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The rs3024839 and rs2227483 Polymorphisms with Immune Pathomechanism Offers a Starting Point for Diagnosis and Susceptibility Testing of Myocardial Infarction

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Abstract

Long-term primary prevention of myocardial infarction faces challenges, but genetic risk assessment may change this dynamic. We sought for genetic risk loci influencing myocardial infarction and underlying pathomechanisms. AS-PCR used for mutation detection (*STAT4*_ rs3024839 and *IL22*_ rs2227483) and confirmed positives by sequencing and Digital PCR. *STAT4* and *IL22* mRNA levels and chromatin accessibility at SNP sites were evaluated. We assessed SNPs for association with myocardial underlying comorbidities as well as their predictive performance ability. The population flow-sorted $CD4^+$ $FOXP3^+$ Tregs and the level of *Foxp3* mRNA were measured and TGF-β1 quantified using ELISA and intracellular staining assay. Immunophenotyping used to identify p53 expression, pro-inflammatory monocytes and circulating endothelial cells. More than 99% samples were positive for mutations. Significant differences in the mutated allele and genotype frequencies were identified at a p value cutoff of 0.05. Analyses identified SNPs as risk factors for comorbid factors with the ability in distinguishing high and low-risk individuals (AUC>0.9). Differentially accessible chromatin regions influencing *STAT4* and *IL22* expression were found in risk loci. Lower circulating

CD4+ FOXP3+Tregs, *Foxp3* expression decline, decreasing TGF-β1 level, increased p53 level, inflammatory state and endothelial dysfunction were further validated. Discovered genotypes open novel opportunities for MI prediction.

Keywords Myocardial Infarction, rs3024839, rs2227483, *STAT4*, *IL22*, pathomechanism

Abbreviations

MI; Myocardial infarction, SNP; Single nucleotide polymorphism, HWE; Hardy-Weinberg equilibrium, ECG; Electrocardiograph, cTnI; Cardiac troponin-I, CK-MB; Creatine kinase MB fraction, STEMI; ST elevation MI, NSTEMI; Non-ST elevation MI, EDTA; Ethylenediaminetetraacetic acid, AS-PCR; Allele-specific polymerase chain reaction, ARMS; Amplification refractory mutation system, dPCR; Digital PCR, FAM; 6-Carboxyfluorescein, VIC; 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein, AUC; Area under the curve, ROC; Receiver operating characteristic, RT-qPCR; Reverse transcription-quantitative PCR, PI; Propidium iodide, TGF-β1; Transforming growth factor-β1, ELISA; Enzyme-linked immunosorbent assay, CECs; Circulating endothelial cells, ORs; Odds ratios, CIs; Confidence intervals, Fst; F-statistics, PCA; Principal component analysis, MFI; Mean fluorescent intensity, CVD; Cardiovascular diseases, GRS; Genetic risk score

Introduction

Up-to-date sobering statistics from the United States and over 10% of the population worldwide (<60 and >60 years old) who experienced heart attack, indicate the reversal of the decreasing trends in mortality and morbidity from myocardial infarction (MI) (1,2). Early declines that have been obtained in the second half of the 20th century are attributable mainly to primary prevention by principal modalities involving therapeutic lifestyle changes and adjunctive drug therapies, but this reduction of adverse events paradoxically has reached a steady-state and in the last years it has not improved significantly (1,3). This could be partially explained by the lack of effective means of identifying individuals at the highest risk for myocardial infarction that has led to the reversal of recent declination on mortality and morbidity of MI and increase of the patients (4). Therefore, corresponding to the previous tremendous success of preventive measures, addressing the primary myocardial infarction prevention through modification of risk factors must continue to be a high priority. Primary prevention, which is designed to commence early in individuals who are asymptomatic, do not develop completely by traditional acquired risk factors as they are age-dependent and are predominantly detected in

the later years of life, which is suboptimal for primary prevention. Therefore modification of MI-risk factors clearly would have to be initiated early in life, and, in some cases, even before adolescence. So if we are to take advantage of the opportunity for primary prevention in MI-predisposed individuals, it would be greatly enhanced with a method of risk detection that detects asymptomatic younger individuals at risk (5,6). With the increasing importance of genetic risk factors in various medical fields, it is vital to evaluate their contribution to disease burden. Importantly, these factors allow for the early evaluation of risk, without dependence on age or other acquired factors. Additionally, it provides the insight to search for clues that will eventually identify the molecular pathways through which the risk is manifested. It has been suggested by geneticists that cardiac events, unlike rare Mendelian disorders, are polygenic in nature and influenced by common variants. Additionally, they hypothesized that these prevalent variants would be single nucleotide polymorphisms (SNP) distributed throughout the genome. SNPs represent the most important and basic form of variation in the genome which are responsible for genetic effects that produce susceptibility to the diseases (5,7,8). Genetic exposures increase the risk of cardiovascular diseases tending to be most impactful in terms of lost healthy life years (9). Therefore, while genetic risk factors are not yet modifiable in practice, knowing the relative contribution of different genetic risk factors on disease burden can help prevent prioritization and design interventions across the lifespan (7). Disease-associated SNPs might affect a sizable number of healthy life years by impact on behaviour of cell signaling (10). To emphasize the importance of this concept, our research looks at two SNPs residing within two genes related to cardiovascular disease—in *STAT4* at 2q32.2 (rs3024839) and in *IL22* at 12q15 (rs2227483). Signal transducer and activator of transcription (STAT) proteins play a central role in heart physiology, e.g. resolution of inflammation by *STAT4*. In addition, IL22 plays a role in inhibiting inflammation in myocarditis, atherosclerosis and MI. On the other hand, the immune–inflammatory axis of *STAT4/IL22* may be implicated in cardiovascular pathogenesis, but its complex interplay remains poorly defined (11-13). We also filtered out previous clinical Association Studies or in vitro analysis with characterizing these variants, however no studies have directly tested this connection and our main review findings emphasize that the biological functions and pathomechanisms of *STAT4* (rs3024839) and *IL22* (rs2227483) SNPs in MI pathogenesis remain uncertain. Nevertheless, relatively few studies have characterized these SNPs functionally in other immune disorders. One of these studies has previously been found that the rs2227483 increase the susceptibility of individuals to develop Atopic Eczema (14).

Additionally, a previous study has indicated that although *STAT4* SNP (rs3024839) is not associated with Type 1 diabetes mellitus in a Brazilian population, it might be associated with this organ-specific autoimmune disease worldwide (15). To deal with this concern, in this work, we have attempted to examine multi-location genetic risk (*STAT4*_ rs3024839 / *IL22*_ rs2227483) in myocardial infarction prediction and more importantly aid in identifying underlying pathomechanisms.

Methods

Study Design and Participants

A case-control study of hospitalized, in-hospital and hospital daycases (with a minimal participants) with myocardial infarction based on a homogenous cohort of Middle East region (South Asian) was conducted at Namazi, Shahid Faghihi, Shahid Beheshti, Al-Zahra and Shahid Hejazi and Kowsar urban Hospitals, as a heart and vascular diseases hospitals affiliated with Shiraz University of Medical Sciences in Shiraz, IRAN. A total of 400 MI patients were selected from the different hospitals and to reduce a bias that may occur when cases and controls differ from the source population, a control group (400 participants randomly assigned) selected from the same source population from which the cases arose. We analyzed MI-hospitalizations and -day care admissions in the consecutive months of 2018 to 2024. We used the STROBE cohort reporting guidelines (16).

Myocardial infarction was confirmed according to the experience of on-duty cardiologists in dealing with MI patients and through recognized by clinical features, including electrocardiographic (ECG) findings (abnormal Q waves in several leads or lead groupings associated with ST deviations or T wave changes in the same leads, as an ST elevation MI (STEMI)), elevated values of biochemical markers (increased cardiac troponin-I (cTnI, median level; 5.3 ng/mL) level plus elevated CK-MB (Creatine kinase MB fraction, average level; 33.11 IU/L), as an non-ST elevation MI (NSTEMI)), and by imaging through echocardiography. Patients with a median age of 57.5 ± 8.5 years were eligible for the study. Patients with ECG abnormalities that mimic MI or whose echocardiography had recognized the abnormal regional myocardial motion and thickening due to one or more of several other conditions (such as mental health disorders), were excluded. Moreover, we analyzed only patients with the first heart attack. Subjects were excluded from the study if any secondary cause of MI was evident, such as trauma, vasculitis and drug use. Other exclusion criteria were defined as severe renal impairment, congenital heart defects, known as structural heart disease and electrolyte disturbances, severe anemia, and COPD or other severe respiratory conditions. Demographic characteristics are shown in Table 1.

For a comparison group, one control subject per case (a sum of 400 age-and gender-matched control subjects with the same median 57.5 ± 8.5 years) were selected randomly from the same catchment area. The controls were healthy with no history of cardiovascular and heart disease (even in first-degree relatives), or any other chronic diseases (i.e. the control group comprised participants who do not have any experimental group's underlying comorbidities). To rule out subclinical heart disease in enrolled controls, a combination of non-invasive imaging techniques including CT scan and echocardiography, and assessment of detailed medical history, blood pressure measurement and body mass index. Controls were selected on a contemporaneous basis with cases, so that similar numbers of cases and controls were enrolled during each month of the study.

Islamic Azad University Kazerun Branch Institutional Review Board assured the protection of the rights and welfare of participants, both in advance and by periodic review and informed consent was documented by means of a written, signed and dated informed consent form.

DNA Extraction and Genotyping

Peripheral venous blood samples were drawn from both patients and control subjects and collected in tubes with ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. Blood samples were drawn at the time of hospital admission. Hospital daycases in this study were blood donors with a backup blood sample deposit in the blood bank of Muhammad Rasulullah Research Tower of Shiraz University of Medical Science (Shiraz, IRAN), for Dr. Naeimi's previous research purpose (17). It is worth noting that these blood samples also had been collected immediately upon admission. The samples were then processed through centrifugation at 2000× g for 10 minutes to obtain the buffy coat. The salt precipitation method was employed to isolate DNA from the buffy coats. In elaboration, the buffy coats were resuspended in 15 ml polypropylene centrifuge tubes containing 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na2EDTA, pH 8.2). The cell lysates were subjected to an overnight digestion at 37°C, using 0.2 ml of 10% SDS and 0.5 ml of a proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na2EDTA). After the digestion was completed, each tube received 1 ml of saturated NaCl, approximately 6M. The tubes were shaken vigorously for 15 seconds and then centrifuged at 2500 rpm for 15 minutes. After the protein precipitation, its pellet remained at the bottom of the tube, and the supernatant containing the DNA was transferred to a new 15 ml polypropylene tube. Room temperature absolute ethanol was added at 2 volumes and the tubes were inverted repeatedly until the DNA precipitated. The precipitated DNA strands

were then collected using a pipette and moved to a 1.5 ml microcentrifuge tube containing 100-200 microliters of TE buffer (10 mM Tris-HCl, 0.2 mM Na2EDTA, pH 7.5). Prior to quantitating, the DNA was allowed to dissolve at 37°C for at least 1 h following extraction. The DNA concentration was measured using a nanodrop spectrophotometer (Biochrom WPA Biowave II, UK). A 260/280 ratio of ~1.8 was used to determine the purity for DNA. The purified DNA was kept in a −80 °C freezer until it was utilized for the purpose of DNA genotyping (18).

DNA samples were genotyped for two selected SNPs), who were satisfied the study eligibility criteria, MAF> 0.01, call rate > 0.95, and presence of Hardy-Weinberg equilibrium (HWE) (p value> 0.05, after Bonferroni correction), using Allele-specific polymerase chain reaction (AS-PCR), also known as amplification refractory mutation system (ARMS) method. Using Oligo7 software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA), primers were designed (Table 2) and their uniqueness assessed via the NCBI BLAST® search engine. The PCR was run on a thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf, Hamburg, Germany), with a final volume of 22 µL for all PCR protocols (19,20).

The first round PCR comprised of 11µL of 2× Master Mix Red (Ampliqon), 1 µL (10 µM) of Forward and Reverse- PCR-Control primer of each gene (a pair of control primers which could not amplify mutant DNA at a given locus was used to confirm that the genomic DNA is, in principle, amplifiable), 8 µL DNase-free water and 1 µL of genomic DNA for the amplification of each gene. After the amplification, 1 µl of PCR products from the primary round was used in the second round PCR amplification (AS-PCR) as a template using forward allele-specific primers and reverse common primer. AS-PCR was performed in a 22 µl reaction mixture consisting of ingredients similar to the first round PCR except for the use of 7 µL DNase-free water. The PCR cycling for the first and second round were the same (Table 2).

PCR Amplicons Recovery, Direct Sequencing and Digital PCR-Confirmation Assay

Thirty microliters of the PCR mixture, which contained the 388bp and 501bp products, were electrophoresed on a 1.5% agarose gel (Yekta Tajhiz Azma, Tehran, Iran). The gel was prepared in a 0.5 × TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM Na2EDTA) and stained with GelRed. The Montage DNA gel extraction kit (Millipore) was utilized to facilitate the rapid extraction of DNA from the agarose gel matrix. Following the removal of excess agarose, the gel slices containing the PCR products, which were less than 200 mL in volume, were transferred to the Gel Nebulizer within the Micropure separator. The device was capped and subjected to

centrifugation for 10 minutes at 14,000×g. The filtrate obtained from the Micropure was sequenced directly, without any further purification or concentration. Qualitative analysis was repeated by NanoDrop readings to measure the DNA yield and purity.

PCR products were directly sequenced using an ABI3130x sequencer (Applied Biosystems, Foster City, CA, USA) with a sequencing reaction undertaken using a standard Big Dye protocol. The master mix for the sequencing reaction was prepared (Big Dye Master Mix preparation) as described in the Table 3 and a final 20μL sequencing reaction was run on the Genetic Analyzer according to the thermal profile in the Table 4 (18).

For standardization and quality management in PCR runs and sequencing analysis, beyond the considerations of DNA quality and quantity, as well as the quality of reagents, primers, tubes, and instrument calibration, false positives were recognized during the PCR runs and sequencing analysis by utilizing non-target controls, i.e., reagent blanks. If the reagent blank demonstrated amplification of the target DNA, the sample results were classified as invalid.

Digital PCR (dPCR) with the aim to be a confirmation assay of sequencing data was developed according to a standardized protocol, to quantitatively measure the amplification of each allele. In brief, primers and VIC/FAM labeled probes on the target variants were kindly supplied in collaboration with Muhammad Rasulullah Research Tower of Shiraz University of Medical Science (Shiraz, IRAN), and the respective fluorescence of the two alleles (the mutated alleles were labeled with 6-Carboxyfluorescein (FAM), whereas the wild type alleles were labeled with 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC)) was measured to differentiate the amplification of each allele using the QuantStudioTM 3D Digital PCR System (Thermo Fisher Scientific). A total volume of 15 μL of reaction mixture (including QuantStudio 3D Digital PCR Master Mix (9 μL), primer and VIC/FAM probes mix (0.5), DNA (1 μL), and nuclease-free water) was loaded into the chambers on chips and underwent the following amplification conditions: 94°C for 10 minutes, 40 cycles at a gradient annealing temperature (58-64 °C) for 1 minute, and 98°C for 10 minutes (1 cycle), followed by holding at 4°C . In addition, a negative control with nuclease-free water as a template was added. Finally, the QuantStudio dPCR software (Thermo Fisher Scientific, Version 3.0.3) assessed the quality of each PCR.

Quantification of *STAT4* and *IL22* mRNA

The cell pellets, consisting of approximately $1\text{-}2 \times 10^6$ cells, were utilized for RNA extraction using the Pars Tous kit (Pars Tous, Iran, Cat No. A101231). The column-

based RNA purification technique was employed to eliminate any contaminating genomic DNA. One microgram of RNA was immediately reverse transcribed using a cDNA synthesis kit (Pars Tous, Iran, Cat No. A101161). Primers specific for *STAT4* and *IL22* were design by NCBI with the following sequences: *Stat4* forward 5' TGCTTTCCCTTGGCGTTTTG 3' and reverse 5' GCTGCCTCCCAGTCTTGATT 3'; *IL22* forward 5' CGACCAGGTTCTCCTTCCCCA 3' and reverse 5' CAGATTTCTGCAGGGCGGCCA 3' (Metabion, Germany). Relative gene expression quantitation with *GAPDH* (forward 5' AAGCTCATTTCCTGGTATG 3' and reverse 5' CTTCCTCTTGTGCTCTTG 3', Metabion, Germany) as an internal reference gene was performed using the Applied Biosystems Stepone Real-Time PCR System (Applied Biosystems, USA) based on the SYBR Green method. Briefly, 10 µL reactions comprising 2 µl cDNA, 5 µl of RealQ Plus 2x Master Mix Green with high ROX™ (Ampliqon), 0.025 µl of each 10 µM primer and 4.5 µl RNase/DNase-free sterile water (Qiagen) were run under the following PCR conditions: initial template denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and combined primer annealing/elongation temperature specific to individual primers at 60 °C and 58 °C for 30 s, respectively. Triplicate reactions were run for each sample and relative expression was analyzed by using $2^{-\Delta\Delta CT}$ method (21).

Evaluation of Alterations in Chromatin Accessibility at SNPs Loci

The alterations in chromatin accessibility at SNP loci (*STAT4* rs3024839 and *IL22* rs2227483), was measured by quantifying target DNA fragments amplified by the AS-PCR, using flow cytometry. Under a simple and straightforward measurement principle, first, the amplified DNA at the SNP of interest was distributed into a 96-well plate. Flow cytometry is then performed on each well, using a fluorescent dye that binds to DNA (Propidium Iodide), and the amount of fluorescence was measured. This fluorescence is directly proportional to the amount of DNA, and thus, the amount of amplified DNA in each well can was used to infer chromatin accessibility at the SNP location in comparison to control. More amplification was considered greater accessibility, while less was suggested as a closed or inaccessible chromatin conformation. The concentration of propidium iodide (PI) used for staining was kept constant for each sample and across experiments.

Predictive model and evaluation of the discriminative accuracy of SNPs

A model was constructed that included genetic polymorphisms (rs3024839 and rs2227483), hypertension, hypercholesterolemia, hyperglycemia, coronary artery disease, and smoking, which indicated significant associations with SNPs in Single-SNP analysis. This model was contrasted with a reference model that encompassed demographic and clinical variables. The area under the curve (AUC) of a receiver

operating characteristic (ROC) curve was employed to evaluate whether the genetic model fit the data better than the reference model. ROC curve analysis was conducted using the R package pROC.

Immunophenotyping, FACS and Differential *Foxp3* Expression Analyses

PBMCs were collected from the interface of Ficoll-Paque and diluted plasma and subsequently transferred into sterile 15 ml tubes. The cells were washed twice with PBS at 1,000 rpm for 10 minutes at a temperature of 22°C. After resuspending in PBS, an aliquot was taken for cell counting. 1×10^6 viable cells from each sample were added to a 96-well U-bottom plate, which was then centrifuged at 1,500 rpm and 4°C for 3 minutes to pellet the cells. The supernatant was carefully pipetted away. To each sample, 100 µl of prediluted anti-CD4 antibody CD4 (negative isolation kit, Invitrogen, Carlsbad, CA) was added and mixed by pipetting. The samples were incubated on ice for 45 minutes. The cells were washed twice with FACS wash and centrifuged at 1,500 rpm for 3 minutes, after which the supernatant was carefully discarded. For the staining of FOXP3, cells were transferred into 1.5 ml microcentrifuge tubes. They were fixed using 1 ml of 1× BioLegend FOXP3 Fix/Perm solution (FOXP3 staining kit, BioLegend) for a duration of 20 minutes at 22°C in the absence of light. Following fixation, the cells were centrifuged at 1,100 rpm for 5 minutes at 22°C, and the supernatant was discarded. The cells were then washed once with 200 ml of FACS wash, with the supernatant being removed. Subsequently, the cells underwent a second wash with 1 ml of FOXP3 Perm buffer at 1,100 rpm for 5 minutes at 22°C, after which the supernatant was discarded. The cells were resuspended in 1 ml of FOXP3 Perm buffer and incubated for 15 minutes in the dark at 22°C. After centrifugation at 1,500 rpm for 5 minutes at 22°C, the supernatant was removed. The cells were then resuspended in 100 ml of Foxp3 Perm buffer, to which 5 ml of anti-FOXP3 antibody was added. This mixture was incubated for 30 minutes in the dark at 22°C. The cells were washed twice with 200 ml of FACS wash (1,500 rpm for 5 minutes at 22°C) and then resuspended in 200 ml of FACS wash, where they were stored at 4°C in the dark until data acquisition. Prior to data acquisition, the cells were filtered through a 50 mm nylon mesh into 5 ml FACS tubes. Cells (at least $1–2 \times 10^5$ events) were analyzed and sorted on FACSCalibur flow cytometer, BD Biosciences (19).

All the above-mentioned reverse transcription-quantitative PCR (RT-qPCR) processes were reiterated for quantifying the level of *Foxp3* expression was additionally done to confirm cytometric data. The primers for PCR were: *Foxp3* forward 5′- ACCTGGAAGAACGCCATC -3′ and reverse 5′- TGTTCGTCCATCCTCCTTTC -3′ (Metabion, Germany).

Determination of TGF-b1

Here, we developed two most popular assay methods to measure Transforming growth factor- β1 (TGF-β1). For intracellular staining, single-cell suspensions were prepared and were fixed and permeabilized with the Intracellular Staining Kit (eBioscience). Cells were then incubated with desired antibodies which were kindly supplied in collaboration with Muhammad Rasulullah Research Tower of Shiraz University of Medical Science (Shiraz, IRAN). Data were acquired from gated CD4+ FOXP3+Tregs on a FACSCalibur flow cytometer (BD Biosciences, USA) and analyzed with FlowJo software. TGF-β1 was then measured in serum using the sandwich enzyme-linked immunosorbent assay (ELISA) method according to a standardized protocol (Fine Test Human TGF-β1 Kit, Wuhan Biotech Co., Ltd., Wuhan, Hubei, China). Absorbance was then read at 450 nm (OD450) by a microplate reader (Bio-Rad).

***IL22*_ rs2227483 carrier patients evaluation for *p53* expression**

For determining p53 protein expression in samples using flow cytometry, briefly, the cells were incubated at room temperature for 10 min using a permeabilization solution, which consisted of 1 mL of 4% paraformaldehyde in PBS (final concentration of 2%) mixed with 1 mL of a 1:10 dilution of Becton Dickinson's FACS lysing solution in distilled water. After incubation, the cells were centrifuged for 5 min, the supernatant was discarded, and the cells were washed (Tween 20/PBS) and centrifuged again for 5 minutes. Once fixed and permeabilized, the cells were incubated with 10 µL of anti-p53 monoclonal antibody (DO-7) directly conjugated to FITC for 30 minutes. After incubation, two sequential washes with Tween 20/PBS were carried out to remove any unbound antibodies. Finally, the cells were resuspended in 500 µL of 1% formaldehyde in PBS and prepared for flow cytometric analysis. Data acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences, USA), and subsequent analyses were carried out utilizing FlowJo software.

Synergy-interacted cofactors evaluation

Inflammation assay

Regarding the inflammation, pro-inflammatory monocytes were distinguished using the CD14 and CD16 markers. To elaborate, PBMCs were washed, centrifuged, and immunolabeled for 30 minutes at 4 °C with FITC-conjugated antibodies. The samples were then promptly analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA), with data interpretation carried out through Cell Quest Pro software.

Detection of circulating endothelial cells

In this study, circulating endothelial cells (CECs) were analyzed by flow cytometry applying CD146 and CD45 antibodies. Firstly, samples underwent an erythrocyte-lysis step, after which the washed pellet was resuspended and combined with the antibodies. Following this, the samples were incubated for 30 minutes at 4 °C, washed using 2 ml of Stain Buffer (BD Biosciences), and subsequently resuspended in FACSFlow solution (BD Biosciences). A specific gating strategy during analysis was employed to quantify CECs, characterized by their expression of CD146 while being negative for CD45.

Statistical analysis

The Kolmogorov Smirnov test was applied to determine normal distribution of data for statistical analysis. Statistical power was assessed using QUANTO (Version 1.2.4; University of Southern California, Los Angeles, CA, USA, http://biostats.usc.edu/Quanto.html) and HWE test for Hardy-Weinberg equilibrium. The strength of the association was evaluated by determining the level of genetic diversity in the population through F-statistics (Fst), which indicates the ratio of observed to expected heterozygous genotypes. A principal component analysis (PCA) was conducted to further examine population structure and identify individuals with shared ancestry. The data were analyzed using the R package smartsnp. Chi-square (χ^2) test was used to compare the groups regarding genotype and the allele frequencies. Logistic regression analysis was performed to estimate odds ratios (ORs) and 95% confidence intervals (CIs) regarding the association between MI and the presence of each polymorphism. Furthermore, a Bonferroni correction for multiple testing was implemented to assess statistical significance at an adjusted p value threshold (p value < 0.05). We adjusted multiple logistic regression models for confounder variables. The expression differences between groups were examined using the Wilcoxon Rank-Sum Test for two groups, with Graph Pad Prism 8 software (GraphPad Software, La Jolla, CA, USA). FlowJo and Cell Quest Pro software was used to generate Flow data plots.

Results

Sample Size and Statistical Power Estimate

Results showed that data were not normally distributed as Kolmogorov–Smirnov test (Asymp. Sig. (2-tailed) = 0.0039) was statistically significant, that is, data were considered with non-normal distributions. We conducted a power analysis with QUANTO v.1.2.4 software to determine if the sample size of 800 individuals was sufficient for detecting significant associations with the MI phenotype. The findings from our statistical power estimation demonstrated that a sample size of 800

achieves a power of 0.89 for identifying associations between *STAT4* rs3024839 and *IL22* rs2227483 with MI in both MI and non-MI groups (Fig.1). This power level is generally recognized as adequate, exceeding the 80% threshold, and is sufficient for uncovering associations between genotype and phenotype.

Testing for Hardy–Weinberg Equilibrium and F-statistics

The HWE test was used to compare the actual genotypes with the expected number based on Hardy-Weinberg equilibrium theory to determine whether the population was in Hardy–Weinberg equilibrium. Under HWE for controls and no HWE for cases, the analysis showed that the deviation from HWE at the polymorphic loci is not significant, thereby confirmed the presence of Hardy-Weinberg equilibrium at the polymorphic rs3024839 (HWE p value $_{Control}$= 0.39) and rs2227483 (HWE p value $_{Control}$= 0.81) sites. We also evaluated separately HWE in the cases. Interestingly, the p-values of the two tests were highly similar (p value $_{rs3024839}$ = 0.41 & p value $_{rs2227483}$ = 0.76), so reporting HWE p values, stated that no deviations from HWE were observed in any of two data sets and both case and control groups are in HWE.

Using the fixation index (Fst), the genetic differentiation was measured to evaluate the relationship of subpopulations. Result of inter population genetic structure FST for polymorphic loci averaged 0.13. Overall, these values (HWE & Fst) suggest a low level of population differentiation. Moreover, the PCA result indicated that sampled individuals comprised one population with no apparent sub-structure (Fig.2). Some outliers could represent migrants.

Genotypic and allelic frequency at rs3024839 in *STAT4* and their association with MI

The PCR effectively amplified a 388 bp amplicon from the *STAT4* gene. Analysis of the data revealed significant differences in both genotypic and allelic frequencies at the rs3024839 locus in *STAT4* when comparing the case and control groups (p=0.004). The frequency of the C allele for the respective polymorphism was statistically higher in the patient group. It is noteworthy that the case group displayed a greater prevalence of the heterozygous (TC) and homozygous mutant alleles (CC) genotypes, while the control group showed a higher occurrence of the homozygous (TT) genotype. The odds ratio (OR) of 2.94 suggests that individuals in the case group have a 2.94 times greater risk of developing myocardial infarction compared to those in the control group (Table 5).

Genotypic and allelic frequency at rs2227483 in *IL22* and their association with MI

The PCR generated a 501 bp amplicon from the *IL22* gene. Analysis of genotypes and risk allele frequency distribution unveiled significant variation in allelic and genotypic frequencies at rs2227483 in *IL22* when comparing patients and controls (p

< 0.001). Notably, the frequency of the A allele of s3218536 was statistically higher in the patients. Moreover, the frequency of heterozygous alleles (AT) and homozygous mutant alleles (TT) were higher in MI patients, whereas homozygous wild type (AA) was more prevalent in the control group. The OR values are indicating that cases have 2.56 fold more risk than controls to develop MI (Table 5).

Direct sequencing of PCR products

As part of the test validation process and post-genome-wide association study analysis that enhanced the capacity to unscramble the potential biological role of danger loci in myocardial infarction, Sanger sequencing was undertaken in order to verify these pathological variants. Direct sequencing results confirmed the homozygous mutant genotype C/C of rs3024839 (in 21 samples), homozygous mutant genotype T/T of rs2227483 (in 70 samples), heterozygous mutant genotype T/C of rs3024839 (in 276 samples) and heterozygous mutant genotype A/T of rs2227483 (in 164 samples), in the affected MI subjects (Fig.3).

We next utilized dPCR as a comparison technique to further validate the accuracy of sequencing data. By comparing the results obtained by both dPCR and sequencing, we observed a concordant result in 99% of samples. In elaboration, all sequencing-homozygous and heterozygous mutations-positive samples were also considered positive by dPCR, while dPCR detected rs3024839 C/C mutation in 23 samples and rs2227483 T/T mutation in 73 samples, i.e., in 2 and 3 additional specimens respectively that were considered wild-type according to sequencing analysis (Fig.4).

Association of SNPs with MI-determinants and -underlying comorbidities

Of the heterozygous and homozygous patients (TC *rs3024839*, AT *rs2227483*, CC *rs3024839*, TT *rs2227483*), 298 had hypertension, 322 had hypercholesterolemia and 317 had hyperglycemia. 258 patients suffered from coronary artery disease and 202 subjects were former smokers (Table 6). The unadjusted regression analysis indicated that the following determinants and underlying comorbidities were found to be significantly associated with the occurrence of the myocardial infarction in patients with TC *rs3024839*, AT *rs2227483*, CC *rs3024839*, TT *rs2227483* genotypes: hypertension (OR: 2.9 , 95% CI: 1.21–4.59, p value=0.004), hypercholesterolemia (OR: 3.01, 95% CI: 1.73–3.52, p value < 0.0001), hyperglycemia (OR: 3.07, 95% CI: 1.93–5.15, p value < 0.0001), coronary artery disease (OR: 2.6, 95% CI: 1.11–2.23, p value < 0.0001) and smoking history (OR: 1.6, 95% CI: 1.1–2.39, p value=0.001). The multivariate adjusted analysis confirmed that these factors, hypertension (OR: 3.37 , 95% CI: 1.39-6.74, p value=0.006), hypercholesterolemia (OR: 2.78, 95% CI: 1.43-5.44, p value < 0.0005), hyperglycemia (OR: 2.72, 95% CI: 1.31-4.76, p value < 0.0005), coronary artery disease (OR: 2.02, 95% CI: 0.92–4.43, p value < 0.0005) and smoking history (OR: 1.07, 95% CI: 0.59–2.03, p

value < 0.001), were associated with the occurrence of the myocardial infarction in patients (Table 6).

Expression Quantitative Trait Loci

The reference gene, *GAPDH*, was checked to see if it amplified genomic DNA. This step ensured that the amplification observed was not due to genomic DNA in the RNA-purified sample. No amplification was found for this gene. Furthermore, the specificity was tested using PCR and gel separation, which confirmed a single band at the expected size of each amplicon. Analysis of relative gene expression data using RT-qPCR and the $2^{-\Delta\Delta CT}$ indicated that *STAT4* expression was elevated in the MI patients, compared with that in the normal controls. The graph in Fig.5A shows that *STAT4* expression in MI patients is > 2 fold higher than in healthy controls. This increase is significant (p value ≤ 0.0001**), indicating that the patients have greater expression levels compared to normal subjects. *IL22* showed significantly lower levels of expression compared to the control group (p value ≤ 0.001*). The $2^{-\Delta\Delta Ct}$ data revealed that *IL22* levels in MI patients were reduced by < 0.69 fold compared to healthy individuals (Fig.5B). There was a connection in gene expression levels between STEMI and NSTEMI for both *STAT4* and *IL22*.

Chromatin accessibility was analyzed via flow cytometry (Fig.6,subfig.A&B). In quantifying both amplified DNA, the mean fluorescent intensity (MFI) changed compared to control, signifying a chromatin state modification. In other words, a significant increase of MFI (p value = 0.021) indicated the accessibility of chromatin at the rs3024839 location while from the significant decrease of MFI at rs2227483 location (p value = 0.002), the condensation was inferred. Taken together, our findings can infer that the differential expression of *STAT4* and *IL22* correlate with increases and decreases in chromatin accessibility in rs3024839 and rs2227483 loci, respectively.

Predictive analysis: ROC curve

The ROC curve results for each model are depicted in Fig.7. ROC curves analysis results demonstrated the genetic model capacity to differentiate between MI patients and controls (excellent predictive value, AUC>0.9), compared with the reference model (good predictive value, 0.8<AUC<0.9).

Regulatory T cells density and deciphering of *Foxp3* expression in SNP carriers

Myocardial infarction is linked to immune system problems. Research shows that low levels of $FOXP3^+$ T cells can significantly affect how the disease progresses. *STAT4* negatively affects the development of $FOXP3^+$ T cells by reducing *Foxp3* expression (22). The exact mechanism by which *STAT4* limits this expression is not fully understood. Additionally, lower levels of *IL22* have been linked to the density

of FOXP3⁺ cells (23). We looked into whether changes in *STAT4* and *IL22* affected the expression of *Foxp3* and the amount of FOXP3⁺ Treg cells. We performed immunophenotyping on blood samples from healthy individuals and those with myocardial infarction. The results showed significant differences in the number of FOXP3⁺ Treg cells between the two groups. In elaboration, flow cytometry and RT-qPCR tests revealed that FOXP3+ cell count and Foxp3 expression decreased in both males and females. In terms of changes in FOXP3⁺ cells counts, with no gender influences, seen a smaller proportion of FOXP3⁺ cells in subjects (men; 8.14% & women; 10.55%) compared to controls (men; 26.01% & women; 22.81%) (Fig.8,subfig.A&B). Moreover, RT-qPCR revealed almost the same reduction of *Foxp3* expression (expression fold-change $_{male} < 0.63$; p value $_{male} \leq 0.001$, expression fold-change $_{female} < 0.54$; p value $_{female} \leq 0.001$) between control and MI patients for both genders (Fig.8,subfig.C). These findings override sex differences and suggest that SNPs in the *STAT4* and *IL22* genes lead to a lower density of circulating FOXP3⁺ cells.

In addition, we analyzed TGF-β1 concentration and found the decreased efficiency of TGF-β1 signaling is related to the deficiency of *Foxp3* expression and FOXP3+ regulatory T cells differentiation in cases compared to controls. There is scientific evidence to suggest that the enforced expression of *STAT4*, such as that resulting from the rs3024839 SNP, can lead to partial insufficiency of TGF-β1 in inhibition of *IFN-γ* expression. Consequently, *IFN-γ*, via *STAT1*, can suppress the *Foxp3* induction caused by *TGF-β1* (24-26). Both quantitative assays showed a good level of concordance with each other and allowed a reliable result in the level of the TGF-β1 being measured. From the flow cytometric results of selected (gated) population of cells (Fig.9A) that reveal a decline in fluorescence intensity in MI samples compared with the control samples, It can be seen that the intracellular TGF-β1 levels were lower in the cases than those in the control group (Fig.9B). In addition, comparing serum TGF-β1 levels of cases with controls revealed a significant difference between groups (p value = 0.001) (Fig.9C). These analyses suggested a negligible sex difference in TGF-β1 level. This data help to confirm that *STAT4* dysregulation limits *Foxp3* expression and FOXP3+ regulatory T cells development.

Increases of p53 in response to *IL22* down-regulation

We employed p53 level increases as a response to *IL22* down-regulation effect of rs2227483 SNP and its pathogenic power, as it has evidenced that the down-regulation of *IL22* directly increases the expression of *p53* and thereby exacerbating MI through myocardial apoptosis (as apoptosis correlated with the clinical severity of heart failure) (27,28). In the present study, the level of p53 was higher in MI

patients (both genders) compared to the control group (Fig.10). We have hypothesized that an increased p53 rate in MI can reflect the pathogenic effect of the affected *IL22* gene. Moreover, although calling for more evidence is needed, rs2227483 SNP, appears to have also the potential to be a biomarker for reporting on p53-dependent apoptosis in myocardial infarction.

Increases of CECs in rs3024839 and rs2227483 SNPs carriers

Ample evidence have indicated that hyperglycemia, inflammation and infection interacted synergistically in the risk of myocardial infarction and endothelial dysfunction is considered one that linked these cofactors in cardiac events, as hyperglycemia is associated endothelial permeability predisposes to inflammation and infection (29). On the basis of these materials, the possible contribution of SNPs in endothelial dysfunction was evaluated in a portion of MI patients with simultaneous suffering from these comorbidities. In this regard, the inflammation was firstly confirmed in SNPs carriers according to the decreased anti-inflammatory monocytes proportion (non-classical) in favor of an increase in the inflammatory monocyte proportion (classical) compared to the control group (Fig.11). No significant gender difference was found. After inflammation confirmation, all carriers who had all three parameters screened for endothelial dysfunction. As predicted, an evident difference was observed in CEC counts comparing controls to both SNPs carrier patients with no gender influences (Fig.12). As previously noted in the literature, in contrast to immature endothelial progenitor cells that originate from the bone marrow, CECs are believed to signify the detachment of mature endothelial cells from the endothelial monolayer due to endothelial injury. Consequently, circulating endothelial cells are regarded as a dependable means of evaluating endothelial function (30).

Discussion

Although recently these improvements are no longer evident, it has been suggested about 25% of the decline in MI mortality has been due to a decrease in its incidence which is attributable mainly to primary prevention, and nearly 75% was due to a reduction in deaths mainly due to more forceful treatment efforts. Although the most important contribution is attributable to modern and effective treatment strategies, prevention of myocardial infarction is highly prioritized, as sudden cardiac death is the first symptomatic event in at least 25% of MI cases (1,3). Seemingly, previous targeting of primary preventive efforts were hampered by insufficient approaches of identifying individuals at the highest risk for an myocardial infarction as most primary prevention efforts have been focused on the major modifiable determinants of risk including social determinants like age, job

status, income, and education, along with risk behaviors such as unhealthy diet, smoking, lack of exercise, and excessive drinking as well as metabolic risk factors like obesity, diabetes, high triglycerides, elevated LDL cholesterol, and low HDL cholesterol levels (4). In many cases, genetic variants impact phenotypes that contribute to disease pathology however no special evidence is available on the application of genomic results in discovering MI prevention targets. Genetic screenings, as noted by Becker et al., focus on populations of asymptomatic individuals, or at subpopulations in which the risk is known to be increased, or in which the specific phase of life merits screening (31). Building upon the genetic screenings, risk prediction based on genetic biomarkers will be extended our understanding that disease risk varies based not only upon acquired factors, but also as a function of inherited factors (e.g., genetic polymorphisms) and tilt the scales for prevention, as the knowledge of an increased risk of a first myocardial infarction might motivate patients and doctors to consider preventive strategies. Khoury and colleagues (32) explain that this process starts after identifying genomic risk factor(s). Its aim is to create potential health applications for use in clinical and public health settings. Ideally, this stage results in a single-gene test or a genome profile that shows high sensitivity, specificity, and predictive value. These tools can help with clinical evaluations like predictive testing, screening, diagnostics, and prognostics. As rapid advances in the genomics of cardiovascular diseases have fostered huge expectations about the future use of detecting susceptibility variants in prevention, diagnosis, and treatment, the contribution of inherited (genetic) disposition was the focus of this research interest. The results demonstrated that with the *STAT4* mutant C allele and *IL22* mutant T allele at 2q32.2 and 12q15 positions, respectively, there are increased levels of *STAT4* and decreased *IL22* expression. In this study an association between altered levels of *STAT4* and *IL22* and risk for development of myocardial infarction was showed and was revealed that these certain SNPs can pass on risk factors such as high blood pressure, high cholesterol, high blood sugar, coronary artery disease and smoking history. Furthermore, these SNPs can lead to endothelial dysfunction, which may predispose patients to synergistic effects of inflammation, hyperglycemia and infection. We have also shown that in the presence of the rs3024839 and rs2227483 SNPs, there are low levels of $CD4^{+}FOXP3^{+}$ Tregs and decreased *Foxp3* expression level. These observations are in line with recent studies demonstrated STAT4 dysfunction may play a role in limiting the antigen-specific expansion of Treg cells (*Foxp3* expression) and those suggested an association between low levels of $CD4^{+}FOXP3^{+}$ Treg cells and lower levels of IL22 and risk for development of coronary events (11,22,23). In

the context of MI, IL22 helps maintain vascular integrity and reduce damage. The studies have found that when IL22 levels are reduced, the protective mechanisms it provides are diminished. Studies using IL22 knockout mice (IL22KO) have shown indicating a heightened inflammatory response. Additionally, the expression of matrix metalloproteinases (MMPs), enzymes that degrade the extracellular matrix, is increased in IL22KO mice, while the expression of tissue inhibitors of MMPs (TIMPs) is decreased. This imbalance in matrix remodeling can weaken the heart's structure and increase the risk of vascular rupture (33). In this study, we also found that the *IL22* downregulation due to rs2227483 polymorphism increases p53 level and likely makes myocardial more sensitive to p53-mediated apoptosis. Various studies have determined that the apoptotic rate correlated with the clinical severity of heart failure. They reported that a strong correlation between the apoptotic rate and macroscopic signs of left ventricular remodeling. Left ventricular remodeling is associated with unfavorable hemodynamic performance and adverse clinical outcomes such as an increasing rate of symptomatic heart failure and sudden cardiac death (28). These findings might represent a new pathomechanism for myocardial infarction that is unrelated to traditional risk factors and is of potential clinical importance because it represents the first evidence from prospective studies that support the notion that Tregs represent a possible target for screening and development of novel interventions in myocardial infarction. In other words, our knowledge of these genetic risk factors and insights on their biologic pathways will be useful in disease prevention efforts and hopefully, by adding them to the traditional risk factors, reaching more effective ways of preventing and controlling MI. Although these variants have not been characterized in prior cardiovascular studies and suggest that they represent novel genetic contributors to MI in the Middle Eastern and South Asian population, the contribution of genetic disposition in prediction of the complex cardiovascular diseases (CVDs) has been the focus of some other research. *MTHFR* polymorphism testing (c.665C → T and c.1286A → C) is often recommended by clinicians when checking for thrombophilia. Research studies, including case–control and cohort studies, have looked at the links between *MTHFR* gene status and several complications. Findings indicated that low MTHFR enzyme activity might lead to high homocysteine levels, which could increase the risk of conditions like venous thromboembolism and coronary heart disease (34). Krarup et al. (35) studied how a genetic risk score (GRS) made up of 45 risk variants affects the occurrence and prediction of myocardial infarction and coronary artery disease in 6,041 Danish people. Their analysis using two models found that the GRS is linked to myocardial infarction based on allele variations. A study by Visel et al.

shows that the risk period for coronary artery disease is important for controlling the expression of cardiac Cdkn2a/b, which is linked to the 9p21 region. This suggests that this area influences how coronary artery disease progresses by affecting how vascular cells multiply. This finding highlights a new mechanism for myocardial infarction that does not relate to traditional risk factors (36). Taken together, these findings along with other prediction studies, may offer a more advanced level of primary prevention interventions for those subjects who are at greater genetic risk. Moreover, present results also aid in the identification of new targets (*STAT4* and *IL22*) for inflammation-targeted therapies which could be useful for reducing MI risk. Hopefully in the near future, advancements in public health practices support the effective use of genomic science. For example, improving prediction models by genetic risk traits related markers (like genetic variation considered in our study), with good discrimination and calibration, can show promising results in the early prediction of MI. Therefore, we conclude and recommend that *STAT* and *IL22* SNPs with high detection rates or low false-positive rates can be considered for improving prediction models at the time of model updating. It is important to emphasize that our study forms a valuable foundation for understanding MI genetic susceptibility, however, there is a need to explore more genes and SNPs related to MI and in future research should consider expanding to diverse world regions to capture more representative patterns. Furthermore, broader investigations corresponding to association between variants and ECG traits and/or angiographic characteristics can generalize findings more effectively. In Addition, the effects of standard treatments like antihypertensives, statins, antiplatelets, and beta-blockers on specific genetic polymorphisms like rs3024839 and rs2227483 are not well-established in the existing literature and the direct influence on these particular SNPs was not a focus of current research, however, it's possible that these SNPs could play a role in how individuals respond to these treatments, so further research is recommended to confirm any links. Besides, the interplay between polymorphic *STAT4* and *IL22* function with infections, inflammation, and diabetes cofactors in MI patients presents a rich field for future research, with the potential to uncover new therapeutic strategies for managing this complex condition.

Conclusion

The both identified genotypes provided considerable discrimination between cases and controls, which recognizes the contribution of genetic information in provision of myocardial infarction prevention services in primary care. Furthermore, the elucidated pathomechanism whereby SNPs mediate their risk may lead to fitting treatment procedures to the unique needs. There is no doubt that the MI prevention

strategies should be linked to the patient's genetic characteristics that may lead a patient to varying clinical consequences and the circumstances surrounding these features. Therefore, based on our results, strategies should focus on providing the framework to the health-care provider who is in the best position to make informed clinical decisions for each of his or her patients. The recognition and implementation of these genetic markers by healthcare providers for their individual patients and policymakers for the health of the general population will contribute to significant improvements in MI prevention as major causes of premature and avoidable morbidity and mortality worldwide.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the approved institutional guidelines of the Islamic Azad University-Kazerun Branch in Iran. All participants signed the informed consent form and their data were anonymized prior to analysis. The Ethics Committee of the Islamic Azad University-Kazerun Branch in Iran approved this study and all experimental protocols (IR.IAU.KAU.REC.1398.045).

Consent for Publication

Not Applicable.

Data availability

The datasets generated and/or analyzed during the current study are available in the [dbSNP] repository [http://www.ncbi.nlm.nih.gov/SNP]" and SNPs can be searched for using the dbSNP ID (rs3024839 (https://www.ncbi.nlm.nih.gov/snp/?term=rs3024839) and rs2227483 (https://www.ncbi.nlm.nih.gov/snp/?term=rs2227483)).

Competing interests

No potential competing interest was reported by the authors.

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Authors' Contributions

N.S., and K.V.K. designed the study and critically reviewed the manuscript. K.N.Z., D.F., M.S., K.H., A.M., A.M., F.A.M., B.G., K.V.K., N.R., K.V.S., and H.E. performed formal analysis. N.S., N.R., and K.V.K. administrated project. K.V.K. wrote the manuscript. Methodology; S.N., K.V.K., and M.S. The final manuscript has been approved by all authors.

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Figure legend

Figure1. Power analysis for rs3024839 and rs2227483 polymorphisms with $\alpha = 0.05$

Figure2. Principal components analysis to identify genetic differentiation among sampled individuals, each point represents one individual, and colors indicate genetic distance between different populations (Persian = Navy Blue Color, Qashqais = Space Blue Color and Lurs = Yale Blue Color)

Figure3. The established PCR's accuracy and specificity were additionally verified using all clinical DNA samples. Sequencing outcomes revealed a 100% concordance rate between the results obtained from PCR and those from the direct sequencing of the PCR products

Figure4. Two-dimensional plots read out from DNA extracted from peripheral venous blood samples by dPCR for presence of the mutant allele and wild type allele of rs3024839 (left graph) and rs2227483 (righ graph) SNPs using FAM & VIC fluorophores. Results demonstrated the presence of mutant (blue) and wild type (red) alleles. Green dots represent chambers with both wild-type and mutant alleles. The yellow dots represent negative chambers for both targets. RFU: Relative fluorescence units

Figure5. The expression of *STAT4* and *IL22* in the MI patients compared with that in the normal controls; the resulting graphs show the significant increase (A) and decrease (B) in expression of *STAT4* and *IL22* in the MI subjects, respectively. The inner reference gene *GAPDH* was used for normalizing the expression levels of genes. The gene expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method. Statistical tests were conducted based on non-parametric Wilcoxon Rank-Sum Test to evaluate differences between groups. A p-value less than 0.05, was considered to be statistically significant. p value ≤ 0.001*, p value ≤ 0.0001**

Figure6. Flow cytometry cytograms (plots of subfig.A) and graphs (subfig.B) with significant mean fluorescence intensity represent chromatin status (rs3024839 p value = 0.021, rs2227483 p value = 0.002). PCR-amplified products at each SNP site labeled with DNA-binding fluorescent dye (PI), were used to analyze chromatin condensation status. Clouds observed in cytograms represent populations of DNA fragments with similar levels of fluorescence. Graph denote means ± Standard Deviation of the mean fluorescence intensity of PI

Figure7. Receiver operating characteristic curves; ROC curves showing the capacity of rs3024839 and rs2227483 polymorphism to discriminate between MI patients and controls (AUC>0.9)

Figure8. SubFigA: Gating strategies for $FOXP3^{+}$ Tregs analysis of men samples; Quarter 1 & 2: Forward Scatter Area vs. Forward Scatter Height; the gates were

created to eliminate artifacts caused by unstable fluidics and cell pairs. After this, dead cells were removed from the process, Quarter 3 & 4: intracellular staining for FOXP3 on gated PBMCs drawn from healthy men (26.01%) and male myocardial infarction patients (8.14%). SubFigB: Gating strategies for $FOXP3^{+}$ Tregs analysis of women samples; Quarter 1 & 2: Forward Scatter Area vs. Forward Scatter Height; the gates were set to allow elimination of artifacts caused by unstable fluidics and cell pairs. The dead cells were then removed from the process, Quarter 3 & 4: intracellular staining for *Foxp3* on gated PBMCs drawn from healthy women (22.81%) and female myocardial infarction patients (10.55%). SubFigC: RT-qPCR used to measure gender-based *Foxp3* expression (p value ≤ 0.001*)

Figure9. Flow cytometry-based intracellular staining and Enzyme-linked immunosorbent assay analyses of TGF-β1. A) FSC and SSC, Flow Cytometry gating for single and live FOXP3+ regulatory T cells; B) Histogram representing intracellular TGF-β1levels in FOXP3+ regulatory T cells; C) Plots show decreased serum level TGF-β1 (p value = 0.001)

Figure10. Level of p53 expression by flow cytometry in male and female MI patients, Upper Panels: The gating strategy for selecting single cells; Lower Panels: Histograms represent the significant enhanced anti-p53 monoclonal antibody-labeled cells in both male (p value <0.01) and female (p value <0.01) MI patients compared to control.

Figure11. Decreased anti-inflammatory monocytes proportion towards an increase in the inflammatory monocyte proportion in interested SNP carriers MI patients, Upper Panel: The gating strategy for selecting monocytes; Lower Panels: Fluorescence graphs, showing: (R1) inflammatory monocytes (CD14++CD16−), (R2) anti-inflammatory monocytes (CD14++CD16+), (R3) anti-inflammatory monocytes (CD14+CD16++), rs3024839 Carriers vs. control (p value <0.01), rs2227483 Carrier vs. control (p value <0.001)

Figure12. Gating strategy for circulating endothelial cells, gating regions show circulating endothelial cells staining positive for CD146 and negative for CD45. Significant differences in rs3024839 carriers compared to the control group, p value =0.045 ; Significant differences in rs2227483 carriers compared to the control group, p value =0.002

Table legend

Table1. Demographic characteristics of participants

Table2. Designed primers for AS-PCR reaction

Table3. Sanger Sequencing PCR reaction setup components

Table4. PCR thermal-cycling parameters for Sanger Sequencing
Table5. Distribution of genotypic and allelic frequencies of rs3024839 in *STAT4* and rs2227483 in *IL22* gene among cases and controls and their possible association with myocardial infarction
Table6. Analysis of the association of TC *rs3024839*, AT *rs2227483*, CC *rs3024839*, TT *rs2227483* genotypes with myocardial infarction determinants and comorbid factors