ORIGINAL ARTICLE

Deployment of next-generation sequencing approach for variant detection in myocardial infarction: A concise investigation

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Abstract

Background: A fatal health issue termed Myocardial Infarction (MI) is characterized by an acute loss of oxygen and blood supply to the heart muscles, ultimately leading to necrosis. This can turn life threatening if left untreated and undiagnosed at early stages. Elevated plasma LDL cholesterol involved in plaque formation and thinning of arterial walls is believed to the main culprit. Till date there are no preventive diagnosis/screening molecular mechanism to identify the responsible markers for this cholesterol metabolism and associated risk factors. *Aim and Objectives:* To identify the predominantly expressed genes associated with MI. The purpose of this work was to aid in the identification of biomarkers for the genetic diagnosis of MI leading to better understanding of the relation between genes involved in coronary heart diseases and their molecular mechanism. *Material and Methods:* This was a case control study in which patients attending the Cardiology Department of Sri Venkateswara Institute of Medical Sciences (SVIMS) recruited and initially evaluated with all biochemical parameters. After taking written informed consent, DNA samples were collected and subjected to NGS sequencing studies; a 17-gene customized MI panel was designed for targeted sequencing. The obtained data was analysed and identified variations within the selected genes were given priority for further investigation. *Results:* Variants in the APOB, MTHFR, WDR12, CELSR2, and MIA3 genes were identified as more predominant in the sequenced individuals and two novel variants were observed from CELSR2 which were not reported previously. *Conclusion:* To ascertain pathogenicity and role in the emergence of MI-related disorders these genes were mapped to online databases. Interestingly we found majority of genes from designed MI panel exhibit a variable effect upon the probability to acquire CAD as well as the severity towards variety of coronary heart diseases.

Keywords: Myocardial infraction, Targeted sequencing, Biomarker development, Cardiovascular Disorders, Variant of Uncertain Significance

Introduction

The rapid loss of oxygen and blood supply to the artery that cause necrosis of the heart muscles is referred as "Myocardial Infarction" (MI) [1]. The cardiac muscles deteriorate when there is no oxygen-rich blood supply, which can be a serious medical emergency if it is not identified and treated quickly [2]. Due to changes in habits, MI is now recognized as a widespread disorder affecting people of all ages. It is thought to be a consequence of an interaction between genetics, lifestyle, and psychosocial factors [3]. Poor diet, inactivity, excessive alcohol use, and tobacco use, increase the risk of high blood sugar, triglyceride, and cholesterol. Young individuals are more vulnerable

to illnesses like MI and cardiac crises [4]. Heart attacks are more common in people and families who are unaware of the risk factors and family history of Cardio-vascular Disease (CVD) [5]. Saraswathi *et al*. showed that quality of life in both urban and rural patients has a significant effect on conditions like stroke, coronary artery disease and MI [6]. Before engaging every individual in the cohort group, we analyzed patients who were referred to the Cardiology Department of Sri Venkateswara Institute of Medical Sciences (SVIMS) as per the inclusion criteria for acute coronary syndromes.

The key objective of this study was to identify the most often expressed genes and their impact on protein alterations that may cause cardiovascular diseases associated with MI in particular cohort populations. The majority of cardiac diseases are caused by increased metabolism of cholesterol and plaque buildup inside the artery walls, which cause thinning and thus obstructs the heart muscles and necessitates life-threatening medical attention. Studies on CVDs have been conducted using single genes and targeted Sanger approaches. However, this study is one of its kind as it employs a custom designed MI panel to assess affected individuals through the use of NGS technology (Table 1). With this approach, we might be able to definitively figure out the origin of alterations at the gene level. The candidate gene selection for this customized NGS panel was based on the comprehensive review of existing literature and bioinformatics analysis, integrating information from Genome-wide Association Studies (GWAS). We carefully selected set of genes implicated in cardiovascular health, atherosclerosis, thrombosis and clinical significance for each candidate gene as mentioned in Table 1, for which patent application 2024410-17059 has been filed.

Clinical involvement of selected gene variants in MI related disorders

Apolipoprotein B (ApoB) is known for its role in lipid metabolism and its variations have been associated with elevated cholesterol levels, contributing to atherosclerosis, a key risk factor for MI [7]. ApoB is implicated in regulating levels of triglycerides (TGs), cholesterol, and low-density lipid protein cholesterol (LDL-C). Elevated LDL-C levels confer the highest absolute risk of MI [8]. MTHFR (Methylenetetrahydrofolate Reductase) polymorphisms in MTHFR have been associated with hyperhomocysteinemia, a condition linked to endothelial dysfunction and increased risk of arterial thrombosis [9]. Melanoma Inhibitory Activity Family, Member 3 (MIA3) is involved in cholesterol metabolism and the formation of arterial plaques by thinning the walls of coronary arteries [10]. Myosin-Binding Protein H (MYBPH) is mostly involved in familial hypertrophic cardiomyopathy characterized by thickening of the heart muscle [11]. WD Repeat Domain 12 (WDR12) and PHACTR1 genes are associated with the angiographic severity of CAD related to atherogenesis [12]. MRPS6, SLC5A3, CELSR2, CXCL12 and KCNE2 genes show SNP associated with risk of early onset myocardial infarction and structural variants in human DNA sequence variation, and may account for some of the unexplained heritability in MI and other common diseases [13-15].

Material and Methods

This study received ethical clearance from Institutional Ethics Committee, SVIMS University, Tirupati, India (reference number: 844(a) dated 23/04/2019). Written informed consent was taken from all participants prior to recruitment. An online

Table 1: Genes selected from GWAS in building targeted NGS panel for MI

Name	Chromo-some	Num Amplicons	Total Bases	Covered Bases	Missed Bases	Overall Coverage	Num Exons	Clinical Significance
CELSR2	chr1	63	11220	10700	520	0.954	34	Cholesterol metabolism, lipid-associated functions
MTHFR	chr1	39	7390	7271	119	0.984	12	Hyperhomocisteinemia, arterial thrombosis
MIA3	chr1	50	8990	8971	19	0.998	29	Atherosclerotic plaque development
MYBPH	chr1	14	2026	2026	0	1	11	Familial hypertrophic cardiomyopathy
CXCL12	chr10	24	5390	5390	0	1	9	Ischaemia
C12 or f43	chr12	17	3051	3017	34	0.989	11	Maturity-onset Diabetes of the young type 3
HNF1A	chr12	22	3637	3584	53	0.985	11	Diabetes Mellitus, MODY3
APOE	chr19	10	1580	1287	293	0.815	8	Hyperlipoproteinemia Type I
WDR12	chr2	17	2542	2542	0	1	13	Coronary artery disease, early-onset myocardial infarction
APOB	chr2	61	14701	14471	230	0.984	29	Elevated cholesterol levels, atherosclerosis
KCNE2	chr21	4	843	843	0	1	2	Atrial fibrillation, congestive heart failure
MRPS6	chr21	6	1050	1048	2	0.998	3	Coronary artery disease
SLC5A3	chr21	44	11663	11661	2	0.99	2	Coronary artery disease, early-onset myocardial infarction
MRAS	chr3	26	5306	5271	35	0.993	9	Cardiofaciocutaneous syndrome
PHACTR1	chr6	20	3181	3181	0	1	16	Epilepsy
MTAP	chr9	24	5081	5033	48	0.991	8	Cardiomyopathy
CDKN2B	chr9	17	4024	4024	0	1	3	Coronary Artery Disease, Coronary Heart Disease, Myocardial Ischemia

Table 2: Clinical information of the 11 patients included in this study

Variable	7772512	7772513	7772502	7772505	7772500	7772514	7772509	7772499	7772503	7772506	7772504
Age (years)	42	70	65	25	55	38	67	37	39	45	49
Gender	M	F	M	M	F	M	M	F	M	F	F
Smoking	1	0	1	1	0	1	0	0	1	0	0
Alcoholic	1	0	1	1	0	1	0	0	1	0	0
BMI (kg/m2)	20.2	20	20.2	23.4	20.6	24	24.2	21.6	29.4	21.5	33.7
24-h SBP (mmHg)	130	120	100	110	130	140	130	130	140	120	110
24-h DBP (mmHg)	80	80	70	70	90	70	80	86	90	80	90
Chol (mg/dl)	184	190	171	214	184	220	186	153	177	174	194
TG (mg/dl)	159	193	99	176	190	82	90	106	140	191	92
HDL (mg/dl)	33	42	40	29	36	56	30	35	38	36	47
LDL (mg/dl)	119.2	109.4	111.2	149.9	110	147.6	129	96.8	111	99.8	128.6
VLDL (mg/dl)	31.8	38.6	19.8	35.2	38	16.4	15	21.2	28	38.2	18.4

BMI: Body mass index, Chol: Cholesterol, TG: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

tool was used to calculate the required sample size and accordingly 74 subjects was the minimum recruitment number with 90% confidence and 5% margin of error. A total of 100 samples (including patients and controls) were selected for this study. All the recruited subjects were aged above 18 years and diagnosed with acute coronary syndromes who were admitted to the Department of Cardiology, Sri Venkateswara Institute of Medical Sciences, Tirupati having acute coronary syndromes including ST-Elevation Myocardial Infarction (STEMI), Non-ST- Elevation Myocardial Infarction (NSTEMI) and unstable angina. Plasma lipid profiles including total cholesterol, High-density Lipoprotein (HDL), Low-density Lipoprotein (LDL), Very Low-density Lipoprotein (VLDL), and triglycerides were measured (Table 2), using commercial kits on DXC600 Beckmann autoanalyzer. To conduct the study, we reviewed GWAS on CVD related conditions and genes most involved in these disorders, and designed a customized MI panel using Illumina design studio software consisting of 17 genes repeated most in the CVD conditions (Table 1).

Out of the 100 samples recruited, only 64 subjects fulfilled the inclusion criteria. Additionally, we recruited 11 unrelated patients for preliminary data analysis for this study and all the details pertaining to age, sex, comorbidities are recorded in Table 2. After obtaining written informed consent from the participants, 5 ml of blood was collected using vacutainers coated with EDTA. DNA extraction

was carried out according to the instructions provided by the manufacturer, utilizing the PureLink Genomic DNA Isolation Kit from Thermo Fisher Scientific. Following the manufacturer's protocol, approximately 100 ng of genomic DNA was used to construct DNA libraries using the Illumina Ampliseq Library preparation kit. The constructed libraries were quantified using the High Sensitivity Genomic DNA Assay on the Qubit 3.0 platform from Thermo Fisher Scientific. The Illumina Miseq platform was employed for sequencing, utilizing the MicroV2 300cycles kit. Sequencing procedures were conducted following the manufacturer's guidelines. Around 1.8 Gb of total data generated after sequencing and raw files were processed for FASTQ conversion and data analysis.

Results

The customized built-in pipelines were employed for data analysis and performed at Medgenome Labs Ltd. in Bengaluru, India. After sequencing, the raw data (BCL files) was obtained; before processing, the raw data was filtered for adapter trimming, duplicate percentage, and guanine-cytosine content and the aforementioned variants were annotated. Targeted genes were analyzed using the GRCh38-hg38 Germline pipeline; samples were prioritized for further investigation based on quality scores, depth, and coverage of variants. We chose only 11 samples with adequate depth and coverage metrics for preliminary analysis from the entire data set to determine the variations that occur most frequently among the 17 targeted genes. We cross-referenced our findings with online clinical databases to investigate the pathogenicity and potential role of SNPs in the genes CELSR2, WDR12, MTHFR, MIA3, and APOB, which were identified after data analysis from all the selected cases. Next-generation Sequencing (NGS) analysis revealed two novel pathogenic variants in CELSR2 from the 11 cases that were sequenced. These heterozygous frameshift deletion variants resulted in an early truncation of the protein 19, amino acids downstream to codon 2397 (p.Phe2397SerfsTer19; ENST00000271332.4). According to ClinVar, dbSNP, OMIM, and ClinGen, both of the detected variations have a pathogenic effect and have not been documented in any databases (Figure 1 & 2). An amino acid substitution of Serine for Alanine at codon 384 (p.Ala384Ser; ENST00000261015.5) has been found as a pathogenic variant in WDR12 on Exon 12 chr2:g.202882755C>; this has not been reported in the topmed databases (Figure 1). The APOB, MTHFR, and MIA3 genes possess variants of uncertain significance that are clinically associated with CVD and MI conditions in individuals who were previously affected. This data is available using the 1000 genomes, gnomAD (v3.1), gnomdAD (v2), and topmed databases, respectively. To understand comprehensively, it was quite mandatory to investigate the impact of these changes on protein modifications and interactions.

Uncertain underlying family history and lifestyle factors impact the majority of MI. This study interprets CVD risk variables in relation to CAD-associated genetic variations by recording the biochemical measurements and lifestyle characteristics from the cases. In order to determine the precise roles that each gene and variant associated with MI play in situations relevant to MI, we concentrated on studying them individually. Our goal was to improve our knowledge of these genes' roles in the development of MI by examining these variations and genes. The main objective of this research was to identify biomarkers linked to MI to improve genetic diagnosis.

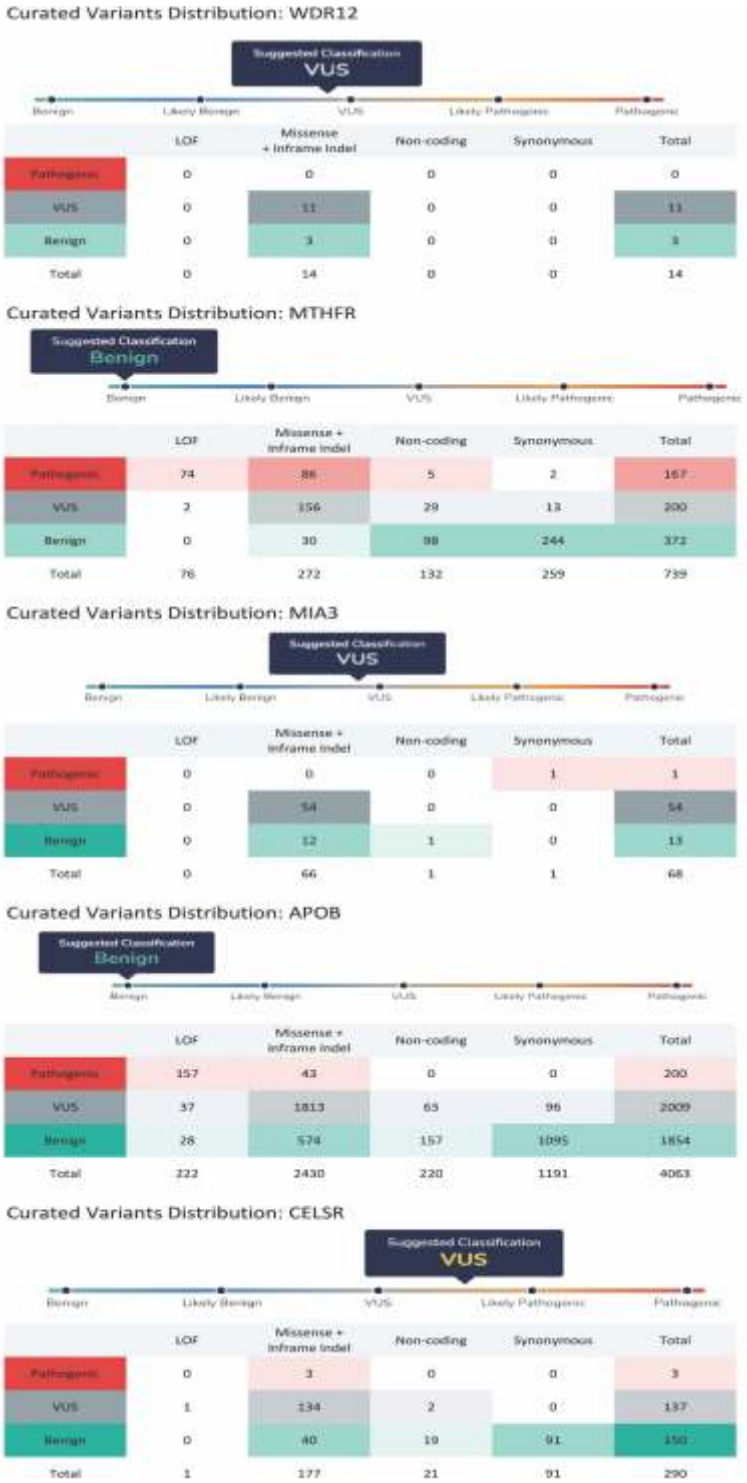

Figure 1: Variant distribution from the genes analysed with their pathogenicity. Gene assessment was carried out using Franklin ACMG Classification. https://franklin.genoox.com/analysis-tool/analysis/variants/11/snp

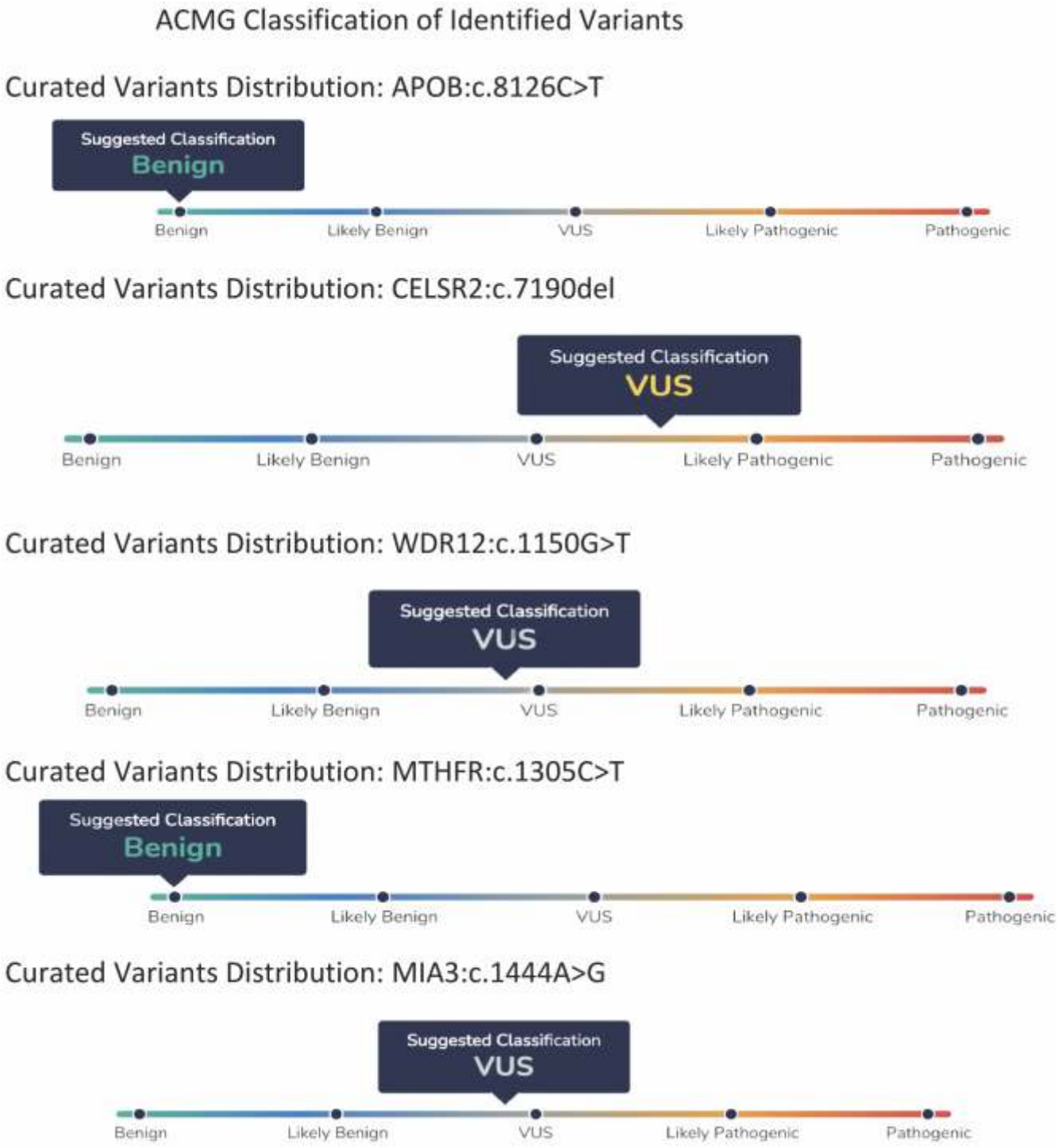

Figure 2: Classification of variants based on pathogenicity. Variant classification is from Franklin ACMG Classification and using assembly: GRCh37/hg38 https://franklin.genoox.com/analysis-tool/analysis/variants/11/snp

Table 3: Distribution of variants from the seventeen analyzed genes in the study design

Sample ID	Chr.	Location	Gene	Variant	SRA Accession Number	BioProject Accession	SRP ID
7772499	chr2	Exon 26	APOB	c.8216C>T	**SRX24532762**	**PRJNA1110856**	**SRP507253**
7772500	chr1	3'UTR	CELSR2	c.*919G>T	**SRX24532977**	**PRJNA1110963**	**SRP507260**
7772502	chr1	Exon 23	CELSR2	c.7190del	**SRX24532978**	**PRJNA1110963**	**SRP507260**
7772503	chr1	Exon 4	MIA3	c.1444A>G	**SRX24532763**	**PRJNA1110856**	**SRP507253**
7772504	chr1	Exon 7	MTHFR	n.1822C>T	**SRX24532980**	**PRJNA1110963**	**SRP507260**
7772505	chr2	Exon 12	WDR12	c.1150G>T	**SRX24532764**	**PRJNA1110856**	**SRP507253**
7772506	chr2	Exon 4	APOB	c.293C>T	**SRX24532765**	**PRJNA1110856**	**SRP507253**
7772509	chr2	Exon 26	APOB	c.6937A>G	**SRX24532766**	**PRJNA1110856**	**SRP507253**
7772512	chr1	Exon 7	MTHFR	n.1822C>T	**SRX24532981**	**PRJNA1110963**	**SRP507260**
7772513	chr1	Exon 23	CELSR2	c.7190del	**SRX24532979**	**PRJNA1110963**	**SRP507260**
7772514	chr2	Exon 4	WDR12	c.239T>C	**SRX24532767**	**PRJNA1110856**	**SRP507253**

Discussion

A homozygous missense variant in the APOB gene in Exon 26 on Chr. 2 was found in 7772499 being benign and 7772509 classified as VUS (Figure 2). This variant c.6937A > G is located in the APOB protein's domain and has been previously reported in patients with familial hypobetalipoproteinemia, hypercholesterolemia, and acute myocardial infarction [16] (Table 3). According to PolyPhen-2 (HumDiv), the variants in silico predictions are most likely harmful, and they are primarily regarded as variants of questionable importance (Figure 1). In the 1000 genomes, gnomAD (v3.1), gnomdAD (v2), and topmed databases, the variants p.Pro2739Leu and p.Ile2313Val have a minor allele frequency. These protein variations are primarily characterized by abnormal LDL, which can contribute to early coronary artery disease, and decreased serum cholesterol. Studies by Martin-Campos *et al.* in 2012 and Damsteegt *et al.* in 2018 [17-18] have linked APOB and CELSR2 to a number of diseases, including hypercholesterolemia, hypobetalipoproteinemia, hypertriglyceridemia, and high LDL levels in cholesterol metabolism. Many disorders, including normotriglyceridemic hypobetalipoproteinemia, hypercholesterolemia due to ligand-defective ApoB, and hypobetalipoproteinemia, can result from mutations in the APOBgene or its regulatory region. Both ApoB levels and plasma cholesterol are impacted by these illnesses and ApoB-100 is the major structural component of VLDL, Intermediate-density Lipoproteins (IDL), LDL, and lipoprotein(a) (Lp(a)) [19]. Further, 7772506 was identified to possess a heterozygous missense mutation in Exon 4 of the APOBgene on Chr2, which causes Isoleucine to be substituted for Threonine at codon 98 (p.Thr98Ile; ENST 00000233242.5).

The discovered variation is located in the PF01347:Lipoprotein amino terminal region domain of the ApoB protein and has previously been described in patients with familial hypercholesterolemia and hypobetalipoproteinemia [20]. In the 1000 genomes, gnomAD (v3.1) and topmed, this variant is most involved in disease associated polymorphism and being previously reported in various studies (Table 3).

A heterozygous base pair deletion in the CELSR2 gene chr1 is detected with NGS analysis in 7772502, and 7772513 that affects the position XX nucleotides downstream of the stopcodon of the CELSR2 (Figure 2). The observed variant has previously been reported in patients affected with acute coronary syndrome, cardiovascular disease, and congenital heart defects with LDL-cholesterol association caused by mutations in the CELSR2 gene [21] (Table 3).

The variant p.Phe2397SerfsTer19 has not been reported in the gnomdAD (v2) databases 1000 genomes, gnom-AD (v3.1) and topmed databases. Based on OMIM and disease database reports CELSR2 variation can be classified as a variant of potential importance/likely disease causing or disease associated (Figure 2). Studies, such as those conducted in 2008 by Samani et al., have demonstrated the role of CELSR2 genes on Chr1 in CAD, mainly by raising plasma LDL levels through cholesterol metabolism [22]. An early diagnosis and identification of metabolic syndrome and understanding of its lifestyle correlates can play a pivotal role in the prevention of various cardio-metabolic illnesses [23].

The MTHFR gene chr1's homozygous missense mutation causes the amino acid NA to be substituted at codon NA in the 7772504 and 7772512 variants. The observed variation has been seen in acute coronary syndrome patients in the past. The domain of the MTHFR protein (NA) contains information on cardiovascular illness and its correlation with other cardiovascular diseases (Table 3). Due to the thinning of coronary artery walls, these diseases may mostly be indirectly implicated in the metabolism of cholesterol and the development of arterial plaques. Variants in the MTHFR gene have been inconsistently linked to CAD [24-25]. At 90.29550%, 90.94000%, 97.48000%, 89.63470%, and 99.44973% minor allele frequency, the identified variations are found in the 1000 genomes, gnomAD (v3.1), gnomdAD (v2), and topmed databases.

The WDR12 gene's heterozygous missense variant in Exon 12 (chr2:g.202882755C>A) resulting in the amino acid substitution of Serine for Alanine at codon 384 (p.Ala384Ser; ENST00000261015.5) was identified in 7772505 (Figure 3). The identified variation, which lies in the WDR12 protein (NA) domain, has previously been reported in patients with cardiomyopathy, dilated coronary arteriosclerosis, coronary artery disease, and myocardial infarction [26]. The minor allele frequency of the p.Ala384Ser variant is 0.01997%, 0.00000%, 0.00000%, and 0.04367% in the 1000 genomes, gnomAD (v3.1), and gnomd-AD (v2) databases, respectively. This variant has not been reported in the topmed databases.

According to PolyPhen-2 (HumDiv), the variant's in silico predictions may be harmful and should be considered probable pathogenic. WDR12 (WD-repeat domain 12) has been linked to early-onset MI in previous studies. There are multiple mechanisms via which WDR12 and CAD are associated, but the main ones are increased plaque vulnera-

bility and coronary artery constriction, which is predominantly caused by the accumulation of fatty deposits within the arterial walls [27]. In vivo gene study showed WDR12 triggers distinct deterioration of cardiac function in adult rat heart and the MI associated WDR12 variant is associated with diastolic dysfunction in human subjects [28]. A VUS that was previously described in patients with CVD and MI situations was also seen in WDR12 for the 7775214 sample on Exon 4 with an amino acid substitution of Alanine for Valine at codon 80 (p.Val80Ala; ENST00000-261015.5). The minor allele frequencies of the p.Val80Ala variant in the 1000 genomes, gnomAD (v3.1), gnomdAD (v2), and topmed databases are 0.05990%, 0.01000%, 0.04000%, 0.00416%, and 0.30570% (Figure 2).

Exon 4 (chr1:g.222628664A>G) of the MIA3 gene contains a homozygous missense variant that, when analyzed with NGS data, resulted in the amino acid substitution of glutamic acid for lysine at codon 482 (p.Lys482Glu; ENST00000-344922.10) in 7772503 (Figure 2) and lies in the domain of the MIA3 protein (NA). In the 1000 genomes, gnomAD (v3.1), gnomdAD (v2), and topmed databases, the p.Lys482Glu variant had minor allele frequencies of 99.56070%, 99.48000%, 99.87000%, 99.39780%, and 99.64251%. This MIA3 variation is categorized as a variant of uncertain significance (Figure 2). MIA3 thins the walls of coronary arteries, which contributes to the metabolism of cholesterol and the formation of arterial plaques. This was in agreement with the study conducted by Wang *et al*. (2011) [29]. ADTRP encodes an Androgen-Dependent TFPI-Regulating Protein through molecular signaling pathway positively regulates the expression of the MIA3 gene through activation of AKT and both are involved in pathogenesis of CAD [30].

Limitations of the study

Given that the sensitivity of the NGS method to detect Copy Number Variations (CNV) is 70–75%, it is recommended that we correlate the results with the clinical symptoms and their significance in other family members in so that we can accurately characterize our predictions in the patients sequenced. When additional family members undergo genetic testing, the variant(s)' relevance or classification can change. Genetic testing is highly accurate, yet occasionally erroneous results can happen for a variety of reasons, and the results aren't always conclusive. Testing may occasionally fail to detect a genetic variant despite its existence. The reason for this might be that rare polymorphisms can cause false positive or negative results. Additionally, this method does not assess variants in untranslated regions, promoters, or intronic variants because the population allele frequencies that are currently available (1000Genome, gnomAD-Exome) are only for the hg19 genome version. Due to the intricate changes in certain genomic areas, this could lead to disparities in variant annotation. A large cohort group of population screening is advised in order to identify a biomarker for genetic diagnosis of CVD and MI. This preliminary analysis data can be used as a design study for a bigger sample size analysis study.

Conclusion

In summary, our findings imply that the majority of genes exhibit a variable effect upon the probability to acquire CAD as well as the severity and variety of coronary heart diseases. Nevertheless, our findings are not entirely in agreement with the hypothesis that these genes represent a reliable genetic risk factor for CAD/MI using this straightforward approach. Further, a comprehensive study with a larger sample size is essential, including an assessment of

alterations in protein interactions to better understand the association between genotypes and coronary heart diseases.

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Data Summary

The data analysed has been represented in the manuscript and the raw data has been submitted to NCBI under the BioProject PRJNA1110856 and PRJNA1110963

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