Introduction: The polymorphisms of beta-fibrinogen gene are now suggested to affect plasma fibrinogen levels and the risk for coronary artery disease (CAD).

Objectives: The present study aimed to evaluate the association between the two polymorphisms of G\A455 and C\A148 with beta fibrinogen gene and the increased risk for premature CAD among a sample of Iranian population.

Patients and Methods: This case-control association study was conducted on 100 consecutive patients suffering premature coronary artery disease as the cases and 100 healthy individuals without any evidence of coronary involvement. Patients were randomly selected and adjusted for gender and age. Determining different genotypic patterns of the G\A455 and C\A148 SNPs were carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis.

Results: The mean level of serum fibrinogen was 354.32±93.85 mg/dL in the case group and 303.43±84.51 mg/dL in the control group, indicating the abnormally increased level of fibrinogen in 68% and 30% respectively with a significant difference (P<0.001). Overall, the mean level of fibrinogen was significantly higher in the smoker group than in the non-smoker group (P=0.032). Regarding the frequency of C/T148 alleles, in the case group, 82% had C allele and 18% had T allele of the polymorphisms. The rate of these alleles in the control group was 83% and 17% respectively with no significant difference (P=0.852). Similarly, the frequency of the alleles A and G of G\A455 polymorphism was 87% and 13% in the case group and 89% and 11% in the control group respectively with no significant difference (P=0.663).

Conclusion: There is no association between G\A455 and C\A148 polymorphisms with beta-fibrinogen gene and presence of CAD. However, the increased level of serum fibrinogen can effectively predict CAD.

Core tip: A case-control association study was performed on 100 consecutive patients – suffering premature coronary artery disease as the case group – and on 100 healthy individual. We found no association between G\A455 and C\A148 polymorphisms with beta fibrinogen gene and presence of coronary artery disease.

Introduction: Coronary artery disease (CAD) is the first cause of death in people older than 35 years in developed countries (1). Preventing CAD and its-related mortality has been identified as a certain dilemma in all countries. CAD is mainly sourced from atherosclerotic process. Along with traditional risk factors for CAD including hypertension, diabetes mellitus, hypercholesterolemia, smoking, and family history of disease, the studies now attempt to identify new metabolic risk profile for CAD (2-4). Most risk factors for CAD predisposing arterial bed to atherosclerosis can impair arterial endothelial function (5). In other words, endothelial dysfunction as an initial process can activate further pathways such as inflammatory and thrombosis cascade, vascular contraction, and oxidation (6). A variety of components such as endothelial cells, platelets, inflammatory proteins, and fibrotic systems can interfere with hemostatic condition and predispose the vascular bed to plaque formation and atherosclerosis plaque (7). All predisposing conditions for atherosclerosis such as hypertension, hyperlipidemia, smoking, diabetes, and even obesity are linked to endothelial dysfunction. Predisposing conditions induce production of inflammatory cytokines and mediators...
such as interleukin-6, tumor necrosis factor-alpha (TNF-α) which may inactive protection and turnover of endothelial cells. This condition may lead to induce liver adipocytes to synthesis of acute phase proteins such as C-reactive protein (CRP), serum fibrinogen and amyloid (8,9). In addition, endothelial dysfunction can promote platelet adhesion and aggregation that is necessary for plaque formation (10).

Fibrinogen as a precursor for fibrin is the main ring in coagulation pathway. Several studies have shown that fibrinogen is an important and independent risk factor for CAD (11,12). Fibrinogen is currently used as a marker of inflammation (13,14). The increase in the plasma fibrinogen concentration is related to the development of CAD through changes in the mechanisms of platelet aggregation. This condition is due to the influence of plasma fibrinogen on quantity of formed fibrin and its accumulation as well as its connection with evolution of atherosclerotic plaque (15). This condition is also with an increase in blood viscosity and increasing the risk of thrombosis (16). Thus, any mutation in the genes encoding fibrinogen may change the likelihood of CAD. Therefore, polymorphisms of the beta fibrinogen gene have been shown to affect plasma fibrinogen levels and the risk for CAD.

Objectives
The present study aimed to evaluate the association between two polymorphisms of G/A455 and C/A148 ON beta fibrinogen gene and the possible increased risk for premature CAD among a sample of Iranian population.

Patients and Methods

Study population
This case-control association study was conducted on 100 consecutive patients suffering premature CAD referred to and hospitalized in coronary care unit (CCU) ward of Peymanieh hospital between 2015 to 2017 as the cases and 100 healthy individual without any evidence of coronary involvement that were randomly selected and adjusted for gender and age (all subjects ranged 30 to 50 years). The case group had the World Health Organization (WHO) criteria for CAD (positive clinical evidence, cardiac enzymatic changes and significant ECG changes for ischemic heart disease). CAD was also defined as a luminal narrowing of greater than 50% in each coronary artery. In contrast, the control group had no any evidence of typical chest pain, abnormal ECG changes, or family history of cardiovascular risk profiles such as hypertension, hyperlipidemia, hyperglycemia, family history of coronary diseases, obesity, or chronic diseases such as rheumatologic disorders, renal insufficiency, or asthma. The latter group had no history of oral contraceptive pill use or postmenopausal condition. Overall, the exclusion criteria were history of inflammatory, autoimmune or malignant disorders, receiving anti-coagulation drugs, history of valvular repair or replacement, history of cardiac or non-cardiac interventions within the last 3 months, history of myocardial infarction within the last one year or unwillingness to collaborate in the study. In this study, all baseline characteristics including demographics, anthropometric parameters and traditional risk factors for CAD were collected by the patients interviewing or clinical assessment of the participants by the study checklist.

Laboratory assessments
Around 5 cc of venous blood sample was taken for laboratory assessments after 7 hours fasting. Of which 3 cc was used for polymerase chain reaction (PCR) and 2 cc used for determining the level of fibrinogen. The samples for PCR were poured into tubes containing citrate and samples for assessing fibrinogen level in plastic tubes containing 3.2% citrate. To increase the accuracy of fibrinogen measurement, only blood donors were selected that their blood lipids were normalized by a medication or diet at the time of testing. To prevent hemolysis and to improve the precision of collection and preparation of samples, sampling was carried out by an expert staff. The PCR samples were quickly transferred to the freezers at the faculty of medicine near the ice and stored at -200°C. Fibrinogen samples were also delivered to the laboratory in less than 1 hour, where they were sanitized by experienced personnel and centrifuged with 1000 rcf for 20 minutes and the extracted plasma was stored at -200°C till final analysis. The plasma fibrinogen level was measured by class clotting time method and using the TEClot Fib Kit 10 fibrinogen kit. The normal level of fibrinogen was considered to be 200 to 400 mg/dL.

Genetic assessments

The DNAs from the peripheral samples were extracted using the melting method [kit of BIONEER Company (Cat. No.: K-30 32)]. To set-up the PCR, the following components were prepared: 1) Master Mix (consisting dNTPS, DNA polymerase, tracking dye, and reaction buffer) named as the BIONEER AccuPower® and manufactured by BIONEER Company, 2) samples of DNAs (5 to 50 ng), 3) specific primers for C/T148 polymorphism as the forward primer with the sequence of 5’- CCT AAC TTC CCA TCA TCC TGT CCA ATT AA-3’ and the reverse primer with the sequence of 3’-TGT CGT TGA CAC CTT GGG ACT TAA CTA G-5’, and 4) specific primers for G/A455 polymorphism as the forward primer with the sequence of 5’- CAG TTA GTG GGA TTT GGA TTA C-3’ and the reverse primer with the sequence of 3’- GGC TGA ACC ATT TTA TCA A-5’. Determining different genotypic patterns of the SNPs were carried out by restriction fragment length polymorphism (RFLP) analysis on the study population carried out by employing specific designed primers digested by restriction enzyme Hind III (for C/T148 polymorphism) and primers digested by restriction enzyme HaeIII (for G/A455 polymorphism). The digested products were then visualized on 3% agarose gel stained with ethidium bromide.
Ethical considerations
This research was performed following the Declaration of Helsinki principles. Informed written consent was obtained from each patient. All information about individuals was coded and kept confidential. This study was approved by the Committee of Shiraz University of Medical Sciences. This study was conducted as a residential thesis in cardiology by Davar Aldavood (#153).

Statistical analysis
For statistical analysis, results were presented as mean ± standard deviation (SD) for quantitative variables and were summarized by absolute frequencies and percentages for categorical variables. Normality of the data was analyzed using the Kolmogorov-Smirnoff test. Categorical variables were compared using chi-square or Fisher’s exact tests when more than 20% of the cells with expected count of less than 5 were observed. The quantitative variables were also compared with t test or Mann-Whitney U test. For the statistical analysis, SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL) was used. Accordingly, P values of 0.05 or less were considered statistically significant.

Results
Comparing baseline variables across the case and control groups (Table 1) showed no meaningful difference in mean age, gender distribution, and history of cardiovascular risk factors. Regarding the frequency of C/T148 alleles, in the case group, 82% had C allele and 18% had T allele of the polymorphisms that the rate of these alleles in the control group was 83% and 17% respectively with no significant difference (P = 0.852). Similarly, the frequency of the alleles A and G of G/A455 polymorphism was 87% and 13% in the case group and 89% and 11% in the control group respectively with no significant difference (P = 0.663) (Table 2). The mean level of serum fibrinogen was 354.32 ± 93.85 mg/dL in the case group and 303.43 ± 84.51 mg/dL in the control group indicating the abnormally increased level of fibrinogen in 68% and 30% respectively with a significant difference (P < 0.001). Overall, the mean level of fibrinogen was significantly higher in the smoker group than in the non-smoker group (P = 0.032). However the observed difference was only revealed in the control group. In total, the incidence of premature CAD in people with hyperfibrinogenemia was 2.12 times higher than those who have normal fibrinogen. Our study showed no significant association between the level of fibrinogen and the incidence of C/T148 alleles (P = 0.401) or G/A455 alleles (P = 0.365).

Discussion
The concentration of fibrinogen is controlled by genetic and environmental factors. These environmental factors include cigarette smoking, obesity, oral contraceptive pill use, trauma and sedentary lifestyle that increase fibrinogen concentration (17). The degree of fibrinogen inheritance varies between 30% to 50% (18). Theoretically, any gene encoding proteins that contribute to fibrinogen metabolism may have an important effect on the genetic adjustment of the serum fibrinogen level (19). There is evidence that fibrinogen, like fibrin and its products, accumulates in atherosclerotic plaques (20). The amount of this accumulation is proportional to the plasma level of fibrinogen (21) Fibrinogen also affects the potential for platelet aggregation through its effect on a number of specific receptors and blood concentrations (22). Regarding all of these effects, it is not surprising that plasma fibrinogen is a risk factor for coronary heart disease (CHD) (23-25), brain stroke, and peripheral artery disease (26). According to increasing prevalence rate of premature CAD in Iran in recent years and also due to the importance of preventing and anticipating the risk of premature CAD, we aimed to assess the relationship of G/A455 and C/T148 polymorphisms of beta-fibrinogen gene with serum fibrinogen levels and the incidence of CAD. Additionally, according to effect of fibrinogen plasma levels on incidence of CHD and also the lack of enough studies on correlation of CAD with beta-fibrinogen gene polymorphisms in Iran, we also sought to assess the relationship of G/A455 and C/T148 polymorphisms of beta-fibrinogen gene with serum fibrinogen levels.

We first showed an association between the plasma fibrinogen level and the risk for CAD. So far, numerous studies showed the correlation between fibrinogen plasma levels and cardiovascular disease, stroke, and atherosclerosis. As shown by Grywacz et al, an association was revealed between the level of fibrinogen and CAD and its severity (27). Robins et al also indicated that the
level of fibrinogen was higher in those with CAD than in control group (28). Ganda et al also found higher levels of fibrinogen in CAD than in non-CAD groups (29).

However, the results on the association of G/A455 and C/T148 polymorphisms with CAD have been paradoxical. Additionally, the relation between the presence of these mutations and serum level of fibrinogen remains unclear. Most studies have shown that individuals with allele A for 455G/A polymorphism exhibit higher fibrinogen levels (30,31), and in some other articles, the pointed allele was related to a higher level of fibrinogen only in smokers (32,33). However, other studies have stated that this relationship is significant only in non-smokers (34). In spite of these studies, it is not yet known whether increased fibrinogen plasma levels are associated with fibrinogen gene polymorphisms or increase the risk of coronary heart disease. As shown by Schmidt et al, a significant relationship between beta-fibrinogen gene polymorphism and carotid atherosclerosis in a population randomly selected in a range of 45 to 75 years was detected. This relationship was also significant, even in absence of neurological signs. However similar to our study, no association between the pointed polymorphism and fibrinogen plasma concentration was found (35). Behague et al highlighted the relationship between beta-fibrinogen polymorphisms with fibrinogen level and CAD severity was demonstrated (32). Likewise, a study in the United Kingdom, like the present study, showed the relationship between 455G/A and fibrinogen levels. However the result of study was similar in smokers and non-smokers (33-35).

In another study, no evidence of an interaction between cigarette smoking and genotypes was found (36). While increased plasma fibrinogen level is a major risk factor for coronary heart disease, various studies have suggested the hypothesis that the carriers of the allele A of G/A455 are likely to have a higher risk for coronary heart disease (37-39). In contrast, some others could not show this association (40,41). There are four possible reasons for such disagreements; 1) not categorizing people into two groups of smokers and non-smokers, 2) the difference in daily tobacco consumption, 3) the difference in sample size, and 4) the difference in age range of study population.

Conclusion
There is no association between G/A455 and C/A148 polymorphisms on beta fibrinogine gene and presence of CAD. Nonetheless, the increased level of serum fibrinogen can effectively predict CAD.

Limitations of the study
During the research, we encountered some problems such as inconsistencies in implementation and time constraints

Authors’ contribution
MM; participated in research design, the writing of the manuscript, and the performance of the research. MM, SHM and AD; contributed to study design, preparation of the manuscript and final revision. AD; consultant of study. All authors read and approved the paper.

Conflicts of interest
The authors declare no conflict of interest.

Ethical considerations
Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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References
Beta fibrinogen gene and CAD


