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ORIGINAL RESEARCH

Evolutionary Action–Machine Learning Model Identifies Candidate Genes Associated With Early-Onset Coronary Artery Disease

Dillon Shapiro, BS; Kwanghyuk Lee, PhD; Jennifer Asmussen, PhD; Thomas Bourquard, PhD; Olivier Lichtarge, MD, PhD

BACKGROUND: Coronary artery disease is a primary cause of death around the world, with both genetic and environmental risk factors. Although genome-wide association studies have linked >100 unique loci to its genetic basis, these only explain a fraction of disease heritability.

METHODS AND RESULTS: To find additional gene drivers of coronary artery disease, we applied machine learning to quantitative evolutionary information on the impact of coding variants in whole exomes from the Myocardial Infarction Genetics Consortium. Using ensemble-based supervised learning, the Evolutionary Action–Machine Learning framework ranked each gene's ability to classify case and control samples and identified 79 significant associations. These were connected to known risk loci; enriched in cardiovascular processes like lipid metabolism, blood clotting, and inflammation; and enriched for cardiovascular phenotypes in knockout mouse models. Among them, *INPP5F* and *MST1R* are examples of potentially novel coronary artery disease risk genes that modulate immune signaling in response to cardiac stress.

CONCLUSIONS: We concluded that machine learning on the functional impact of coding variants, based on a massive amount of evolutionary information, has the power to suggest novel coronary artery disease risk genes for mechanistic and therapeutic discoveries in cardiovascular biology, and should also apply in other complex polygenic diseases.

Key Words: coronary artery disease ■ evolutionary action ■ gene-based associations ■ machine learning ■ myocardial infarction

Coronary artery disease (CAD) remains the global leading cause of death. Greater than 20 million adults are diagnosed with CAD, and approximately 650 000 people in the United States die annually from some form of heart disease.[1,2] CAD stems from a combination of genetic and environmental factors; therefore, understanding which individuals are most at risk for the development of disease could facilitate earlier lifestyle and pharmacological interventions. Past studies have estimated genetic heritability for CAD to be between 40% and 60%[3,4] and identified >100 loci associated with CAD and its related phenotypes, including variants primarily related to cholesterol metabolism, vascular remodeling, inflammation, and angiogenesis.[5–7] Recent population scale analyses with hundreds of thousands of samples such as the coronary artery disease genome wide replication and meta-analysis plus the coronary artery disease genetics consortium,[8,9] UK Biobank,[10,11] and the trans-omics for precision medicine program[12] have replicated the association of several previously reported common variants. However, these associations often have uncertain implications due to their frequent localization in noncoding regions of the genome. As a complement to common variant associations, whole exome sequencing studies[13–15] have identified

Correspondence to: Olivier Lichtarge, MD, PhD, Baylor College of Medicine, One Baylor Plaza, T921, Houston, TX 77030. Email: lichtarg@bcm.edu

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CLINICAL PERSPECTIVE

What Is New?

- A novel exome-wide association method using evolutionary information and machine learning reveals multiple genes with functional evidence linked to early-onset cardiovascular disease.
- Criteria for successful experiments support contributions from known and suggested coronary artery disease–associated risk genes in important biological pathways.

What Are the Clinical Implications?

- The protein-coding variation characterized in this study further expands our knowledge of genetic contributions to coronary artery disease risk and prioritizes new avenues for mechanistic interrogations, polygenic risk assessment, and therapeutics.

Nonstandard Abbreviations and Acronyms

EA	Evolutionary Action
EAML	Evolutionary Action–Machine Learning
EOMI	early-onset myocardial infarction
MCC	Matthew correlation coefficient
MIGen	Myocardial Infarction Genetics
STARNET	Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task

rare mutations with the strongest links to heart disease risk and age of onset, including lipid metabolism genes *LDLR*, *LDLRAP1*, *APOB*, and *PCSK9*, which are causal for familial hypercholesterolemia and are associated with dramatic increases in low-density lipoprotein cholesterol.[16–20] However, rare variant studies are typically underpowered due to sample sizes that are magnitudes smaller than those used in traditional array-based approaches.[21] Consequently, fewer variants and genes from rare variant-based approaches meet the statistical significance threshold to be of interest for follow-up analyses, leaving a gap in the identification of novel CAD risk factors.

Although current genome-wide association studies (GWAS) findings explain approximately 40% to 50% of estimated heritability in CAD, with common and rare variants accounting for ≈20% and ≈2% to 4%, respectively,[22,23] one possible source of missing heritability is the potential for variants to have nonadditive effects on disease risk[24] based on their functional impact. Standard modeling techniques rely on strict, linear assumptions about genetic inheritance,[25–28] and approaches that consider nonlinear and nonadditive interactions have shown improvements in some cases.[29–31] Although recent studies have begun to explore the impact of variation beyond the level of independent single nucleotide polymorphisms (ie, rare variant burden, epistatic interactions between genes/variants, network interactions),[25,26,32] association methods could be augmented by the fact that variants have different levels of impact on protein structure and function across evolution. Together, this argues that new approaches are needed to identify more direct, functional connections between genetic factors and CAD.

To address these issues, we developed a novel Evolutionary Action–Machine Learning (EAML) framework that scores the relative contribution of a gene's mutations in distinguishing individuals affected by a complex trait from healthy controls.[33] By incorporating the Evolutionary Action (EA) functional impact score,[34] derived from a systematic analysis of evolutionary information on protein sequence variations and divergences,[35] into association testing and focusing on protein-coding variants, we include a larger portion of variance in our association modeling that is directly related to biological importance. The use of EA has been previously demonstrated in blinded community challenges to detect deleterious coding variants[36] and in identifying genes associated with Alzheimer disease,[33,37,38] cancer,[32,39–41] autism spectrum disorder,[42] and antibiotic resistance.[43] Additionally, the use of machine learning allows us to address nonlinear patterns of variation within potential risk genes. Here, we have used EAML to search for novel risk genes in early-onset myocardial infarction (EOMI), an outcome of CAD where genetic inheritance is a major risk component. First, we flagged potential risk genes using EAML on 7426 samples with EOMI and healthy samples from the Myocardial Infarction Genetics (MIGen Consortium).[13] We then assessed these EAML candidates against known CAD-related risk genes and traits and further characterized them through clustering with known risk genes in a protein–protein interaction network. Finally, we prioritized EAML candidates by aggregating evidence related to GWAS, relative risk, mouse knockout data, expression quantitative trait loci (eQTLs), and PubMed co-occurrences. EAML recovered the most important known biological associations through direct overlap and pathway enrichments, but also prioritized novel candidates through multiple computational criteria. These results suggest an increasingly important role for genes that regulate lipid metabolism, inflammation, blood clotting, and the cell cycle and open new directions for mechanistic and therapeutic research in CAD.

METHODS

Data Disclosure

This study protocol (H-37394) was approved by the Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals.

Our analyses were based on the following 3 data sets: the ATVB (Atherosclerosis, Thrombosis, and Vascular Biology) study, the OHS (Ottawa Heart Study), and the PROCARDIS (Precocious Coronary Artery Disease) study. These studies were approved by the institutional review boards of all participating institutions, and information about informed consent from the study participants can be found in the study homepages (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000814.v1.p1, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000883.v1.p1, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000806.v1.p1).

Because of the sensitive nature of the data collected for this study, requests to access the data sets from qualified researchers may be sent to the database of Genotypes and Phenotypes (dbgap-help@ncbi.nlm.nih.gov).

Software Availability

EA scores of missense variants are publicly available via web server (http://eaction.lichtargelab.org/). EAML code is publicly available on GitHub (https://github.com/LichtargeLab/EAML), including easy installation and a toy data set for testing.

Data Collection

Whole-exome sequencing data were obtained from 3 previously established studies within the MIGen Exome Sequencing Consortium: the ATVB study (dbGaP accession: phs000814),[44] the OHS (dbGaP accession: phs000806),[45] and the PROCARDIS study (dbGaP accession: phs000883).[46] In ATVB, both cases and controls were selected from across 125 coronary care units in Italy between 1994 and 2007. Cases were defined as patients hospitalized for a first myocardial infarction (MI) <45 years of age, and controls were age- and sex-matched individuals without a history of thromboembolic disease, a subclass of cardiovascular disease that shares many of the underlying risk factors with CAD. In OHS, cases were selected through the Ottawa Heart Institute and defined as patients diagnosed with CAD (defined as MI, coronary artery bypass, or angiographic stenosis >50%) <55 years of age in men and <65 years of age in women, whereas controls were men >65 years of age and women >70 years of age without a history of cardiovascular disease and selected through newspaper and television advertising. In PROCARDIS, cases were selected from hospitals in the United Kingdom and defined as patients with MI, unstable or stable angina, or coronary revascularization <66 years of age. Controls were age- and sex-matched individuals without personal or sibling history of cardiovascular disease, recruited from the same centers through self-administered questionnaires. The cardiovascular disease history of the control samples is defined according to the case definition in each respective study. Available clinical characteristics of each cohort were limited to those provided by the studies in dbGaP, including case–control numbers and reported sex distributions. The age cutoffs and sample selection criteria were predetermined by the individual studies, and detailed descriptions of sample selection criteria are available in each cohort's original publication.

Variant Quality Control

To remove potentially low-quality variant sites, we filtered variant sites based on the average genotype quality, average depth of coverage, missingness, and Hardy-Weinberg Equilibrium test. Variants with an average genotype quality <20, average depth of coverage <8, missingness >2%, or Hardy-Weinberg Equilibrium *P* value of controls $<5\times10^{-5}$ were removed from the data set. All variant filtering steps were performed using the BCFtools software.[47]

Sample Quality Control

We performed multiple steps to identify outlier samples before analysis. First, we inferred ancestry by using principal component analysis to map samples with 1000 genomes and excluded samples that were inferred as non-European (Figure S1). Second, we removed samples with an excess of coding variants (>17 000), an excess of missing variants (>1300), or an excess of singletons (>20), because these can suggest low-quality sequencing (Figure S2). We carefully considered these thresholds through the visualization of sample statistics. Next, we inferred sex with the ratio of heterozygous to homozygous variants on the X chromosome and removed samples with a mismatch between inferred and self-reported sex (Figure S3). Finally, we estimated kinship coefficients between samples and removed samples with third-degree relatives (kinship coefficient >0.1; Figure S4). Principal component analysis, sex, and relatedness filtering were performed using the *peddy* Python package.[48]

Variant Annotation

Variants (single nucleotide variants and indels) were annotated using the hg19 RefSeq reference and the Annotate Variation annotation tool.[49] Nonsynonymous variants were annotated with the EA equation,[34] receiving a variant impact score between 0 and 100. We assigned loss-of-function variants such as frameshift indels and stop-gain variants a maximal EA score of 100.

Statistical Analysis

Rare Variant Association Analyses

As a control experiment and to replicate previous studies, we performed a rare variant burden test using the optimal sequence association test method[25] in the efficient and parallelizable association container toolbox package (https://genome.sph.umich.edu/wiki/EPACTS). We selected variants with a minor allele frequency <1% and collapsed variants across a gene using EA impact score thresholds to compare optimal sequence association test performance more directly to EAML. We used 3 different variant groupings: (1) all nonsynonymous variants, (2) partially deleterious variants based on having an EA score >30, and (3) deleterious variants based on an EA score>70.

EAML Pipeline

Quantifying Functional Genetic Burden

For each gene, we first calculated an aggregate score of all variant level effects into 1 metric for 6 underlying genotype–phenotype association hypotheses. For this, we developed a function dubbed the EA probability (pEA). This is defined as:

$$pEA = 1 - \prod_{j=1}^{k}\left(1 - \frac{EA_j}{100}\right)^{zyg} \quad \forall EA > C \qquad (1)$$

where k is the total number of variants in a gene, j is the index over those variants, EA_j is the EA score from a given variant, and zyg denotes the zygosity of variant j (0 denotes wild-type, 1 denotes heterozygous, and 2 denotes homozygous). C denotes 3 different thresholds of EA, specifically 0, 30, and 70. The 6 underlying hypotheses are delineated by terms C and zyg. First, the thresholds defined by C correspond to how the degree of predicted variant impact associates with disease status: (1) any missense variant (EA >0), (2) moderate-to-high impact variants (EA >30), or (3) deleterious variants (EA >70). Second, the *zyg* term avoids a priori assumptions about a gene's inheritance pattern, allowing for an association in either an autosomal dominant (zyg >0) or recessive (zyg >1) manner. Mutations are separated into 6 pEA learning features based on the assumptions made by C and zyg. Finally, these features are aggregated into a nx*6* design matrix for each gene, where n is the number of samples.

Model Architecture

The learning architecture consisted of 9 different classifiers, representing standard models used in modern machine learning problems, combined in an averaging ensemble. These classifiers include Association Rules (PART,[50] JRip [51]), Function Optimizations (Multilayer Perceptron,[52] Naïve Bayes,[53] Logistic Regression,[54] and K Nearest Neighbors[55]), Decision Trees (Random Forest[56] and J48[57]), and meta-classifiers (Adaboost[58]). All classifiers were implemented in Weka with default hyperparameters (https://www.cs.waikato.ac.nz/ml/weka/).

Association Testing

To evaluate the association of each gene, each member of the 9-classifier ensemble was trained on 90% of the input data set to classify disease cases from healthy controls. Performance was evaluated on the leftover 10%, generating a classification score for a given gene. This process was repeated in a 10-fold cross validation, with the scores averaged across folds, to minimize data set overfitting.

The classification performance of an individual gene was used as a surrogate for the magnitude of association with the given phenotype. This was estimated using the Matthew correlation coefficient (MCC), defined as:

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}} \qquad (2)$$

The MCC scores from all 9 classifiers were averaged and used to produce a final ranking of all genes. We then computed Z scores and the corresponding 1-tailed Z test P values for each average MCC relative to the full distribution of gene MCC scores. Finally, we implemented a false discovery rate correction using the Benjamini-Hochberg method, and genes with a false discovery rate–corrected P value <0.1 were selected as candidate risk genes. More details of the EAML approach can be found in Data S2 (Figure S5).

Mouse Phenotype Analysis

To assess biological causality between EAML genes and cardiovascular phenotypes, we queried the Mouse Genome Informatics database of assayed mouse knockout models.[59] To test whether EAML candidates are linked to cardiovascular phenotypes more often than random genes, we counted the number of candidates with a mouse model reporting a cardiovascular system phenotype. Then, we randomly sampled gene sets of the same size and repeated the counting procedure to generate a random background distribution. We then calculated the Z score and P value of the candidate gene list's enrichment in comparison with the random background distribution.

STRING Network Analyses

All protein–protein interaction networks were constructed using the STRING protein-protein interaction database version 11.0, downloaded from https://version-11-0b.string-db.org/. Edges with a combined score (from all evidence types)>400 were included in network analyses.

Graph information diffusion[60–62] was used to assess how closely related EAML genes are to target gene sets within a protein–protein interaction network. Graph information diffusion propagates signal from an input gene set across the network and reported the signal received by every gene. The closeness of the input and target gene sets was then reported as a receiver operating characteristic and the area under the curve (AUC). Because there is a large class imbalance with many more genes labeled as unrelated to MI, AUC can be an inflated measure of performance. To control for this, we randomly sampled gene sets with the same size and degree distribution as the target gene set and repeated the graph information diffusion analyses. AUCs were measured for these random gene sets, then used as a background distribution for which the *Z* score of the true target gene set was measured. A *Z* score >1.96 (*P*<0.05) suggested that the input genes and target genes were functionally related.

Odds Ratio Calculation

To identify the directionality of each candidate gene's/variant's contribution to relative risk, which is not revealed by EAML, we calculated a crude allelic odds ratio for each candidate gene and each protein-coding variant within each candidate gene. Statistical significance was calculated using the Fisher exact test. We corrected for multiple testing using the Benjamini-Hochberg false discovery rate correction and assigned statistical significance with an adjusted *P* value threshold of 0.01.

Gene-Level eQTL Mapping

Data on healthy tissue eQTLs were acquired from the Genotype-Tissue Expression (GTEx) Project database.[63] We considered any eQTL reported in any tissue with a *P* value $<1\times10^{-6}$. Cardiovascular eQTLs were identified using the STARNET (Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task) study.[64] The data from this study contain both normalized RNA expression and genotyped DNA across 7 cardiovascular tissue types. We used a set of eGenes reported by the initial study, which are genes containing at least 1 *cis*-eQTL.

RESULTS

EAML Identified 79 EOMI-Associated Disease Candidate Genes Using Evolution-Based Machine Learning

To discover novel risk genes associated with EOMI using evolutionary information, we aggregated whole exome sequencing data from 3 cohorts within the MIGen (Table S1). These data included 3736 individuals diagnosed with EOMI who also underwent coronary angiography. The controls included 3690 healthy subjects with no history of thromboembolic or cardiovascular disease. After annotating the cohort variants with gene, transcript, and EA scores, we performed quality control to exclude potentially false-positive variants as well as individuals of non-European descent, mismatched sex, and sequencing outliers (Figure 1A; see Methods). We then analyzed the cohorts with EAML, an ensemble-based pipeline that evaluates each gene's ability to classify cases from controls using EA scores and supervised machine learning.

For each gene and individual within the cohort, EAML first calculates a probability of functional impact by aggregating EA scores for 6 different groups of variants, each associated with their own unique underlying hypothesis (Figure 1B). We defined the groups by the EA magnitude and inheritance pattern, allowing EAML to selectively evaluate the importance of each gene based on different patterns of functional impact and variation. Each gene-based feature matrix was then used as the input for an ensemble of supervised classifiers, and the average MCC[65] score across classifiers was calculated for each gene. Averaging was used to determine the consensus among model types while also reducing false positives and focusing on specificity over sensitivity. Finally, each gene was ranked and prioritized based on its average MCC score. For our analysis of the EOMI cohort, we applied EAML in an unbiased fashion, using all variants scored with EA regardless of allele frequency. Using EAML, we identified 79 genes (Table S2) passing the false discovery rate–corrected *P* value threshold of 0.1 and having a positive MCC score (Figure 1C) after 10-fold cross-validation on 16912 genes with non-0 discriminatory power (Figure S6A and S6B). For comparison, we performed a rare variant association analysis, optimal sequence association test, on the same data and identified a single gene meeting statistical significance ($P<5\times10^{-6}$), the widely known CAD risk gene *LDLR* (Figure S6C). These data show how EAML prioritizes potential EOMI risk genes using evolutionary information and ensemble machine learning, recovering more genes for computational and experimental validation than current state-of-the-art association methods. The remainder of the study was focused on these 79 candidate genes.

EAML Candidates Are Enriched in Cardiovascular Disease Gene Sets and Related Phenotypes

To assess the ability of EAML to recover genes associated with cardiovascular phenotypes and their biomarkers, we first tested the 79 EOMI candidate genes for phenotype enrichment within the GWAS Catalog database using the functional mapping and annotation portal.[66] We found significant enrichments for CAD ($P=5.20\times10^{-11}$) and MI ($P=3.48\times10^{-6}$) as well as for multiple lipid biomarkers and related phenotypes (Figure 2B), which included triglyceride:high-density lipoprotein (HDL) ratio ($P=9.63\times10^{-5}$), hypertriglyceridemia

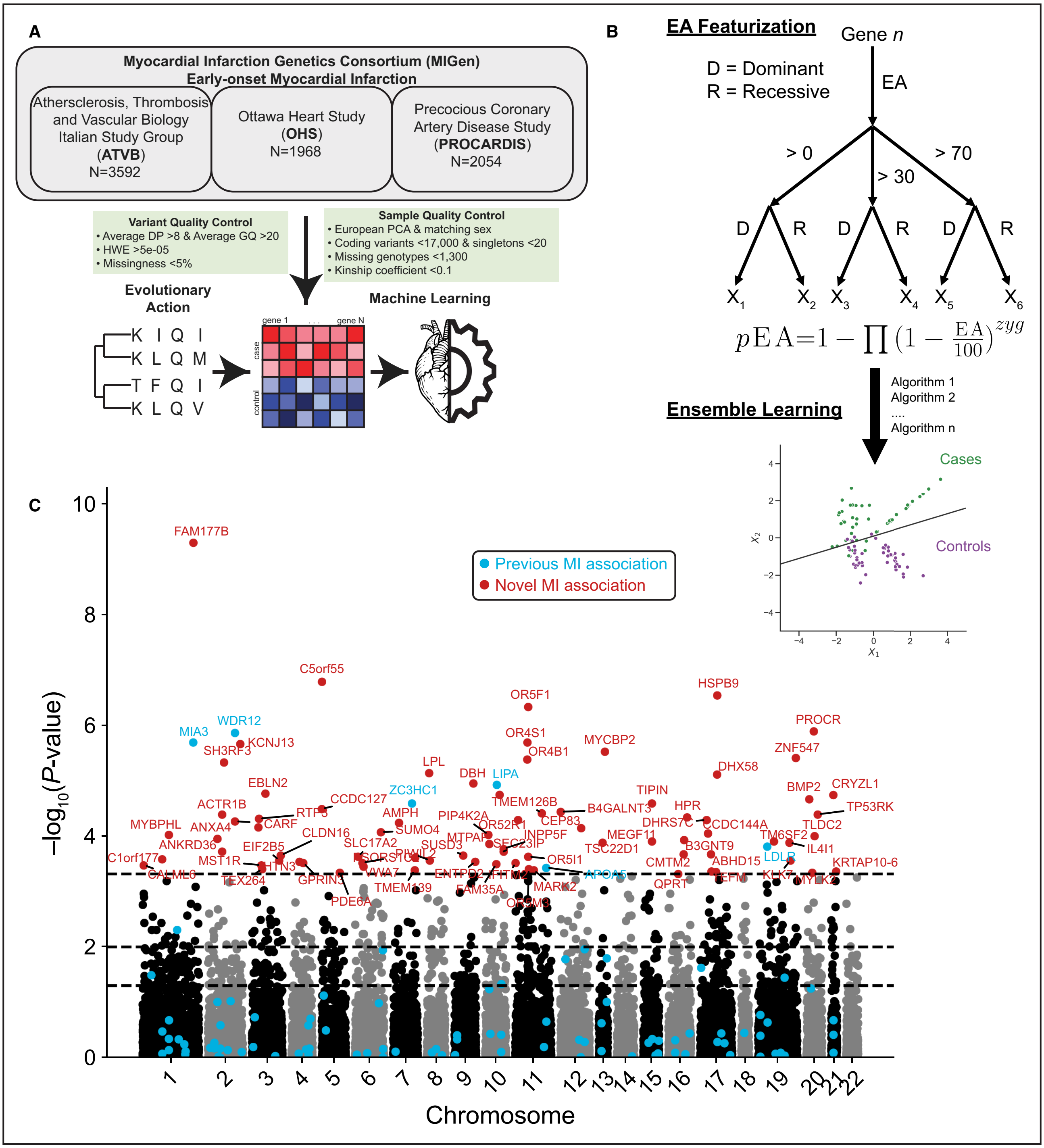


Figure 1. Overview of study design and Evolutionary Action-Machine Learning (EAML) results.
A, Workflow of sample selection, quality control, and EAML analysis. Samples are individuals from 3 separate Myocardial Infarction Genetics studies with whole exome sequencing performed by the Broad Institute. After variant and sample quality control, all single nucleotide variants are given an Evolutionary Action (EA) functional impact score based on the residue's evolutionary importance and amino acid substitution. These scores are used to train an ensemble machine learning model that ranks each gene's disease association. **B**, Schematic of how EAML uses variants and inheritance hypotheses to identify genes associated with early-onset myocardial infarction risk. **C**, Manhattan plot of EAML results. Red dots are genes found by EAML (adjusted *P*<0.1), and blue dots are genes found in previous myocardial infarction genome-wide association studies. DP indicates depth of coverage; GQ, genotype quality; HWE, Hardy-Weinberg Equilibrium; and PCA, principal component analysis.

(P=1.05×10^{-4}), type 2 diabetes (P=1.21×10^{-4}), and antithrombotic agent use (P=3.00×10^{-4}). Notably, *LDLR*, *APOA5*, and *LPL* occurred repeatedly in these enrichments; all of which are extremely important in lipid metabolism and the underlying pathology of cardiovascular disease. Next, we compared the 79 EOMI candidate genes against an aggregated set of reference genes from curated MI associations in GWAS Catalog[67] (n=75) and clinical MI variants in the ClinVar clinical variant database[68] (n=36) (Table S3). The EAML candidates significantly overlapped (n=6) with the union of the 2 reference gene sets (Figure 2B, P=8.55×10^{-6}: *WDR12*, *MIA3, LIPA*, *LDLR*, *APOA5*, and *ZC3HC1*). Because most of the candidates did not overlap with the GWAS Catalog or ClinVar reference gene sets, we evaluated the degree to which our candidate genes colocalized with previously reported CAD GWAS loci. A total of 19 candidates (Table S4) fell within 500 kb of a CAD GWAS Catalog locus (Figure 2C; P=0.0018), showing that our EAML prioritized genes are related to previously identified CAD loci. These data show how the 79 EOMI candidate genes replicate previously reported cardiovascular disease associations and are enriched in known CAD biological processes.

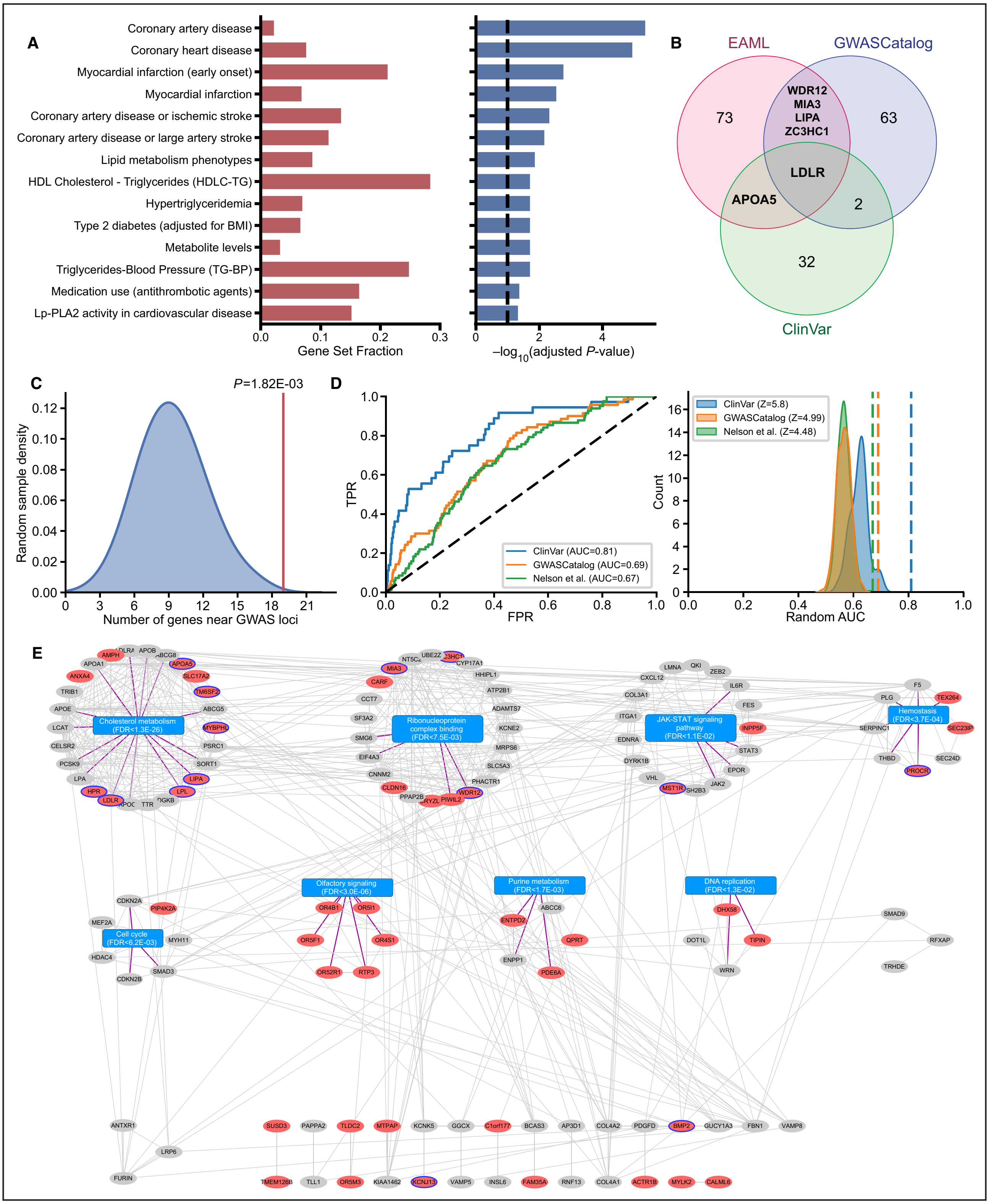
EOMI Candidate Genes Exhibit Significant Network Relatedness and Connectivity to Cardiovascular Risk Genes and Biology

To investigate the mutual interactions of the EOMI candidates, we assessed connectivity of the 79 genes across the STRINGv11 protein–protein interaction network. We found significant connectivity between 23 EOMI candidates (P=1.36×10^{-3}) (Figure S7A), although EAML does not make any a priori assumptions about network connectivity. These genes fall into local network clusters enriched for cholesterol and lipid homeostasis, olfaction, and vascular smooth muscle contraction. Next, to assess the relative network proximity of our 79 EOMI candidate genes to the GWAS Catalog and ClinVar reference gene sets, we used a graph-based information diffusion algorithm (nDiffusion)[62] that measures the closeness of interactions between 2 gene sets in any input network (eg, STRING). The 79 EOMI candidate genes are highly and significantly (Z scores=5.80, 4.99, 4.48) connected to the 2 reference gene sets, as well as a third CAD-associated gene set[11] (Figure 2D, Figure S7B, Table S3). We observed the highest connectivity between EAML genes and ClinVar reference genes (AUC=0.81, Z=5.80). These data show that the 79 EAML genes are significantly connected to known CAD biology in the context of a protein–protein interaction network, implicating potential novel associations and biology.

To further understand how the EOMI candidate genes are related to known cardiovascular risk genes, we built an interaction network between our 79 genes and the GWAS Catalog and ClinVar reference gene sets. The resulting candidate-reference gene hybrid network was highly enriched for protein–protein interactions (P<1.0×10^{-16}) and allowed us to visualize first neighbor connections between the 79 candidates and the reference genes (Figure 2E). We then performed Markov clustering on the hybrid network to identify densely connected regions and subsequently tested for pathway enrichment in the clusters containing at least 1 EOMI candidate gene. Of the 22 Markov clusters, 16 contained at least 1 EOMI candidate. Pathway enrichment of the clusters with g:Profiler[69] identified CAD-relevant biology including lipid metabolism, inflammation, blood coagulation and platelet degranulation, purine and nicotinate/nicotinamide metabolism, and transcriptional regulation (Table S5). The genes related to lipid metabolism fall into the largest cluster, including notable cardiovascular disease risk factors, namely *LDLR*, *APOA5*, *LIPA*, and *LPL*.[13,17,70] Additionally, this cluster contains *HPR* and *ANXA4*, both of which may indirectly impact lipid metabolism through interactions with apolipoprotein L1 (apoL-I)–containing HDL and phospholipids, respectively. It is also noteworthy that *MYBPHL* has been shown to regulate ventricular and atrial conduction and is associated with dilated cardiomyopathy.[71] Other pathways of interest include a cluster with 2 EOMI candidates, *INPP5F* and *MST1R*, linked to inflammation and a cluster related to blood coagulation and platelet degranulation with 3 candidates, *PROCR*, *SEC23IP*, and *TEX264*. The inflammation cluster centers around JAK/STAT signaling, an important inflammatory pathway that is an established target for modulating cardiovascular risk.[72] Additionally, *PROCR* has an essential role in regulating anticoagulation through protein C levels[73] and has previously been associated with CAD and venous thromboembolism.[74,75] These data show that EOMI candidates encompass important biological processes essential to cardiovascular disease through links to known CAD genes.

EOMI Candidates Have Significant Relative Effects on Cardiovascular Disease Risk

To characterize the relative risk associated with each of the 79 candidate EOMI genes, we calculated allelic odds ratios (ORs), aggregating all nonsynonymous variants within each gene. Forty-nine of the 79 EAML candidates were significantly associated with disease status (adjusted P<0.01), of which 15 were associated with increased risk (OR >1), and 34 were associated with decreased risk (OR <1) (Figure 3A). Genes linked to familial hypercholesterolemia like *LDLR* (OR, 1.28) and *APOA5* (OR, 1.2) showed the strongest associations with increased risk, alongside *CALML6* (OR,



1.25). Genes associated with increased risk also included other known CAD risk factors, *WDR12*, *CARF*, *DHX58*, and *LIPA* with OR values of 1.18, 1.15, 1.14, and 1.09, respectively. Surprisingly, most genes were associated with protection from disease, including *MIA3* (OR, 0.87) and *PROCR* (OR, 0.81), despite both genes being known for associations with increased risk of CAD and thromboembolisms, respectively.

Next, to map the risk of each of the 79 EOMI candidates more finely, we tested if specific variants within

Figure 2. Enrichment of Evolutionary Action–Machine Learning (EAML) candidates for direct overlap or interactions with known cardiovascular associations in clinical and genome-wide association studies (GWAS) data.

A, Enrichment for traits with genome-wide associations, performed using the functional mapping and annotation web portal. The left bar plot illustrates the fraction of genes for each enriched trait that overlaps with EAML genes. The right bar plot shows the adjusted *P* value for each enrichment. **B**, Overlap between EAML candidates and myocardial infarction-associated genes from ClinVar and GWAS Catalog databases. **C**, A density plot illustrating enrichment for EAML candidates within 500 kb of established coronary artery disease GWAS loci. The density plot represents the colocalization between GWAS loci and randomly sampled gene sets, and the red line represents the observed colocalization with EAML. The *P* value was calculated using a *Z* test. **D**, Receiver operating characteristic curves for network diffusion from EAML candidates to ClinVar- and GWAS-mapped genes (left). Distributions of areas under the curve (AUCs) based on 100 randomly sampled, degree-matched target gene sets (right). Dashed lines represent the experimental AUCs. **E**, Network-based clustering and functional enrichment of EAML candidates and previously reported myocardial infarction genes. The network was built using 79 EAML candidates and 103 known coronary artery disease risk genes from GWAS Catalog and ClinVar that interacted with one another in STRING version 11 (confidence >0.4). Modules were created using the Markov clustering algorithm with an inflation parameter of 3. Functional enrichment was performed for each cluster using g:Profiler. Red nodes represent EAML genes, and blue borders represent PubMed comentions with cardiovascular disease. BMI indicates body mass index; Lp-PLA2, lipoprotein-associated phospholipase A2; FDR, false discovery rate; FPR, false positive rate; and TPR, true positive rate.

each gene were associated with disease risk. We found 8 variants to be significantly associated with increased risk and 9 variants associated with decreased risk (adjusted $P<0.01$) (Figure 3B). All associations were among common variants with allele frequencies ranging from 0.03 to 0.50 and included variants within genes with previously identified noncoding associations like *MIA3* (K605R, EA=14; E881G, EA=56), *ZC3HC1* (R363H, EA=34), and *DHX58* (Q425R, EA=8). In *MIA3* and *MST1R* (Figure 3C), we identified additional variants with opposing EA scores and identical ORs, potentially due to linkage disequilibrium between the variants. In *FAM177B*, we identified a variant (I3S) that corresponded with increased risk (OR, 1.18) and has recently been associated with CAD in a gene-based meta-analysis along with the substitution Q523R in *MST1R*.[76] These data illustrate that the 79 candidate genes are clinically important by increasing or decreasing the relative risk of EOMI and reveal variants of potentially novel mechanistic interest.

Cardiovascular *cis*-eQTLs Are Enhanced in EOMI Candidate Genes

In addition to being impacted by variation that directly affects protein function, a gene may affect disease risk through regulatory variation. Importantly, regulatory variation can modify the penetrance of protein coding variants.[77,78] To determine if the EOMI candidates contain evidence for *cis*-eQTLs, we queried each candidate gene in the GTEx database[63] for *cis*-eQTL associations within cardiovascular tissues. We found that 67 EOMI candidates show overlapping eQTLs in at least 1 cardiovascular tissue (Figure S8A). Among the candidate genes, neither *APOA5* nor *KCNJ13* showed evidence of significant changes in differentially expressed gene levels, despite their previous strong associations with CAD. However, GTEx contains samples without any specific phenotypes, and these data may not represent how expression is regulated in the context of cardiovascular disease. To identify EOMI candidates containing regulatory associations specific to cardiovascular disease, we searched the eQTL summary statistics from STARNET,[64] a RNAseq-based study of 600 patients with cardiovascular disease that contains 8 291 095 eQTLs mapped to 14 174 genes across 7 cardiovascular tissues. Fifty-three EOMI candidate genes intersected with reported eQTLs in at least 1 of the 7 tissues (Figure S8B). This included EOMI candidate genes that overlapped with the GWAS Catalog and ClinVar reference gene sets, except for *KCNJ13*. Six genes overlap with at least 1 eQTL in all 7 tissues, namely *CCDC127*, *CCDC144A*, *DHX58*, *PIP4K2A*, *TEX264*, and *TIPIN* (Table S6). It is noteworthy that *APOA5* and *LDLR* are linked to eQTLs specific to liver tissue, which play an important role in regulating triglyceride metabolism[79,80] and plasma lipid clearance.[81] These data show that many EOMI candidates are associated with differentially expressed gene levels in cardiovascular tissues, further supporting their importance for CAD progression.

EOMI Candidates Are Enriched for Cardiovascular Effects in Mice

To evaluate whether alterations in EOMI candidates directly drive or modulate biological changes that impact cardiovascular health in animal models, we turned to the Mouse Genome Informatics database.[59] In total, there are 18 122 human genes with mouse orthologs present in the Mouse Genome Informatics database, and 2845 of these exhibit a cardiovascular system phenotype in at least 1 mouse model. Of the 79 EOMI genes tested, 66 were altered in at least 1 mouse model within the database. Of these, we found significant alterations in 19 genes associated with a cardiovascular system phenotype ($P=5.04\times10^{-3}$) when compared with randomly sampled gene sets (average number of cardiovascular phenotypes for random sets=9.3; Z=3.04). Notable overlapped genes are *LDLR*, *LPL*, and *LIPA*, as well as genes with strong common variant associations with CAD, namely *MIA3* and *DHX58*. We also found that

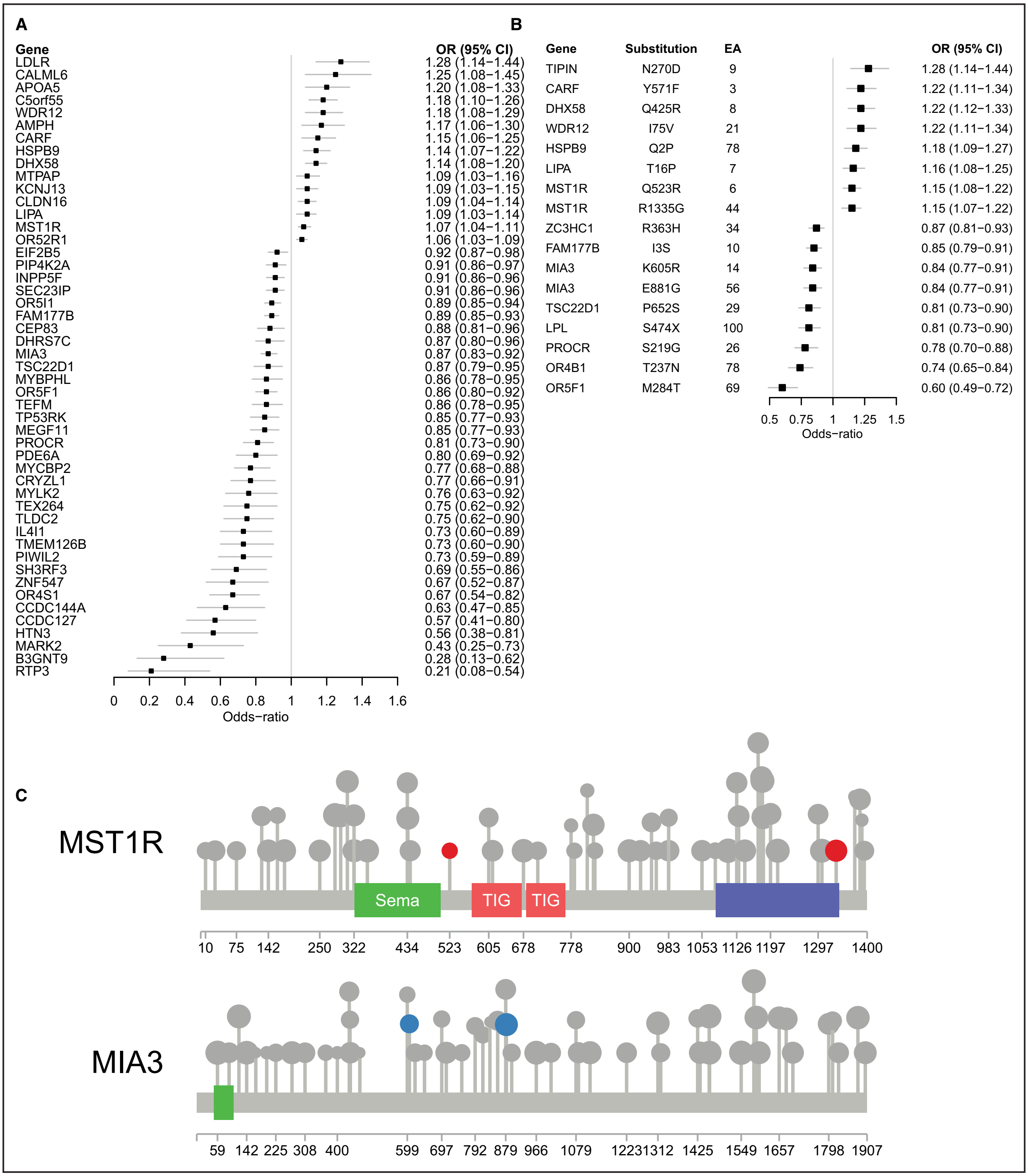


Figure 3. Estimated contributions of Evolutionary Action–Machine Learning (EAML) candidate variants to myocardial infarction (MI) risk.

A, Aggregate odds ratios (ORs) of EAML candidates based on nonsynonymous single nucleotide variants (SNVs) (adjusted *P*<0.01). **B**, ORs of nonsynonymous SNVs with adjusted *P*<0.01 in EAML candidates. **C**, Lollipop plot of nonsingleton missense SNVs in *MST1R* and *MIA3*. Circle size corresponds to Evolutionary Action (EA) score, and colored SNVs are significantly associated with early-onset MI in variant-level OR analysis (adjusted *P*<0.01). Red color corresponds to OR >1, and blue corresponds to OR <1.

4 genes without previously reported MI associations (*BMP2*, *TEFM*, *MARK2*, and *DBH)* affected heart physiology and inflammatory response. Despite having no direct CAD associations, *BMP2* has been shown to be essential for proper cardiac development.[82–84] *MST1R* models exhibit increased acute inflammation, whereas models targeting *LEXM*, *B4GALNT3*, and *MARK2* exhibit abnormal T-cell morphology. These data show the phenotypic relevance of the EOMI genes in the context of a model organism, supporting their predicted importance in cardiovascular disease.

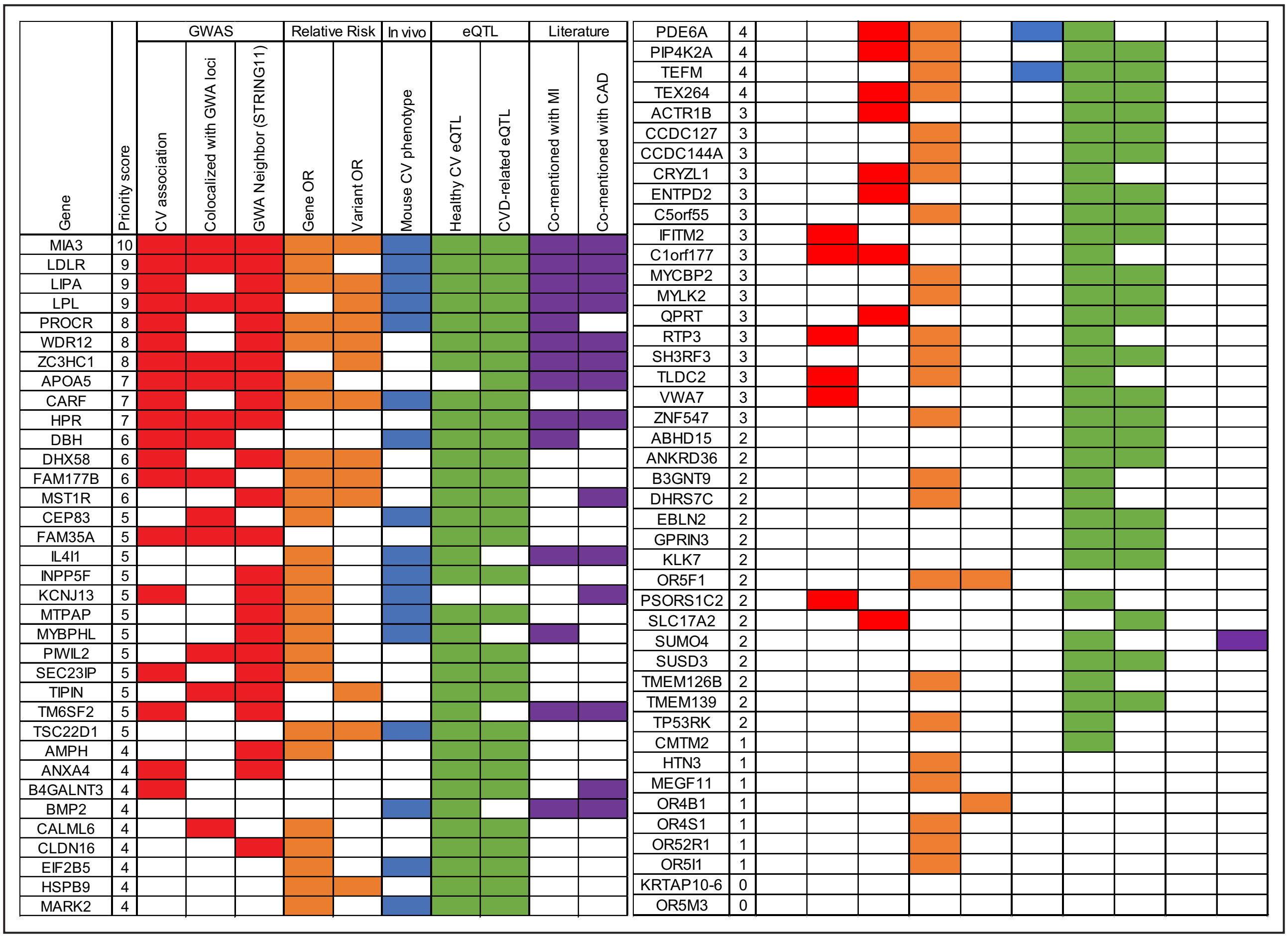
EAML Prioritizes Genes of Biological Importance in Cardiovascular Disease

To prioritize EOMI candidate genes that are most likely to hold biological significance in cardiovascular disease risk and mechanism, we combined the results from our validation analyses to build a gene prioritization score (Figure 4)[85,86] based on 10 equally weighted experiments in 5 overall categories: GWAS, relative risk, in vivo, eQTL, and literature. For the GWAS category, we aggregated criteria related to known cardiovascular associations, colocalization with an established GWAS loci (Figure 2C), and direct interaction with a GWAS-reported gene in STRING (Figure 2E). For the relative risk category, positive criteria include having a statistically significant allelic OR (adjusted P<0.01) or containing any variant with a significant OR (Figure 3). The in vivo criteria include whether the gene showed cardiovascular phenotype evidence in the Mouse Genome Informatics database. The eQTL category contains criteria for the presence of cardiovascular *cis*-eQTLs in GTEx or STARNET (Figure S7). Lastly, the literature category contains evidence of comentions with either CAD or MI in PubMed titles and abstracts. Based on these criteria, *MIA3* was the top gene with positive evidence in every category, followed by 13 genes with priority scores between 6 and 9: *LDLR*, *LIPA*, *LPL*, *PROCR*, *WDR12*, *ZC3HC1*, *APOA5*, *CARF*, *HPR*, *DBH*, *DHX58*, *FAM177B*, and *MST1R*. The top 8 genes all contain established links to CAD biology, whereas the subsequent 6 genes possess mostly circumstantial evidence. Among the potentially novel gene findings with little to no prior literature association with CAD or MI, the highest prioritized candidates were *CARF* and *HPR*, with a score of 7. *CARF* lies within the same region as *WDR12*, another previously reported CAD gene. Both genes are targeted by *cis*-eQTLs within the same locus[87] and contain variants (rs72932557, rs35212307) that are associated with similar levels of increased MI risk. *CARF* was also identified as a key driver in a gene regulatory network derived from the STARNET cohort.[64] *HPR* has functional evidence linking it to cardiovascular biology through hemoglobin and HDL.[88] Next, *DBH*, *DHX58*, *FAM177B*, and *MST1R* had gene prioritization scores of 6. *DBH* is essential to noradrenaline production, with liver-specific eQTLs (Table S6) and knockout mouse models showing defects related to general development, heart morphology, and circulating hormone levels.[89] *DHX58* has evidence from 2 previous CAD association studies, 1 of which identifies the same risk-increasing coding variant as our study (rs2074158; OR, 1.22; P=9.58×10^{-6}).[70,90] *FAM177B* was scored as the top EAML candidate gene, yet there are no direct studies linking it to MI or CAD. However, our analysis suggests it is associated with a protective effect (OR, 0.89 [95% CI, 0.85–0.93]; P=2.×10^{-5}), and intergenic variants have been associated with coronary artery bypass grafting.[91] Another interesting and novel candidate with a gene prioritization score of 5 is *INPP5F*. It has not previously been associated with cardiovascular phenotypes and fails to colocalize with established risk loci, yet we find evidence supporting an association with CAD. The *INPP5F* gene-level (OR, 0.91 [95% CI, 0.86–0.96]; P=6.2×10^{-4}) risk appears mostly driven by the protective variant rs318805 (OR, 0.88 [95% CI, 0.0.82–0.94]; P=3.3×10^{-4}), which aligns with the functional role it plays in inhibiting *STAT3* and its antiapoptotic/proangiogenic activity.[92,93] These data show that by aggregating different types supporting evidence, we reaffirm genes with known CAD associations and prioritize genes with novel clinical and functional insights.

DISCUSSION

Previously, we presented a novel genomic analysis framework, EAML, that combines ensemble machine learning with a continuous functional impact score for coding variants for discovering genotype–phenotype associations.[33] Here, for cardiovascular disease and EOMI, we identified 79 genes with underlying mutational patterns specific to EOMI, of which 60 were not previously linked to CAD, to the best of our knowledge.

Consistent with past works, our study shows that lipid metabolism continues to be the pathway most strongly associated with CAD risk. Mutations in *LDLR*, *LPL*, and *APOA5* are known for their direct links to lipid metabolism and trafficking, with rare variants often being causal for familial hyperlipidemias.[94–96] Although no individual variants in *LDLR* or *APOA5* in the MIGen cohort are significantly associated with MI status, presence of any mutation in either of the genes shows an association with increased risk (ORs: *LDLR* 1.28 and *APOA5* 1.20). In addition to these established risk genes, we identified *HPR*, *MYBPHL*, *AMPH*, *ANXA4*, and *SLC17A2*, all of which have little to no direct evidence linking them to CAD. In particular, *HPR* was ranked in the top 10 genes in the prioritization table, has appeared in multiple studies associated with both



Gene	Priority score	GWAS			Relative Risk		In vivo	eQTL		Literature	
		CV association	Colocalized with GWA loci	GWA Neighbor (STRING11)	Gene OR	Variant OR	Mouse CV phenotype	Healthy CV eQTL	CVD-related eQTL	Co-mentioned with MI	Co-mentioned with CAD
MIA3	10	🟥	🟥	🟥	🟧	🟧	🟦	🟩	🟩	🟪	🟪
LDLR	9	🟥	🟥	🟥	🟧		🟦	🟩	🟩	🟪	🟪
LIPA	9	🟥		🟥	🟧	🟧	🟦	🟩	🟩	🟪	🟪
LPL	9	🟥	🟥	🟥		🟧	🟦	🟩	🟩	🟪	🟪
PROCR	8	🟥		🟥	🟧	🟧	🟦	🟩	🟩	🟪	
WDR12	8	🟥		🟥	🟧	🟧		🟩	🟩	🟪	🟪
ZC3HC1	8	🟥	🟥	🟥		🟧		🟩	🟩	🟪	🟪
APOA5	7	🟥	🟥	🟥	🟧				🟩	🟪	🟪
CARF	7	🟥		🟥	🟧	🟧	🟦	🟩	🟩		
HPR	7	🟥	🟥	🟥				🟩	🟩	🟪	🟪
DBH	6	🟥	🟥				🟦	🟩	🟩	🟪	
DHX58	6	🟥		🟥	🟧	🟧		🟩	🟩		
FAM177B	6	🟥	🟥		🟧	🟧		🟩	🟩		
MST1R	6			🟥	🟧	🟧		🟩	🟩		🟪
CEP83	5		🟥		🟧		🟦	🟩	🟩		
FAM35A	5	🟥	🟥	🟥				🟩	🟩		
IL4I1	5				🟧		🟦	🟩		🟪	🟪
INPP5F	5			🟥	🟧		🟦	🟩	🟩		
KCNJ13	5	🟥		🟥	🟧		🟦				🟪
MTPAP	5			🟥	🟧		🟦	🟩	🟩		
MYBPHL	5			🟥	🟧		🟦	🟩		🟪	
PIWIL2	5		🟥	🟥	🟧			🟩	🟩		
SEC23IP	5	🟥		🟥	🟧			🟩	🟩		
TIPIN	5		🟥	🟥		🟧		🟩	🟩		
TM6SF2	5	🟥		🟥				🟩		🟪	🟪
TSC22D1	5				🟧	🟧	🟦	🟩	🟩		
AMPH	4			🟥	🟧			🟩	🟩		
ANXA4	4	🟥		🟥				🟩	🟩		
B4GALNT3	4	🟥						🟩	🟩		🟪
BMP2	4						🟦	🟩		🟪	🟪
CALML6	4		🟥		🟧			🟩	🟩		
CLDN16	4			🟥	🟧			🟩	🟩		
EIF2B5	4				🟧		🟦	🟩	🟩		
HSPB9	4				🟧	🟧		🟩	🟩		
MARK2	4				🟧		🟦	🟩	🟩		
PDE6A	4			🟥	🟧		🟦	🟩			
PIP4K2A	4			🟥	🟧			🟩	🟩		
TEFM	4				🟧		🟦	🟩	🟩		
TEX264	4			🟥	🟧			🟩	🟩		
ACTR1B	3			🟥				🟩	🟩		
CCDC127	3				🟧			🟩	🟩		
CCDC144A	3				🟧			🟩	🟩		
CRYZL1	3			🟥	🟧			🟩			
ENTPD2	3			🟥				🟩	🟩		
C5orf55	3				🟧			🟩	🟩		
IFITM2	3		🟥					🟩	🟩		
C1orf177	3		🟥	🟥				🟩			
MYCBP2	3				🟧			🟩	🟩		
MYLK2	3				🟧			🟩	🟩		
QPRT	3			🟥				🟩	🟩		
RTP3	3		🟥		🟧			🟩			
SH3RF3	3				🟧			🟩	🟩		
TLDC2	3		🟥		🟧			🟩			
VWA7	3		🟥					🟩	🟩		
ZNF547	3				🟧			🟩	🟩		
ABHD15	2							🟩	🟩		
ANKRD36	2							🟩	🟩		
B3GNT9	2				🟧			🟩			
DHRS7C	2				🟧			🟩			
EBLN2	2							🟩	🟩		
GPRIN3	2							🟩	🟩		
KLK7	2							🟩	🟩		
OR5F1	2				🟧	🟧					
PSORS1C2	2		🟥					🟩			
SLC17A2	2			🟥					🟩		
SUMO4	2							🟩			🟪
SUSD3	2							🟩	🟩		
TMEM126B	2				🟧			🟩			
TMEM139	2							🟩	🟩		
TP53RK	2				🟧			🟩			
CMTM2	1							🟩			
HTN3	1				🟧						
MEGF11	1				🟧						
OR4B1	1					🟧					
OR4S1	1				🟧						
OR52R1	1				🟧						
OR5I1	1				🟧						
KRTAP10-6	0										
OR5M3	0										

Figure 4. Prioritization of Evolutionary Action–Machine Learning candidates.
Criteria include (1) mapped to previously reported cardiovascular (CV) genome-wide association studies (GWAS), (2) located within 500 kb of a previously reported GWAS locus, (3) first-neighbor interaction with a mapped GWAS gene in the STRING protein-protein interaction network, (4) gene-based odds ratio (OR) with adjusted *P*<0.01, (5) at least 1 variant with OR with adjusted *P*<0.01, (6) associated with CV phenotype in Mouse Genome Informatics database, (7) reported expression quantitative trait locus (eQTL) association in CV healthy tissue in the Genotype-Tissue Expression database, (8) reported eQTL association in CV tissues in the STARNET (Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task) study, (9) text or abstract comention with myocardial infarction (MI), and (10) text or abstract comention with coronary artery disease (CAD). Each category is equally weighted, and the priority score is the sum of all categories. Colored fields indicate positive evidence for the given gene. CVD indicates cardiovascular disease; and GWA, genome-wide association.

low-density lipoprotein[97] and total cholesterol levels,[98] and is expressed exclusively in the heart and liver in GTEx samples. The *HPR* protein is known for its role in innate immune protection against trypanosomes through association with apolipoprotein L1–containing HDL particles,[99] yet our results suggest that its role may extend beyond that in terms of impact on CAD risk. Additionally, *MYBPHL* has previously been characterized for its function in cardiac conduction through atrial cardiomyocytes.[100] There is also strong evidence associating the *MYBPHL*-containing locus 1p13.3 with decreased low-density lipoprotein cholesterol,[101] which aligns with our data that *MYBPHL* mutations are associated with a protective effect on EOMI risk (Figure 3A). Although it is well known that lipid metabolism is important in cardiovascular health, EAML further supports this by placing multiple associated genes in a functional context.

Following lipid metabolism, inflammation is the other major mechanism involved in all stages of atherosclerotic progression, from plaque formation[102,103] to post-MI recovery.[104] Three EAML genes, *INPP5F*, *MST1R*, and *DHX58*, were linked to inflammatory functions. First, *INPP5F* has a gene prioritization score of 5 and lacks previous CAD evidence (Figure 4), yet showed an allelic association with EOMI risk and was directly linked to *STAT3* (Figure 2E),[92] an important inflammatory signaling regulator. *INPP5F* encodes the *SAC2* protein, an inositol 4-phospatase involved in the endocytic recycling pathway.[105,106] *Inpp5f*$^{-/-}$ mice exhibit increased susceptibility to stress-induced cardiac hypertrophy, and cardiac-specific overexpression led to a decreased hypertrophic response.[107] Through its inhibition of *STAT3*, *INPP5F* may play an important role in JAK/STAT immune signaling, which is activated in response to acute MI.[72] When acutely activated, this key inflammatory pathway invokes a cytokine cascade that exerts a cardioprotective effect through the regulation of myocyte survival, whereas chronic activation of JAK/STAT signaling leads to cardiac remodeling and a decline in heart function.[108] *INPP5F* likely plays a poorly studied role in these processes. A second potentially novel risk gene, *MST1R* (scored at 6), is a receptor tyrosine kinase that regulates wound healing and plays a role in both chronic and acute inflammation through macrophage recruitment.[109,110] Our analyses showed that *MST1R* is connected in STRING to *EPOR*, a regulator of *JAK2* (Figure 2E), and contains a single nucleotide polymorphism that is directly associated with increased EOMI risk (R1335G; OR, 1.15; EA=43.52) (Figure 3B). *MST1R* has recently been identified by gene-based association tests for CAD,[76] and *MST1R* null mice have impaired nitric oxide levels and increased tissue damage in response to acute stress.[111] Third, *DHX58* represents a previously reported yet understudied association in the context of CAD.[70,90] Although it does not link to the same immune pathways, evidence has shown that the downstream MAVS (mitochondrial antiviral signaling protein) regulates inflammation and fibrosis through NF-κB and MAPK with reduced expression of *MAVS* being associated with improved cardiac function.[112] When linked to the increased relative risk from *DHX58* coding mutants in our own study (Figure 3), this suggests that *DHX58* has direct functional importance in CAD.

Our findings also highlighted genes related to other mechanisms important to cardiovascular health, including blood clotting, purine metabolism, and nicotinamide adenine dinucleotide (NAD) metabolism. EAML genes linked to these pathways include *PROCR*, *SEC23IP*, *TEX264*, *PDE6A*, *ENTPD2*, and *QPRT*. The blood clotting cluster centers around *PROCR*, which contains a variant we report as protective (rs867186; OR, 0.78), matching what was previously reported in a GWAS within the ATVB cohort.[113] This same variant has also been previously associated with increased levels of protein C,[114] increased levels of factor VII,[115] increased risk of venous thrombosis,[116] and decreased risk of CAD.[74] Also included in the blood clotting cluster are *SEC23IP* and *TEX264*, both of which are novel risk genes that appear to be involved in endoplasmic reticulum–related functions. *SEC23IP* is involved in coat protein complex II (COPII)-mediated endoplasmic reticulum-to-Golgi trafficking,[117] which is essential for the secretion of clotting-related factors, whereas *TEX264* is involved in endoplasmic reticulum-phagy in response to nutrient stress.[118,119] *SEC23IP* has also previously been associated with type 2 diabetes[120] and HDL cholesterol levels.[121] A final interesting cluster is one primarily enriched for purine and NAD metabolism, containing 3 novel EAML candidates: *PDE6A*, *ENTPD2*, and *QPRT*. Although both pathways are less studied in the context of cardiovascular health, uric acid (a product of purine metabolism) has been associated with long-term cardiovascular risk,[122,123] and experimental evidence has shown that NAD+ elevation can protect against cardiovascular outcomes in preclinical models.[124,125]

Although the EAML method shows many advantages such as the incorporation of a gene-based impact score derived from evolutionary history and the use of ensemble machine learning, we could not consider hyperparameter optimization for each individual classifier. Due to the algorithmic complexity of training 9 classifiers for 18 000 genes, optimization would lead to an exponential increase in computing time and required computing power, without a guaranteed performance improvement. To address this, we performed 10-fold cross-validation for each individual classifier to minimize bias and overfitting/underfitting of the models by repeatedly testing each gene and averaging the results, and we strengthened our confidence in the resulting candidate genes through multiple independent validation experiments. Specifically, we illustrate that these genes are reliably linked to CAD and have performed an extensive review of published evidence, showing that many of these genes have biologically relevant functions. Therefore, our observations suggest that hyperparameter tuning was not a significant issue in the identification of CAD-associated genetic risk factors using EAML in the current study.

In conclusion, this study provides new insights into cardiovascular genetics by extending exome-wide associations with the combination of evolutionary information and supervised machine learning. Although EAML is still limited to analyzing individual genetic risk factors as in standard GWAS methods, it is broadly applicable to case–control whole exome studies. In addition to solidifying the role of several common genetic risk factors, EAML discovered both novel and previously known risk genes that had only been associated with CAD through noncoding variation. Furthermore, several EAML candidates without direct cardiovascular associations were closely related to established CAD risk loci, and linked together in gene clusters enriched in biological functions related to known disease mechanisms. Lastly, our study illustrates that protein-coding variation has a significant impact on complex disease risk. Our findings have the added benefit of being directly targetable in future mechanistic studies and are applicable to polygenic risk methods, which can further inform cardiovascular causes.

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Affiliations

Department of Molecular and Human Genetics (D.S., K.L., J.A., T.B., O.L.), and Computational & Integrative Biomedical Research Center (O.L.), Baylor College of Medicine, Houston, TX.

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Disclosures

None.

Supplemental Material

Data S1–S2
Tables S1–S6
Figures S1–S8

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