism of *IL17A* gene (HGNC:5981) and −94 ins/del (rs28362491; NC_000004.12:g.102500998_102501001ATTG) polymorphism in the *NFKB1* (HGNC:7794) gene. PCR and RFLP methods were established and used for genotyping of noted polymorphisms. PCR procedure was performed in a 15 μl total reaction volume including 200 μM of each dNTPs, 300 ng genomic DNA, 2 mM of $MgCl_2$, 10× PCR Buffer, 1 U Taq DNA polymerase and 1.0 μM of each primer (10 pM concentration). Then the related restriction enzyme added to PCR products and incubated at 37 °C overnight in a dry block. The structures of primers; required restriction enzyme; recognition site of restriction

Table 2
Primer sequences, restriction endonucleases, and PCR product lengths of the studied polymorphisms.

SNPs	Primer sequences	Restriction enzyme	products length (bp)
***IL17A* G197A**	Forward -5′- AACAAGTAAGAATGAAAAGAGGACATGGT -3′ Reverse -5′-CCCCCAATGAGGTCATAGAAGAATC -3	*Xag*I (*Eco*NI)	AA:102 AG: 102, 68, 34 GG:68, 34
***NFKB* -94 ins/del ATTG**	Forward -5′-TGGGCACAAGTCGTTTATGA -3′ Reverse -5′-CTGGAGCCGGTAGGGAAG-3′	*Van*91I (*Pfl*MI)	ins/ins: 285 ins/del: 285, 240, 45 del/del: 240,45

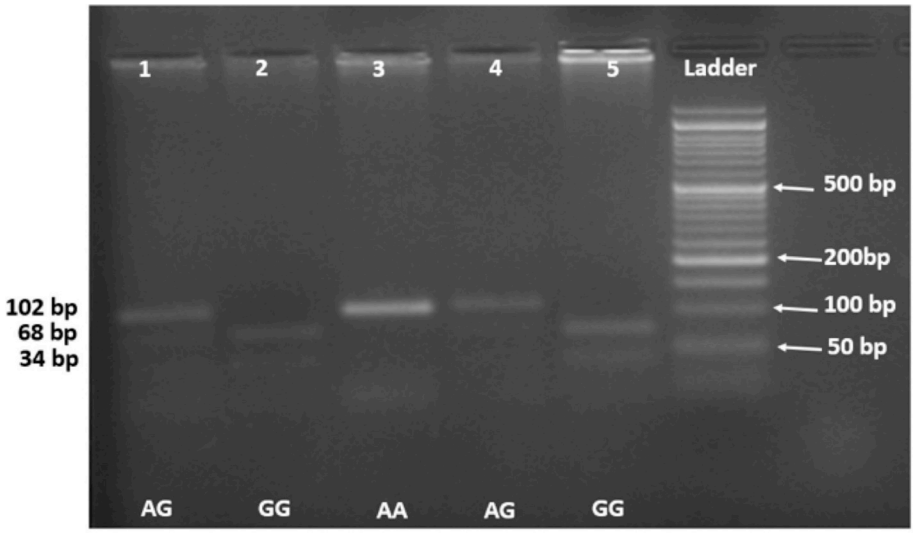

Fig. 1. PCR-RFLP products corresponding to the *IL17A*197G polymorphism showing AG (lanes 1 and 4), GG (lanes 2 and 5) and AA (lane 3) genotypes.

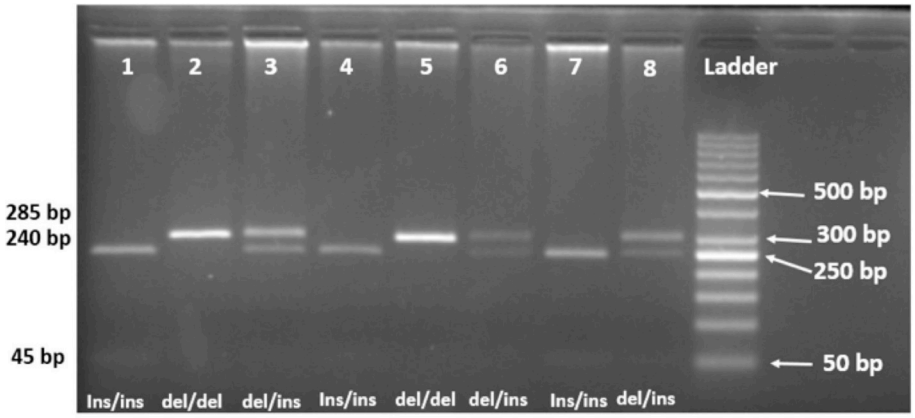

Fig. 2. PCR-RFLP products corresponding to the *NFKB1*-94 ins/del ATTG polymorphism showing ins/ins (lanes 1,4 and 7), del/del (lanes 2 and 5) and del/ins (lanes 3,6 and 8) genotypes.

enzyme and length of cleaved products are shown in Table 2. The cleaved product by restriction enzyme were ran on a 3.5 agarose gel containing safe stain and visualized by UV light at 254 nm (Figs. 1 and 2).

2.4. Chemokine measurement

CXCL1 serum levels in 126 MI patients and 50 normal subjects which were measured by CXCL1 ELISA assay are reported in our

Table 3
The genotypes and allelic distribution of *IL17A* G197A and *NFKB1* -94 ins/del ATTG in patients and controls.

Genotypes and alleles	MI patients N (%)	Controls N (%)	P value	OR	RR
***IL17A*: G197A Genotypes**			0.29		
AA	29 (14.4)	20 (10.1)		**Reference**	**Reference**
AG	77 (38.3)	88 (44.2)		0.60 (0.32–1.15)	0.79 (0.59–1.05)
GG	95 (47.3)	91 (45.7)		0.72 (0.38–1.36)	0.86 (0.66–1.13)
Alleles			0.67		
A	135 (33.6)	128 (32.2)		1.07 (0.79–1.43)	1.03 (0.89–1.19)
G	267 (66.4)	270 (67.8)		**Reference**	**Reference**
***NFKB1*-94 ins/del ATTG Genotypes**			0.92		
ins/ins	90 (46.6)	93 (47)		**Reference**	**Reference**
ins/del	80 (41.5)	84 (42.4)		0.98 (0.65–1.50)	0.99 (0.80–1.23)
del/del	23 (11.9)	21 (10.6)		1.13 (0.59–2.19)	1.06 (0.77–1.46)
Alleles			0.81		
ins	260 (67.4)	270 (68.2)		0.96 (0.71–1.30)	0.98 (0.84–1.14)
del	126 (32.6)	126 (31.8)		**Reference**	**Reference**

Table 4
The Associations of clinicopathological manifestations of MI with *IL17A* G197A genotype and allele frequencies in patients.

Clinical manifestations (N)	*IL17A* G197A N (%)			$\chi 2$ P	OR	Regression P	*IL17A* G197A N (%)		χ^2 P	OR	Regression P
LVH (151)	GG	GA	AA	0.1	AA: ref	GA: 0.46	G	A	0.04*		0.44
No (130)	69 (53.1)	42 (32.3)	19 (16.6)		GA: 1.93 (0.34–11.02)	GG: 0.85	180 (69.2)	80 (30.8)		Reference	
Yes (21)	6 (28.6)	10 (47.6)	5 (23.8)		GG: 0.84 (0.14–5.08)		22 (52.4)	20 (47.6)		0.72 (0.31–1.66)	
Apical hypo kinesia (133)											
Apical hypo kinesis (133)	GG	GA	AA	0.06	AA: ref	GA: 0.23	G	A	0.03*		0.15
Negative (120)	61 (50.8)	43 (35.8)	16 (13.4)		GA: 0.39 (0.08–1.85)	GG: 0.09	165 (68.7)	75 (31.3)		Reference	
Positive (13)	4 (30.8)	4 (30.8)	5 (38.4)		GG: 0.26 (0.061.22)		12 (46.2)	14 (53.8)		0.53 (0.22–1.27)	
Systolic dysfunction (127)	GG	GA	AA	0.14	AA: ref	GA: 0.67	G	A	0.06		0.99
Normal (102)	52 (51)	37 (36.3)	13 (12.7)		GA: 0.70 (0.14–3.52)	GG: 0.66	141 (69.1)	63 (30.9)		Reference	
Abnormal (mild + moderate + severe + very severe) (25)	9 (36)	9 (36)	7 (28)		GG: 0.71 (0.15–3.29)		27(54)	23(46)		1.00 (0.43–2.30)	

previous study (Pordel et al., 2018). The data were used to assess the correlation between CXCL1 levels in serum and the polymorphisms in *IL17A* and *NFKB1* genes.

2.5. Statistical analysis

Statistical analysis of data by chi-square test was performed using SPSS18 (version 18, SPSS Inc, Chicago, IL. USA) software. We also performed a logistic regression model (Enter method) to assess the risk of MI associated with each genotype and allele of *IL17A* G197A as well as −94 *NFKB1* ATTG ins/del. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Genotypes and alleles distributions

The genotype distributions and allelic frequencies of studied polymorphisms are shown in Table 3. The two studied polymorphisms in patients and controls groups were in Hardy-Weinberg equilibrium. The genotyping of *IL17A* G197A was successful for 201 patients and 199 controls but genotyping for *NFKB1* -94 ins/del ATTG polymorphisms was successful in 193 patients and 198 controls, for both of which we found no significant differences in the frequencies between patients and controls (Table 3).

3.2. Associations between polymorphisms of the IL17A G197A and NFKB1 -94 ins/del ATTG with clinicopathologic characteristics of patients

IL17A G197A polymorphism was significantly associated with left ventricular hypertrophy (LVH), apical hypokinesia and systolic cardiac dysfunction in Chi-Square test, however, the results were not confirmed in the regression analysis (Table 4). On the other hand, NFKB1 -94 ins/del polymorphism was associated with BMI lower than 25; high blood pressure and a previous history of coronary artery bypass grafting in patients. Regression analysis confirmed the association between del allele and lower BMIs in patients with MI. Also patients with del allele were more likely to need coronary artery bypass graft (CABG) during the course of treatment (Table 5). In a curious attempt to identify the effect of carrying different alleles from both polymorphisms, we analyzed the allelic combinations in relation to the clinical data of patients. Interestingly, the patients with major alleles at both sites, were less likely to have apical hypokinesia and Diabetes Mellitus (Table 6). Moreover, patients with minor alleles at both sites were more likely to show a familial history of MI (Table 6).

3.3. Associations between polymorphisms of the IL17A G197A and NFKB1 -94 ins/del ATTG with CXCL1 chemokine levels

We investigated the association between plasma levels of CXCL1 and the studied polymorphisms in patients and controls (Fig. 3). Due to the low number of patients in each cluster of genotype and scattered branches in the plasma CXCL1; we used Kruskal-Wallis test for data analysis. We found a significant correlation between plasma levels of CXCL1 and *NFKB1* -94 ins/del ATTG polymorphism in patients but not controls (P = 0.01, Fig. 4).

4. Discussion

In this study, we found that patients with BMI lower than 25 had increased minor allele (del) of *NFKB1* -94 ins/del polymorphism compared to patients with high BMI (equal or more than 25). Previous studies have shown that lower BMI in individuals with a heart attack is associated with worse prognosis and significantly higher mortality (Haridasan et al., 2016; Oreopoulos et al., 2008). On the other hand, it is suggested that fat adipose tissue produces hormones that may have a protective effect on heart function (Kershaw and Flier, 2004). A recent study showed that individuals with cardiovascular disease who have high plasma levels of leptin (more than 2000 pg/ml) and BMI higher than 28 have a better prognosis than others (Simiti et al., 2016). Therefore, the association of *NFKB1* del allele with lower BMIs is noteworthy and can be evaluated as a potential prognostic factor in future studies. We also found that the patients who underwent cardiac artery bypass graft (CABG) surgery had significantly higher frequency of minor allele of *NFKB1* -94 ins/del polymorphism. CABG is used commonly in individuals who have stenosis in more than two coronary

Table 5
The Associations between clinicopathological manifestations of MI and the frequencies of NF-κB1-94 ins/del in patients (only significant associations are shown).

Clinical manifestations (N)	NF-KB1-94 ins/del N (%)			χ2 P	OR	Regression P	NF-KB1-94 ins/del N (%)		χ2 P	OR	Regression P
BMI [104]	ins/ins	ins/del	del/del	0.09	ins/ins: ref	ins/del: 0.06	ins	del	0.04*		0.048*
≤ 24.9 (52)	19 (36.5)	24 (46.2)	9 (17.3)		ins/del:0.41 (0.16–1.05)	del/del: 0.11	62 (59.6)	42 (40.4)		Reference	
> 25 (52)	30(57.7)	17(32.7)	5 (9.6)		del/del:0.33 (0.08–1.30)		77 (74)	27 (26)		0.52 (0.27–0.99)	
Hypertension (155)	ins/ins	ins/del	del/del	0.04*	ins/ins: ref	ins/del: 0.10	ins	del	0.01*		0.078
negative (108)	58 (53.7)	40 (37)	10 (9.3)		ins/del:2.39 (0.85–6.71)	del/del: 0.12	156 (72.2)	60 (27.8)		Reference	
positive (47)	15 (31.9)	24 (51.1)	8 (17)		del/del:3.05 (0.74–12.48)		54 (57.4)	40 (42.6)		1.85 (0.93–3.68)	
Previous revascularization (169)	ins/ins	ins/del	del/del	0.14	ins/ins: ref	ins/del: 0.42	ins	del	0.03*		
No (144)	71 (49.30)	58 (40.3)	15(10.4)		ins/del:1.61 (051–5.04)	del/del: 0.26	200 (69.4)	88 (30.6)		Reference	
PCI (20)	9 (45)	9 (45)	2 (10)		del/del:2.34 (0.53–10.43)		27 (67.5)	13 (32.5)		1.16 (0.51–2.66)	0.73
CABG (5)	0 (0)	3 (60)	2 (40)				3 (30)	7 (70)		5.49 (1.05–28.80)	0.044*

vessels as opposed to PCI procedure in individuals with stenosis in one or two arteries that supply blood to the heart.

We found that patients with hypertension were more likely to have del (minor) allele of NF-κB1 -94 ins/del. Del allele and del/del genotype are associated with increased inflammation (Koc et al., 2014). Previous studies have shown the relation between blood pressure and immune inflammation. Angiotensin II, a member of the blood pressure modulating renin-angiotensin system, induces the expression of NF-κB (Phillips and Kagiyama, 2002; Benigni et al., 2010). Increased activity of renin-angiotensin system due to inflammatory conditions or genetic background are shown to be associated with MI (Schieffer et al., 2008). Angiotensin II directly affects blood vessels and can cause generation of neoantigens recognized by T cells, can induce synthesis and release of IL-6 by macrophages and accumulation of white blood cells in the plaque or kidneys which significantly reduces in IL-17A −/− mice (Haridasan et al., 2016; Oreopoulos et al., 2008; Schieffer et al., 2008). Therefore, genetic variation in the genes downstream of important inflammatory/regulatory responses, such as NF-κB1, is the common trail in many pathways contributing to MI. In this regard, we also found that patients with del/del genotype produced significantly higher levels of CXCL1. Del allele and del/del genotype are associated with increased IL-6 levels (Koc et al., 2014). A recent study found that patients with coronary artery disease carrying del/del genotype have higher serum levels of IL-6 compared to those with other genotypes (Lai et al., 2015). IL-6 and CXCL1 are target genes of NF-κB transcription factor, thus −94 ins/del ATTG functional polymorphism of NF-κB1 can be effective in expression and plasma levels of both cytokines among many others.

Our results indicated a trend of increase in the frequency of the minor allele of the *IL17A* G197A in MI patients with apical hypokinesia and also those with left ventricular hypertrophy (LVH). Hypokinesia is defined as decreased movement in some parts of heart walls within each heartbeat. An inadequate blood supply leads to wall motion abnormalities; a condition such as myocardial infarction. Hypokinesia can reduce ejection fraction, cardiac output and by reducing the blood supply to the organs can cause organ failure. Patients with wall motion abnormality have lower cardiac output and higher CRP than individual without the disorder (Savoia and Schiffrin, 2007). Also increased levels of acute phase CRP protein and soluble form of tumor necrosis factor receptor in patients with LVH are reported (Takei et al., 2009; Bo et al., 2012; Salles et al., 2007). The A allele in this polymorphism is associated with higher promoter activity which may participate in production of higher levels of IL-17A and sustaining inflammation. Interestingly, as shown in Table 6, the presence of both minor alleles (del/A) in an individual significantly increased the risk of apical hypokinesia compared to all other combinations. These results once again underscore the importance of inflammatory IL-17A pathway in the pathogenesis of MI (Cicala et al., 2007).

Cytokines related to the innate and adaptive immune responses have critical roles in the inflammatory responses that predispose individuals to cardiovascular diseases (Zheng et al., 2016). Myocardial infarction is one of the deadly consequences of cardiovascular dysfunction and IL-17A pathway appears to be involved in both early and late inflammatory responses leading to myocardial infarction and ischemic reperfusion injury. This cytokine causes activation of NF-κB transcription factor which is the main transcription factor involved in inflammatory responses. Our study showed that variations in the genes upstream and downstream of IL-17A pathway may play a role in the severity or predisposition to MI.

Table 6
The Associations of clinicopathological manifestations of MI with combination of *IL17A* G197A and *NFKB1*-94 ins/del allele frequencies in patients (only significant associations are shown).

Clinical manifestations	*IL17A* G197A/NF-KB1-94 ins/del N (%)				$\chi 2$ P	OR (CI 95%)				Regression P
Apical hypokinesis	ins/A	ins/G	del/A	del/G	0.02*	ins/A	ins/G	del/A	del/G	ins/A: 0.006*
Negative	54 (23.5)	103 (44.8)	14 (6.1)	59 (29.6)		323.83 (0–132)	1039.40 (15.91–67890)	reference	92.905 (1.25–6880)	ins/G: 0.001*
Positive	7 (31.8)	6 (27.3)	5 (22.7)	4 (18.2)						del/G: 0.039*
Diabetes Mellitus					0.12					ins/A: 0.278
Negative	55 (23.9)	106 (46.1)	15 (6.5)	54 (23.5)		27.58 (0.07–11117)	471.4 (1.36–197355)	reference	4.22 (0.01–1681)	ins/G: 0.046*
Positive	23 (29.4)	24 (30.9)	8 (10.3)	23 (29.4)						del/G: 0.637
Familial History of MI					0.18					ins/A: 0.012*
Negative	58 (26.3)	92 (41.9)	12 (5.5)	58 (26.3)		60.749 (2.46–1499)	27.153 (1.35–545)	reference	17.635 (1.11–280)	ins/G: 0.031*
Positive	20 (22.2)	39 (43.4)	11 (12.2)	20 (22.2)						del/G: 0.042*

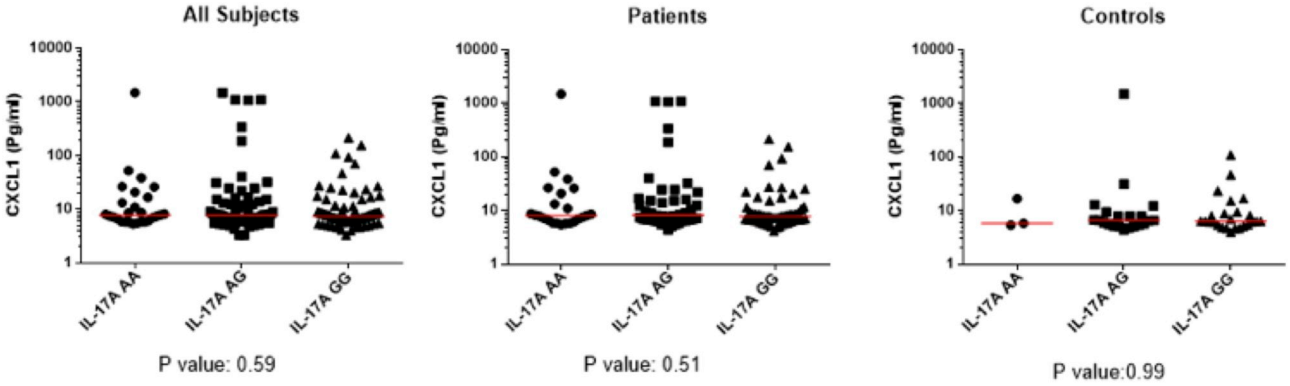

Fig. 3. Comparison of CXCL1 plasma levels based on the three genotypes AA (n = 27), AG (n = 64) and GG (n = 82) of *IL17A* G197A polymorphism.

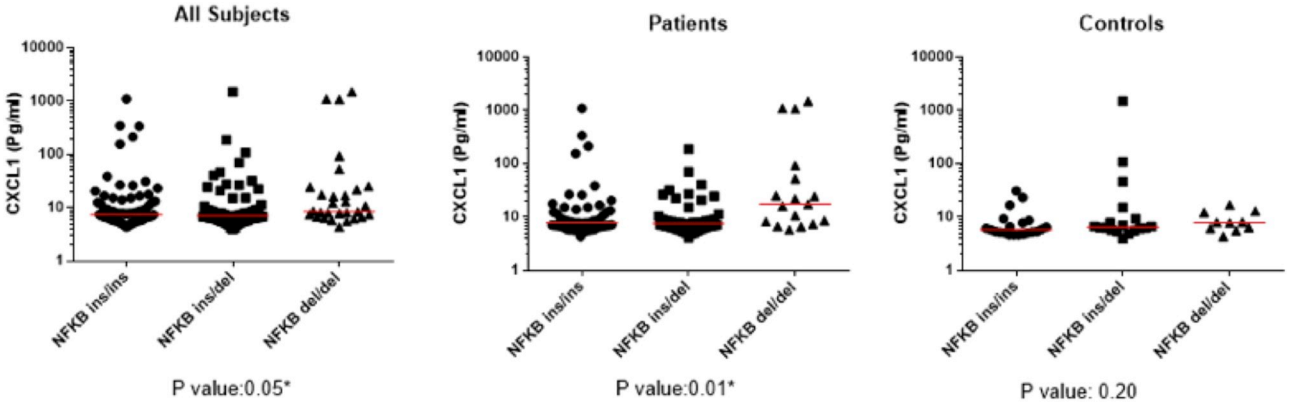

Fig. 4. Comparison of CXCL1 plasma levels based on the three genotypes ins/ins (n = 75), ins/del (n = 64) and del/del (n = 29) of *NFKB1*-94 ins/del ATTG polymorphism.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.obmed.2019.100098.

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