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ARTICLE

Development of a comprehensive cardiovascular disease genetic risk assessment test

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ABSTRACT

Purpose: Despite monogenic and polygenic contributions to cardiovascular disease (CVD), genetic testing is not widely adopted, and current tests are limited by the breadth of surveyed conditions and variant interpretation burden. To address these limitations, a comprehensive clinical genome CVD test with semiautomated interpretation was developed.

Methods: Monogenic conditions and risk alleles were selected based on the strength of disease association and evidence for increased disease risk, respectively. Non-CVD secondary findings genes, pharmacogenomic (PGx) variants, and CVD-associated polygenic risk scores (PRS) were also assessed for inclusion. Test performance was modeled using 2594 genomes from the 1000 Genomes Project and further investigated in 20 previously tested individuals.

Results: The CVD genome test comprises a panel of 215 high-confidence CVD gene-disease pairs, 35 non-CVD secondary findings genes, 4 risk alleles or genotypes, 10 PGx genes, and a PRS for coronary artery disease. Modeling of test performance using samples from the 1000 Genomes Project revealed approximately 6% of individuals with a monogenic finding in a CVD-associated gene, 6% with a risk allele finding, 1% with a non-CVD secondary finding, and 93% with CVD-associated PGx variants. Assessment of blinded clinical samples showed concordance with prior testing. An average of 4 variants were reviewed per case, with interpretation and reporting time ranging from 9 to 96 minutes.

Conclusion: A genome-sequencing-based CVD genetic risk assessment test can provide comprehensive genetic disease and genetic risk information to patients with CVD. The semiautomated and limited interpretation burden suggest that this testing approach can be scaled to support population-level initiatives in phenotypically enriched populations.

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Introduction

Identifying the genetic underpinnings of cardiovascular disease (CVD), including both monogenic and polygenic factors, can improve patient risk stratification, refine clinical diagnoses, and inform targeted treatment opportunities.[1-4] Guidelines recommend genetic testing for patients presenting with cardiomyopathy, arrhythmias, dyslipidemias, and aortopathies, but such testing is substantially underutilized. A recent retrospective cohort study found that only 0.8% to 1.6% of patients newly diagnosed with CVD, and for whom guidelines recommended testing, received any kind of genetic investigation.[5] For those patients who do receive genetic testing, the results may be affected by interlaboratory assay variability (eg, panel vs exome), gene selection, and variant interpretation and classification.[6] Inconsistent testing practices in the CVD population can exaggerate genetic testing inequities and reduce the likelihood of reimbursement.

A growing body of evidence supports the use of genome sequencing (GS) as a first-line test, particularly in the neonatal acute care population[7] and in children with signs and symptoms of a genetic disease.[8] We reasoned that a GS-based CVD-focused genetic risk assessment test may simplify CVD genetic testing practices for both general cardiologists and subspecialists by providing a single, multifaceted, high-throughput testing platform for adult patients for whom genetic testing is recommended based on suspicion of a genetic cause for CVD inclusive of family history. This phenotype-enriched and genomically broad testing strategy could offer a pragmatic option to identify high-confidence, medically actionable variants at-scale in large clinical populations. Test selection and patient identification have been identified as barriers to cardiac genetic testing[9]; thus, this broad, single-test approach for any CVD patient who is eligible for genetic testing, specifically patients diagnosed with cardiomyopathy, arrhythmias, aortopathies, and dyslipidemia,[10,11] could lead to appropriate increases in utilization by simplifying test selection. Additionally, testing for a range of CVD phenotypes may provide additional genetic risk information that otherwise would have been missed but nonetheless informs patient care.

Here, we describe the development of the TruGenome CVD Test, including monogenic CVD and risk allele evaluations, the selection of evidence-supported pharmacogenetic (PGx) alleles, identification of a population-sensitive polygenic risk score (PRS), and approaches to reduce test interpretation and reporting time. We also report the frequency of findings in unrelated individuals in the 1000 Genomes Project cohort, providing an estimate of CVD risk and enabling an assessment of test outcomes across multiple genetic ancestries. Finally, we explore test interpretation burden and compare findings from the TruGenome CVD Test to findings in a set of clinical samples from 20 previously tested individuals with CVD. The approach and outcomes reported here may inform future integration of GS applications in the diagnosis and management of CVD and other disease areas.

Materials and Methods

CVD gene panel development

Conditions and phenotypes relevant to adults with CVD in a cardiac clinic setting were considered for inclusion in the CVD gene panel. Congenital heart defects were not considered given the complexity of genomic variation associated with congenital heart defect and their frequent initial presentation in childhood. Cerebrovascular disease was also not assessed based on the likely initial neurologic presentation of these conditions. Aortopathy, arrhythmia, cardiomyopathy, and dyslipidemia were included based on recommendations for genetic testing for adults with 1 of these clinical diagnoses.[10,11] Thrombophilia and hypertension were included given the established link with known monogenic disease and risk alleles with potential implications for medical management.

Genes associated with aortopathy, arrhythmia, cardiomyopathy, dyslipidemia, hypertension, or thrombophilia were identified through comprehensive literature review, evaluation of publicly available and internal gene-disease curations and assessment of commercially available CVD gene panels. Nuclear DNA and mitochondrial DNA genes associated with primary mitochondrial disease, which can include CVD phenotypes and present in adulthood, were identified from the Mitochondrial Disease Compendium[12] with additional review of the literature.

For monogenic conditions, gene-disease pairs were evaluated for both the quantity and quality of evidence supporting the purported gene-disease relationship (GDR). A modified version of the ClinGen Gene-Disease Validity framework was utilized and a minimum classification of definitive or strong (D/S) was implemented as the threshold for inclusion of a gene-disease pair. Briefly, for each GDR, the ClinGen framework awards points for both genetic and experimental evidence supporting the relationship and assigns a classification to the GDR based on a weighted matrix. Genetic evidence is awarded a maximum of 12 points and experimental evidence a maximum of 6 points. To reach a classification of D/S, the total score from both genetic and experimental evidence must reach 12 points. If there is not sufficient genetic evidence to reach 6 points, even with the maximum score for experimental evidence, the final classification cannot reach D/S. Scoring of evidence for GDR curation of mitochondrial DNA genes in association with primary mitochondrial disease was based on internal expertise and guidance from the ClinGen Mitochondrial Diseases Gene Curation Expert Panel. Gene

curation took place between October 2021 and July 2022 and gene panel inclusion decisions were made based on information available at the time of curation.

Gene-disease pairs with D/S GDR classifications from prior internal Illumina Laboratory Services (ILS) gene curations, ClinGen or groups that use the ClinGen framework were included in the CVD gene panel. Gene-disease pairs with a refuted or disputed relationship per internal curations, ClinGen, or groups using the ClinGen framework were not included. All remaining nuclear DNA gene-disease pairs, including those with no publicly available GDR classification, or with a moderate or lower GDR classification from any group, were curated using a modified version of the ClinGen Gene-Disease Validity framework[13] (Supplemental Figure 1). The evaluation of evidence was dependent on the classification of the GDR (ie, definitive, strong, moderate, etc) and whether the GDR source was from (1) ClinGen, (2) a group that utilizes the ClinGen framework (including ILS), (3) a group that uses an approach based on the ClinGen framework, or (4) a group that uses an alternate methodology. GDRs with a D/S classification from a group using a ClinGen framework-based approach were verified by 2 clinical scientists to evaluate if the evidence cited for the GDR met the classification criteria. A literature search was performed for select GDRs to assess if new evidence warranted an update to the available curation. GDRs deemed to have sufficient new evidence in the literature to potentially change the GDR classification were assessed using an expedited curation process, which consisted of an initial assessment of genetic evidence supporting the GDR. Experimental evidence was only evaluated for gene-disease pairs with sufficient genetic evidence scores ($\geq$6 points) to potentially be classified as D/S. *DMPK* (HGNC:2933) and *CNBP* (HGNC:13164), associated with and Myotonic Dystrophy type 1 (OMIM 160900) and type 2 (OMIM 602668), respectively, were included without additional gene curation given the established association between these genes and myotonic dystrophy, which can include cardiac arrhythmias and cardiomyopathies in their phenotypic spectrum.

Removal of common variants in CVD and non-CVD secondary finding genes from case analysis

To reduce variant triage burden during interpretation, common variants in the in silico CVD gene panel and non-CVD secondary findings genes that were unlikely to be disease causing given the penetrance and prevalence of the associated disease were proactively excluded from case analysis. Disease prevalence and average penetrance were estimated for each of the associated diseases as follows: disease prevalence and average penetrance were collected from the literature, GeneReviews,[14] and Orphanet[15] for each of the associated diseases. When specific penetrance estimates were not available from the literature, a permissive default penetrance of 80% and 40% was assigned to autosomal recessive and autosomal dominant conditions, respectively.[16] Similarly, conditions without available disease prevalence information were assigned a default prevalence of 1 in 250,000 to avoid removing variants inappropriately. This prevalence cutoff was informed by definitions of rare disease based on prevalence from the US Rare Disease Act[17] and the European Union.[18] Next, allele count and allele number, defined as the allele count in genotypes for each alternate allele and the total number of alleles in called genotypes, respectively, were extracted from the current version of gnomAD at the time of these analyses (gnomAD v2.1.1[19]) for variants that were likely to affect the protein sequence and were present with an overall minor allele frequency (MAF) $< 5\%$. MAF population cutoffs were then calculated using the following equations for diseases with autosomal dominant or X-linked and autosomal recessive inheritance, respectively.

$$AD\ or\ X-linked\ autoscore = \mathrm{Log}_{10}\frac{\left[\left(1-\left(1-95\%\ \mathrm{CI\ lower\ bound\ of}\ \frac{\mathrm{AC}}{\mathrm{AN}}\right)^2\right)*\mathrm{Penetrance}\right]}{\mathrm{Prevalence}}$$

$$AR\ autoscore = \mathrm{Log}_{10}\frac{\left[\left(95\%\ \mathrm{CI\ lower\ bound\ of}\ \frac{\mathrm{AC}}{\mathrm{AN}}\right)^2*\mathrm{Penetrance}\right]}{\mathrm{Prevalence}}$$

Variants with a score > 0 were deemed too common to be pathogenic or likely pathogenic (P/LP) based on the estimated disease prevalence and penetrance and were removed from the interpretation and reporting workflow for case analysis. Of a total 1,666,138 unique variants from gnomAD v2.1.1 in the CVD panel and the American College of Medical Genetics and Genomics (ACMG) secondary findings genes, excluding CVD-associated genes, 428,380 variants were removed with an average number of 2532 variants removed per gene (range 41-60,826).

Evaluation of CVD-risk alleles

Relevant risk alleles for adult cardiac clinic patients were identified through literature review, prior internal laboratory experience and consultation with medical genetics experts. Risk allele assessment was based on the ClinGen Low Penetrance/Risk Allele Working group guidance.[20] Each risk allele was evaluated with respect to the size of case-control cohorts reported in the literature, replication of the association between the risk allele and disease phenotype across studies, and any potential ascertainment or analysis bias. A minimum >2-fold odds ratio (OR) point estimate of increased disease risk was set as the threshold for including a risk allele, and the terminology of Established Risk Allele, Likely Risk Allele, and Uncertain Risk Allele was adopted from the ClinGen Low Penetrance/Risk Allele Working group guidance to classify the level of evidence supporting each evaluated association. Genotypes that combined more than 1 risk allele were labeled as Established Risk Genotypes, Likely Risk Genotypes, or Uncertain Risk Genotypes.

Identifying and evaluating gene-drug pairs for pharmacogenomic testing

Gene-drug pairs for PGx testing were selected based on guidance from the US Food and Drug Administration (FDA), the Clinical Pharmacogenetics Implementation Consortium (CPIC), and the Association for Molecular Pathology (AMP). Guidance from the ACMG informed the reporting strategy. Variants in genes in the FDA table of PGx associations[21] with published CPIC guidelines[22] (as of May 2022), and with evidence of a clinical and functional impact were selected for the test. These include tier 1 (recommended) and tier 2 (optional) variants from AMP publications,[23] together with variants with sufficient literature evidence as reviewed and confirmed by CPIC. Variants with uncertain clinical impact or function were excluded. Gene-drug pairs with relevance to CVD were identified based on review of the drug indications (as detailed by CPIC and the FDA) and were confirmed through consultation with a CVD pharmacogenomic subject matter expert. Both cardiovascular- and noncardiovascular-related pharmacogenomic findings were considered reportable based on the test design principle of providing all actionable clinical results that support cardiovascular patient care.

Evaluating PRSs for CVD

PRSs for CVD phenotypes, including coronary artery disease (CAD) and atrial fibrillation, were investigated for potential inclusion in the test. The extent and robustness of the PRS validation, strength of risk reclassification, and clinical utility were assessed for each score. For publications describing a CVD-related PRS the following were assessed: size and diversity of the population tested, the number of markers used in the PRS calculation, and the performance characteristics of the score. CAD PRSs were determined to have sufficient evidence to support their integration into clinical testing.

Three CAD PRS scores were evaluated for inclusion: the GPS CAD,[24] metaGRS,[25] and an ancestry-specific Allelica PRS score (Allelica, Inc).[26] The latter was chosen based on its overall performance metrics and integration feasibility given the availability of a validated software package and Docker image. A detailed description of the development and validation of the Allelica, Inc ancestry-specific CAD PRS is available elsewhere.[26] Briefly, the Allelica, Inc CAD PRS calculates genetic ancestry for each individual and a corresponding ancestry specific PRS is applied. Computed ancestry groups include African, American Admixed, East Asian, European, and South Asian. Elevated risk of CAD is defined as an individual with $\geq$2-fold increased relative risk of developing CAD compared with the remainder of the individuals in their ancestry group. The proportion of individuals in each ancestry group with a $\geq$2-fold increased risk ranges from 0.22 to 0.17 across computed ancestry groups. The OR per standard deviation (SD) for the 5 ancestry-specific PRSs ranges from 1.323 to 1.579, and the range of areas under the receiver operating characteristic curve was 0.69 to 0.78.

To inform PRS reporting, we reviewed the literature for best practices for the return of genetic risk information from a PRS[27] with particular attention to communicating PRS as population-level risk stratification uncertainty in the context of a test focused on reporting patient-specific risk information and the evidence for using PRSs for clinical decision making with respect to disease risk assessment and management.

Assessment of CVD-related findings in the 1000 Genomes Project cohort

Genome sequence data from a total of 2594 unrelated 1000 Genomes Project individuals were analyzed across the CVD test components described above. Small variants from the NYGC BWA/GATK joint call data set[28] were used to assess the burden of reportable CVD gene panel findings and non-CVD secondary findings. Small variants,

copy-number variants (CNVs), and gene-specific callers from DRAGEN v4.0.3 (Illumina, Inc)[29] were used to assess risk alleles and PGx findings. Variants in the mitochondrial genome were excluded from this analysis given the absence of information on heteroplasmy levels.

Potentially relevant monogenic variants were annotated using Nirvana (illumina.github.io/NirvanaDocumentation/) and filtered such that only those with a MAF < 1% and either classified as P/LP in ClinVar with a 2* status or with the predicted functional effect of protein truncation were included in the data set. Protein-truncating variants were defined as those that resulted in a frameshift, canonical splice disruption, or stop-gain in genes in which loss of function (LOF) is an established mechanism of disease. The determination of LOF as a disease mechanism was based on review of ClinGen resources (eg, haploinsufficiency scores, GDR curation summary statements, and applicable Variant Curation Expert Panel guidelines) and previously reported internally classified P/LP LOF variants in relevant genes.[30] Protein-truncating variants were reviewed to confirm that they would not be expected to escape nonsense-mediated decay based on their position within the protein. The MANE select transcript was chosen as the clinically relevant transcript, except in those cases in which a gene had more than 1 MANE transcript. For these genes, the clinically relevant transcript was chosen based on consensus use in ClinVar, the Human Genome Mutation Database, and MANE. In the cases in which there was no consensus, a transcript was chosen based on expression pattern, literature review, and protein length, such that the longest isoform expressed in the tissue of interest was preferred (selected transcripts are available upon request). Truncating variants in *TTN* were included only if they were in exons that are highly expressed in cardiac tissue (defined as exons with a percent spliced in of >90%) based on the established association with dilated cardiomyopathy.[31] Variants identified in more than 1 genome were manually reviewed to confirm their inclusion as likely reportable variants. Genomes with more than 1 variant identified in the same gene were also manually reviewed to rule out the presence of a single complex heterozygous allele.

The number of genomes in the data set with 1 or more variants in genes associated with autosomal dominant or semidominant conditions, the number of genomes with 2 variants in genes associated with autosomal recessive conditions, the number of unique likely reportable variants and the proportion of truncating variants that were not in ClinVar as 2* P/LP, and the number of likely reportable variants across ancestry groups were calculated.

CVD risk allele status was also determined for each genome in the data set. For each genome, the DRAGEN v4.0.3 small variant and CNV gVCFs were filtered to the 7 risk allele sites in the TruGenome CVD test. For each site, genotypes were mapped to a predefined matrix to determine the presence or absence of risk alleles.

Finally, a PGx analysis was conducted for each genome. PGx star alleles were called using the DRAGEN v4.0.3 small variant and CNV output. Star alleles were mapped to a matrix of functional or numerical scores to determine the impact for each drug-gene pair.

Proof-of-concept evaluation using previously tested clinical samples

Institutional review board approval for activities involving human samples was received from WIRB-Copernicus Group (Protocol 20241866). Informed consent was not required. To demonstrate successful test implementation from sample receipt, through analysis, interpretation and reporting, DNA from 20 individuals with a suspected hereditary CVD were tested with the TruGenome CVD Test. Individuals with indications for testing consistent with the intended use population for the test were selected from a cohort of previously tested patients at Greenwood Genetics Laboratory. Clinical testing was performed using phenotype-focused next-generation sequencing (NGS)-based panels (eg, hypertrophic cardiomyopathy and aortic dysfunction/dilation) or a comprehensive cardiac panel of more than 100 genes. Samples from individuals in the Greenwood Genetics Laboratory cohort with a range of CVD-associated phenotypes and a P/LP variant identified by clinical testing were prioritized for inclusion. ILS was blinded to prior test outcomes until after the completion of the TruGenome CVD analysis.

Variants in the CVD gene panel and non-CVD secondary findings genes were triaged and interpreted by 2 PhD clinical scientists and 1 genetic counselor, who tracked the number of variants requiring triage per case and time for interpretation, review, and reporting. Variant curations were only performed for variants determined by the analyst to have a high likelihood of being classified as P/LP and reported. Only cases with P/LP classified variants in the CVD and/or non-CVD ACMG secondary finding gene panels were reviewed by the genetic counselor. Interpretations and classifications were reviewed by a laboratory director (A.K.) who also tracked test review and reporting time. The presence of risk alleles, the PRS outcome (elevated or not elevated), and the presence of PGx variants were autogenerated by the analysis pipeline using 2 internally developed software services integrated with the variant analysis system (TruSight Software Suite, Illumina, Inc).

The number of individuals with a finding in the CVD gene panel, select CVD risk alleles, non-CVD secondary findings, elevated CAD PRS, and both CVD- and non-CVD-related PGx information was calculated.

Results

Design of the TruGenome CVD Test

Test development focused on CVD phenotypes with a high prior probability of a genetic etiology and relevance in an

adult cardiac clinic population, particularly aortopathy, arrhythmia, cardiomyopathy, dyslipidemia, CAD, hypertension, and thrombophilia. The final test design includes 5 components: a CVD gene panel, CVD risk alleles, non-CVD secondary findings, a CAD PRS, and PGx findings. Each component integrates reporting thresholds set to support the return of high-confidence, medically actionable genetic information at-scale in a phenotypically enriched CVD population (Figure 1).

CVD-gene panel

A total of 339 gene-disease pairs related to the relevant CVD phenotypes were identified from the literature, commercial gene panels, and publicly available GDR databases. One hundred thirty-nine of these 339 gene-disease pairs were included without further assessment based on a D/S GDR classification from ClinGen, ILS, and groups that use the ClinGen Gene-Disease Validity framework. Forty-five gene-disease pairs were excluded based on a refuted or disputed GDR from ClinGen, ILS or a group that uses the ClinGen framework. Using an expedited curation methodology, the remaining 155 gene-disease pairs were assessed, and an additional 76 gene-disease pairs were included, yielding a final panel of 215 gene-disease pairs (Supplemental Figure 2, Supplemental Table 1).

Cardiomyopathy was the most common CVD phenotype among the gene-disease pairs (66/215, 30%). Fifty GDRs (23%) were associated with more than 1 CVD phenotype, the majority of which included cardiomyopathy and arrhythmia (36/50, 72%). Curation of gene-disease pairs without a publicly available or ILS GDR classification led to the inclusion of 16 gene-disease pairs, associated with cardiomyopathy (7 genes), dyslipidemia (4 genes), hypertension (4 genes), and arrhythmia (1 gene). Additionally, internal review and recuration of publicly available curations led to the inclusion of 63 gene-disease pairs, representing 29% of the gene-disease pairs on the final panel (Figure 2).

To support test scalability and to prioritize the return of medically actionable information, variants identified in genes on the CVD gene panel were required to meet a P/LP variant classification threshold for return, consistent with ClinGen guidance supporting return of P/LP variants in gene-disease pairs with a D/S classification.[32]

Secondary findings gene panel

The ACMG recommends patients be offered testing for a minimum list of gene-disease pairs deemed to be medically actionable in the context of genomic-sequencing-based diagnostic tests.[33] These nondiagnostic results, or "secondary findings", include genes associated with inherited cancer risk, CVD, and metabolic conditions. In alignment with these ACMG recommendations, analysis of secondary findings genes on the ACMG list is included as an optional component of the TruGenome CVD Test. Of the 81 genes recommended for evaluation at the time of test development, 46 genes were included in the CVD panel, and the remaining 35 non-CVD genes were incorporated for testing as secondary findings. Variants identified in these 35 genes required a classification of P/LP consistent with reporting of the CVD panel and ACMG recommendations.

CVD-risk alleles

A risk allele is defined as a genetic variant, which may or may not be common in the population, that is associated with an increased probability of an individual developing a disease when compared with the baseline risk observed in a control population without the allele.[20] The evaluation of CVD-associated risk alleles was framed using recommendations from the ClinGen Low Penetrance/Risk Allele Working group.[20] The risk allele reporting threshold included classification as an Established Risk Allele or an Established Risk Genotype. Relevant risk alleles or genotypes in 6 genes were identified through a comprehensive literature review, assessment of risk alleles previously evaluated in ILS in support of a rare undiagnosed disease genome test, and through consultation with medical genetics experts. Two risk alleles and 2 risk genotypes met criteria for reporting: the Factor V Leiden variant in *F5* (HGNC:3542), the prothrombin variant in *F2* (HGNC:3535), the E2/E2 genotype in *APOE* (HGNC:613), and homozygosity or compound heterozygosity for the G1 and G2 alleles in *APOL1* (HGNC:618). (Table 1). Heterozygosity for the E2 or E4 allele in *APOE* and heterozygosity of the NM_000219.6(KCNE1):c.253G>A p.(Asp85Asn) variant (HGNC:6240) were evaluated and did not meet the threshold for reporting based on the absence of replicated case-control studies supporting an associated >2-fold increased risk of CVD and long QT syndrome, respectively.

Clinical pharmacogenomic report

PGx variants endorsed by the FDA to have a potential impact on therapeutic management recommendations, safety or response, or pharmacokinetic properties[21] and with published CPIC guidelines[22] as of May 2022, were reviewed for evidence of clinical and functional impact. Guidance from the AMP on allele selection was also reviewed.[23] Variants in 10 genes associated with a total of 38 drugs were validated for inclusion (Table 2). Variants in *CYP2C19* (HGNC:2621), *CYP2C9* (HGNC:2623), *CYP4F2* (HGNC:2645), *SLCO1B1* (HGNC:10959), and *VKORC1* (HGNC:23663) are associated with drugs with implications for the management of CVD conditions. The remaining genes are associated with drugs that may be prescribed to individuals with cardiovascular events, for example, immunosuppressants after organ transplantation, antidepressants, and pain medications. Chemotherapy, HIV treatment, and proton pump inhibitors were also included. PGx reporting follows ACMG recommendations.[34]

PRS for CAD

Comprehensive review of available PRSs and the evidence supporting their validity and potential clinical utility supported the inclusion of a CAD PRS into the test design (see Supplemental Methods). After comparing the performance

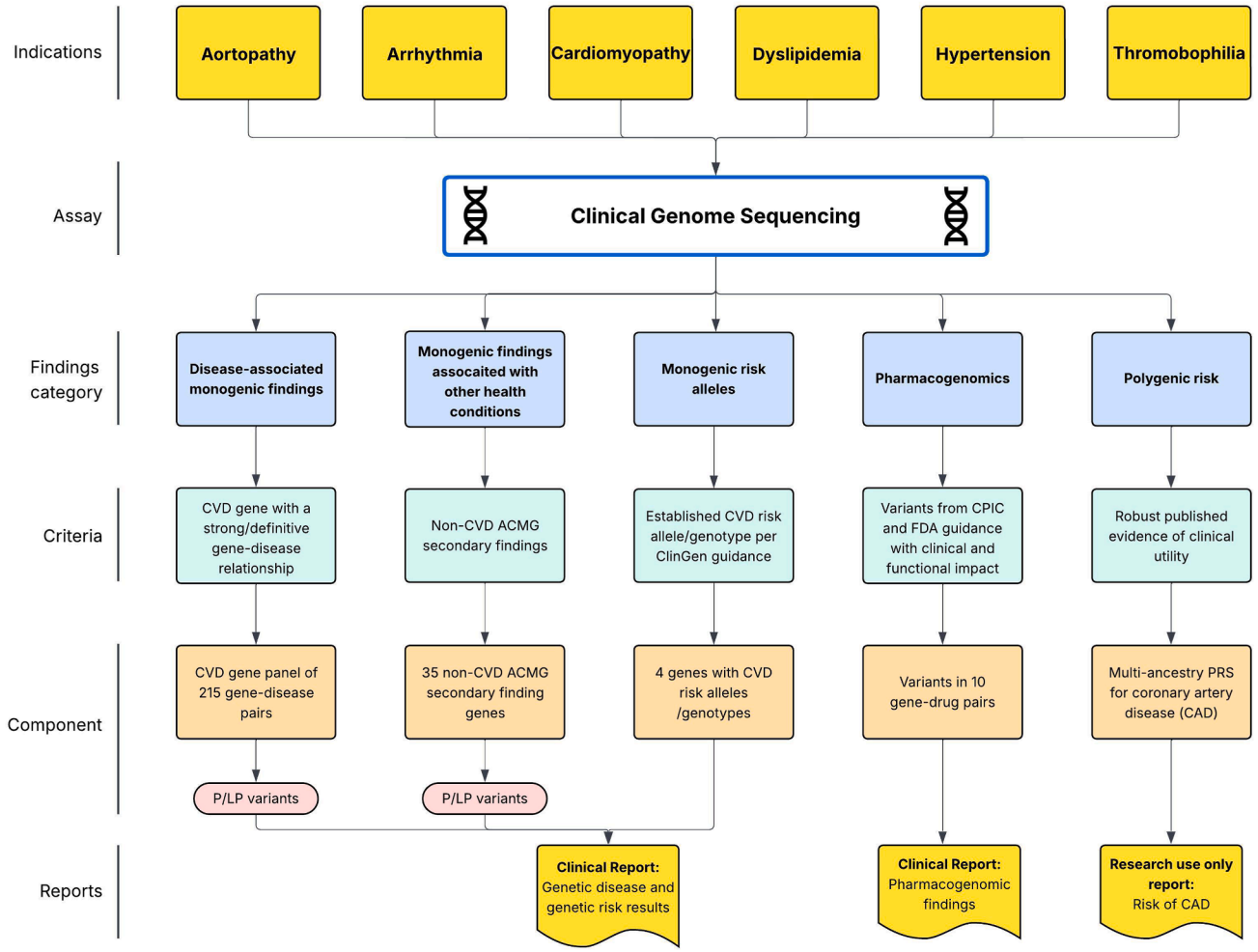

Figure 1 TruGenome CVD test design and deliverables. Test design focused on CVD phenotypes relevant to cardiac clinic patients. Monogenic conditions and risk alleles were evaluated for the strength of evidence for increased disease risk. CVD PRSs and PGx findings were also explored for inclusion. The final composition of the test includes 5 components: a 215 gene-disease pair CVD panel, the 35 remaining non-CVD ACMG secondary findings genes, 4 risk alleles or genotypes (see Table 1), PGx findings across 10 gene-drug pairs (see Table 2), and a PRS for CAD. Test deliverables include a clinical report for genetic disease and genetic risk findings, an RUO CAD PRS report and a clinical report of PGx findings.

metrics and integration feasibility, an ancestry-specific CAD PRS developed by Allelica, Inc was selected for use.[26] A bespoke CAD PRS report was developed which provides a binary outcome (elevated or not elevated). The elevated threshold was defined as an estimate that the tested individual is at 2-fold increased risk of developing CAD compared with the remainder of the individuals in their ancestry group. The upper and lower bounds of the confidence interval are reported to highlight uncertainty in the threshold estimate. Language regarding uncertainty of PRS predictions was included, inclusive of the importance of genetic variants which may not be included in the PRS calculation, medical history, family history, and lifestyle factors (Supplemental Figure 3). Given the uncertainty of the clinical utility of PRS scores the TruGenome CVD Test CAD PRS is provided as a Research Use Only (RUO) finding, and the associated report highlights that the result is not validated for use in the diagnosis, prevention, or treatment of CAD.

Evaluation of test performance in the 1000 Genomes Project cohort

To assess TruGenome CVD Test performance, the frequency of findings across the monogenic CVD genes, risk alleles, and PGx components was evaluated in 2594 unrelated individuals from the 1000 Genomes Project cohort. PRS performance was not assessed because the 1000 Genomes Project data were used in the development of the Allelica multiancestry CAD score.[26] For this analysis, likely reportable variants were defined as rare variants (MAF < 1%) in genes on the CVD and/or secondary findings gene panels that have a P/LP ClinVar 2*

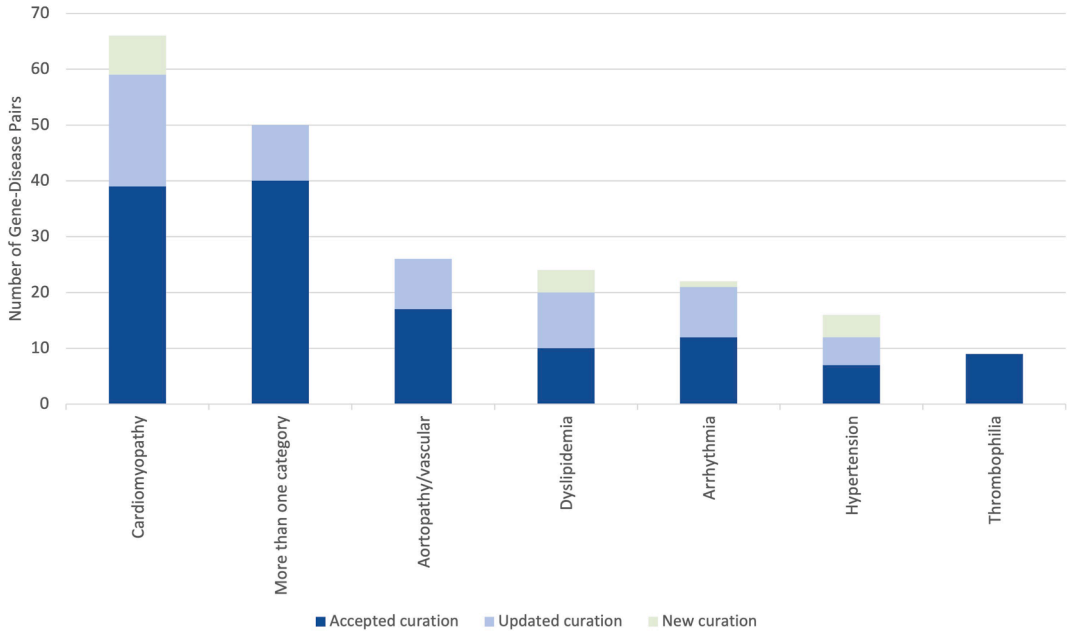

Figure 2 Number of included gene-disease pairs in each CVD phenotype by curation source. All included gene-disease pairs were classified as strong or definitive using the ClinGen Gene-Disease Validity Framework. The gene-disease pairs were sourced from 1 of 3 sources: (1) an accepted publicly available or internal curation, (2) an updated publicly available or internal curation, or (3) a new internal curation. Updated curations and curations of gene-disease pairs with no current publicly available or internal curation data led to the inclusion of 79 gene-disease pairs on the 215 gene-disease pair CVD panel.

classification or were predicted to have the functional effect of protein truncation (and thus have a high probability of being classified as LP/P) for which this variant type is an established disease-causing mechanism.

One hundred thirty-five genomes (6%) had a single likely reportable variant ($n = 131$ genomes) or 2 likely reportable variants ($n = 4$ genomes) identified in the nuclear genes associated with autosomal dominant or semidominant conditions on the CVD gene panel. No genomes had 2 likely reportable variants in a gene associated with autosomal recessive disease. The common NM_000371.4(TTR):c.239C>T p.(Thr80Ile) variant (HGNC:12405) associated with hereditary transthyretin amyloidosis was identified in 28 (21%) genomes. A total of 105 unique variants were identified, of which 37 were P/LP ClinVar 2*, and 68 were predicted truncating variants. Likely reportable variants were identified most often in African populations, which was attributable to hereditary transthyretin amyloidosis NM_000371.4(TTR):c.239C>T p.(Thr80Ile) ($n = 20$) and predicted truncating variants ($n = 17$).

Twenty-four genomes (0.9%) had a single likely reportable variant in 1 of the non-CVD secondary finding genes associated with autosomal dominant or semidominant conditions. These included 22 unique variants, 83% of which were P/LP ClinVar 2* with the remainder predicted to be truncating. Secondary findings were identified in 17 of the 27 1000 Genomes Project population groups, with Northern and Western European ($n = 6$) and African ($n = 7$) populations accounting for most of these findings.

A risk allele was identified in 159 genomes (6%) (Supplemental Table 2). The most frequently identified risk alleles were homozygosity or compound heterozygosity for the G1 and G2 alleles in *APOL1* (4% of genomes) and heterozygosity for the factor V Leiden variant in *F5* (1% of genomes).

Almost all genomes (2586, >99%) had at least 1 CVD-relevant PGx finding. Each genome had on average 2 PGx findings (range 0-5) in genes associated with a drug that has direct CVD implications and 2 PGx findings (range 0-5) in the remaining genes on the PGx test. Overall, 93% of genomes had 1 or more PGx findings in a gene associated with a drug with CVD treatment implications (Figure 3, Supplemental Table 3).

TruGenome CVD testing compared with prior genetic testing

The TruGenome CVD test was performed on a blinded cohort of 20 individuals with a clinical diagnosis of CVD who had previously received genetic testing (Table 3). Most individuals (16/20, 80%) were between the ages of 40 to 60 years old, and approximately half had cardiomyopathy as a component of their CVD phenotype (11/20, 55%).

The average number of variants triaged in genes on the CVD panel and in non-CVD secondary findings genes per case was 4 (range, 1 to 10 variants). Of the 77 variants triaged across the 20 cases, most were single-nucleotide variants (SNVs; 67/77, 87%) and in genes on the CVD gene panel (59/77, 77%).

Table 1 Cardiovascular-disease-risk alleles

Gene	Allele/Genotype	Associated Risk	Classification	Supporting Evidence
F5 (HGNC:3542)	Heterozygosity or homozygosity for NM_000130.4(F5):c.1601G>A p.(Arg534Gln),NC_000001.11:g.169549811C>T	Thrombophilia susceptibility (OMIM 188055)	Established Risk Allele	Odds ratio >2 for increased risk for thromboembolism Replication across studies
F2 (HGNC:3535)	Heterozygosity or homozygosity for NM_000506.5(F2):c.*97G>A,NC_000011.10:g.46739505G>A	Thrombophilia susceptibility (OMIM 188050)	Established Risk Allele	Odds ratio > 2 for increased risk for thromboembolism Replication across studies
APOE (HGNC:613)	Homozygosity for E2 allele [rs429358 (T:T) and rs7412 (T:T)][a]	Familial dysbetalipoproteinemia (OMIM 617347)	Established Risk Genotype	Odds ratio > 2 for increased risk for cardiovascular disease Replication across studies
APOL1 (HGNC:618)	Homozygosity or compound heterozygosity for G1 and G2 alleles[b]	Chronic kidney disease (OMIM 612551)	Established Risk Genotype	Odds ratio > 2 for increased risk for chronic kidney disease with associated hypertension Replication across studies

[a]The E2 allele consists of 2 variants: NM_000041.4(APOE):c.388T>C p.(Cys130Arg)], NC_000019.10:g.44908684T>C and NM_000041.2(APOE):c.526C>T (p.Arg176Cys), NC_000019.10:g.44908822C>T.
[b]The G1 allele consists of 2 variants: NM_003661.4(APOL1):c.1152T>G p.(Ile384Met, NC_000022.11:g.36265988T>G and NM_003661.4(APOL1):c.1024A>G p.(Ser342Gly), NC_000022.11:g.36265860A>G. The G2 allele is NM_003661.4(APOL1):c.1164_1169del (p.Asn388_Tyr389del), NC_000022.10:g.36662042_36662047del.

The time to triage, interpret, and report each case, including laboratory director review and sign out, ranged from 9 to 96 minutes. For cases that did not require a curation and without a P/LP variant ($n = 12$), the average time was 20 minutes (range 9 to 48 minutes). For those with a P/LP variant that had been previously curated, the range was 19 to 43 minutes ($n = 3$). The range was 58 to 96 minutes for cases with 1 or more reportable P/LP variants requiring curation ($n = 5$). All variants that underwent curation based on the analyst's determination of a high likelihood of P/LP classification were reported. No suspicious variants of uncertain significance (VUS) were identified.

Seven P/LP variants were identified in genes on the CVD panel (*FBN1* (HGNC:3603), *MYBPC3* (HGNC:7551) x3, *SERPINC1* (HGNC:775), *TTN* (HGNC:12403) x2), and 1 P/LP variant was identified in a non-CVD secondary finding gene (*RYR1* (HGNC:10483)) (Table 4). One or more PGx variants in genes associated with drugs determined to have direct CVD implications were identified in all 20 individuals.

All P/LP variants in the CVD gene panel identified on the TruGenome CVD test had been previously identified and reported by the clinical genetic testing performed by Greenwood Genetic Center, except a pathogenic variant in the *SERPINC1* gene, which was only detected by the TruGenome CVD test. Variants in *SERPINC1* are associated with antithrombin III deficiency. This individual had a Comprehensive Cardiac NGS Panel clinical test at Greenwood Genetic Center based on a diagnosis of obstructive cardiomyopathy and *SERPINC1* was not assessed.

Discussion

A GS-based CVD genetic risk assessment may address genetic testing inconsistencies, underutilization and underdiagnosis in CVD by providing a single, scalable platform for use in cardiology settings in which patients present with cardiovascular disease in a phenotype-enriched, clinically ascertained population. Although not intended as a traditional diagnostic test for individuals with a narrowly defined phenotype, nor as a population screen, this test offers a pragmatic intermediate-use option to identify high-confidence, medically actionable variants in a broad clinical population. Here, we report the development and validation of such a test comprising 215 high-confidence CVD gene-disease pairs, 35 non-CVD secondary findings genes, 4 risk alleles or genotypes, 10 PGx genes, and a PRS for CAD. This approach reduces the risk of false negative results that can occur when using focused NGS gene panel testing[35] and enables the evaluation of complex CVD presentations that may have multiple genetic drivers. A broad testing approach also provides the option to identify ancillary genetic risk information, including medically actionable secondary findings and PGx information.

Table 2 Gene-drug pairs included in pharmacogenetic testing

Gene	Associated Drug(s)	Variant/Star Allele(s) Included
CYP2C19 (HGNC:2621)	Citalopram, Clopidogrel, Doxepin, Escitalopram, Lansoprazole, Omeprazole, Pantoprazole, Voriconazole	*2, *3, *4.001, *4.002, *5, *6, *7, *8, *9, *10, *17, *35
CYP2D6 (HGNC:2625)	Amitriptyline, Atomoxetine, Clomipramine, Codeine, Desipramine, Doxepin, Imipramine, Fluvoxamine, Nortriptyline, Paroxetine, Tamoxifen, Tramadol, Trimipramine	*2, *3, *4.013, *4, *5, *6, *7, *8, *9, *10, *12, *13, *14, *15, *17, *21, *29, *31, *36, *40, *41, *42, *49, *56, *59, *68, copy-number variants, tandem and hybrid variants
CYP3A5 (HGNC:2638)	Tacrolimus	*3, *6, *7
CYP2C9 (HGNC:2623)	Celecoxib, Flurbiprofen, Fosphenytoin, Ibuprofen, Meloxicam, Phenytoin, Piroxicam, Warfarin	*2, *3, *5, *6, *8, *11, *12, *13, *14, *15
VKORC1 (HGNC:23663)	Warfarin	NC_000016.10:g.31096368C>T [c.-1639G>A (rs9923231)]
CYP4F2 (HGNC:2645)	Warfarin	*3
DPYD (HGNC:3012)	Capecitabine, Fluorouracil	c.[1236G>A;1129-5923C>G] (rs56038477; rs75017182), c.1679T>G (rs55886062), c.1905+1G>A (rs3918290), c.2846A>T (rs67376798), c.557A>G (rs115232898)
HLA-B (HGNC:4932) (*57:01 proxy: *HCP5*)	Abacavir	NC_000006.12:g.31464003T>G (n.852T>G) (rs2395029)
SLCO1B1 (HGNC:10959)	Simvastatin, Atorvastatin, Rosuvastatin	*5
TPMT (HGNC:12014)	Azathioprine, Mercaptopurine, Thioguanine	*2, *3A, *3B, *3C, *4

Gene curation is a necessary, and sometimes overlooked, step in robust clinical test development. The level of evidence supporting the association between a gene-disease pair affects whether an identified variant in a gene would be expected to increase risk for the associated disease. The gene curation efforts described here expanded the CVD gene panel from 139 gene-disease pairs with previously curated D/S classifications to a total of 215 gene-disease pairs. Clinical laboratories, population genomic studies, and other institutions performing genomic analysis for individuals with CVD may consider utilizing this panel as a resource. Additions and subtractions are expected to be necessary as new information becomes available about these and other genes.

Polygenic risk scores have shown utility for population-based risk stratification, but their applicability to clinical medicine as individual level risk estimates across diverse ancestry groups is under investigation.[36] Data from a recent studies support benefits and changes in patient management with the return of combined CAD PRS and monogenic disease risk information.[37] However, more evidence is required to understand the clinical utility of the application of PRSs across ancestry groups and to inform best practices for PRS reporting to patients and providers. This ambiguity was a factor in our decision to return the CAD PRS finding as an RUO finding. Evidence generated from clinical trials integrating CAD PRSs as a component of a comprehensive genome-based test may help to fill these gaps and inform future integration of PRSs into clinical practice.

Although the intended use of the TruGenome CVD Test is for adult patients with a diagnosis of CVD that may be genetic, the test development approach described here can inform the development and implementation of GS-based CVD risk assessment tests for broader populations. The rate of 6% of genomes having 1 or more likely reportable variant in the CVD gene panel aligns with expectations based on disease prevalence and penetrance.[38] Based on our test performance evaluation in a small cohort of individuals with CVD, an average of 4 variants required triage per case, and the average time for interpretation and reporting for cases without a P/LP variant was 20 minutes. This is in contrast to previously published rapid exome or GS studies in which mean sample-to-report turnaround times range from 5 to 43 days, with laboratory processing time inclusive of interpretation averaging approximately 14 days.[39-42] This is an imperfect comparator but, nonetheless, suggests that TruGenome CVD interpretation is relatively low-burden and unlikely to be the primary bottleneck to the provision of testing. Taken together, these observations suggest that the TruGenome CVD Test and similar broad GS-based CVD risk assessment tests are likely amenable to population scale implementation, given that most individuals would not have a P/LP variant identified and therefore would not require time intensive interpretation and reporting. Additionally, given the low anticipated proportion of individuals with 1 or more P/LP variants, large-scale testing would not be expected to lead to a high burden of additional clinical follow-up and cost.

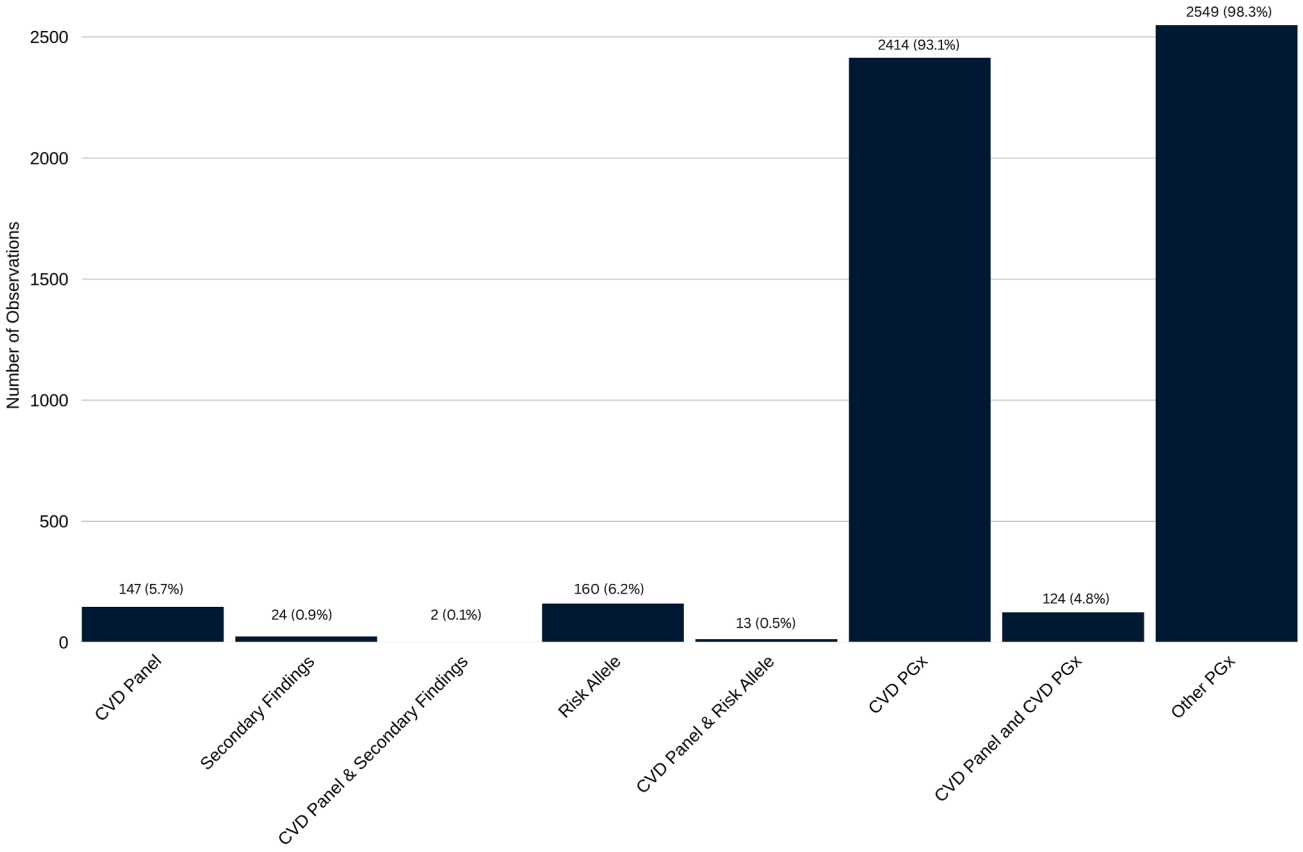

Figure 3 Genetic disease, risk and PGx findings in an unselected popuation. To ascertain TruGenome CVD Test performance, the frequency of monogenic disease, risk allele, and PGx findings was evaluated in 2,594 unrelated individuals in the 1000 Genomes Project cohort. Six percent of genomes had a finding in a CVD-associated gene, 6% had a risk allele finding, 0.9% had a non-CVD-associated ACMG secondary finding, and 93% had at least 1 CVD-associated PGx variant.

This work has several limitations. The TruGenome CVD test definition was intentionally restricted to genes that met stringent gene-disease evidence criteria. Given historical challenges with VUS recategorization, and the increased possibility for the delivery of false positives, the conservative yet broad testing strategy described here may be an efficient approach for large-scale testing in individuals with CVD-associated phenotypes. This, of course, carries the trade-off of providing less detailed analyses of genes of potential interest. In some cases, the more traditional testing approach of including all variants may be desirable, eg, in scenarios in which a provider has a particular expertise where it could be beneficial to interrogate genes with moderate evidence of disease association and return VUS with limited evidence. The CVD gene panel content reported here may not represent all relevant gene-disease pairs with a D/S association because available evidence to evaluate GDRs is continually generated. Similarly, there are likely other CVD risk alleles that could meet the threshold for inclusion that were either not evaluated or did not have sufficient evidence to support inclusion at the time of test development. The proportion of genomes with a PGx finding in a gene-drug pair with direct CVD implications was not interpreted in the context of the dosing algorithm for warfarin, which incorporates the alleles tested in *CYP2C9, VKORC1* and *CYP4F2* in combination to modify warfarin dosing.[43] The proof-of-concept evaluation of $n =$

Table 3 Age, phenotypes, and clinical testing details for samples from individuals at risk for genetic CVD

Variable	Number of Individuals
Age (years)	
20-40	2
40-60	16
>60	2
Phenotype(s)	
Aortopathy/aneurysms	5
Arrhythmia only	1
Cardiomyopathy and arrhythmia	3
Cardiomyopathy and/or heart failure	8
Hyperlipidemia	3
Clinical genetic testing	
Aortic dysfunction/dilation panel	5
Cardiomyopathy panel	7
Comprehensive cardiac panel	5
Familial hypercholesterolemia panel	2
Long QT syndrome panel	1

Table 4 Findings in individuals with a suspected diagnosis of genetic CVD

Category of Result	Individuals with a Reportable Finding *N*, (% of Total)
CVD gene panel	7, (35)
CVD risk allele	
F5 (HGNC:3542)	0, (0)
F2 (HGNC:3535)	1, (5)[a]
APOE (HGNC:613)	0, (0)
APOL1 (HGNC:618)	1, (5)[b]
Non-CVD ACMG secondary findings	1, (5)
Elevated CAD PRS	0, (0)
Pharmacogenomic finding(s)	
CVD-related	20, (100)
Non-CVD related	16, (80)

[a]Heterozygosity for the c.*97G>A risk allele variant in F2 was identified.

[b]The *APOL1* genotype G1/G2 was identified.

20 patients was intended to demonstrate feasibility of end-to-end implementation of the TruGenome CVD Test, including triage, interpretation, and reporting within a real-world workflow. These data are complementary to—but distinct from—planned large-scale clinical analyses such as the CardioSeq Study, which will evaluate test performance, diagnostic yield, and clinical utility across diverse populations and care settings, and there is a small chance that these findings are not generalizable to other laboratory workflows. Finally, time estimates in the test performance evaluation were based on the use of internally developed software for the reporting of risk alleles, the CAD PRS, and PGx findings without manual data review.

GS as a platform for CVD genetic risk assessment in adult patients seen in cardiology settings with phenotypes for which genetic testing may be appropriate may increase access to high-confidence, clinically meaningful genomic information. The TruGenome CVD Test is optimized for throughput and specificity and designed to support genome-based risk assessment in a phenotype-enriched population with elevated likelihood of inherited disease. This hybrid approach enables scalable return of actionable results in general cardiology contexts and may complement more traditional diagnostic evaluations when warranted. This approach transferrable to other disease indications and supports the use of genetic data across the lifespan of an individual. As the cost of sequencing continues to decrease and the number of potentially actionable findings across the genome increases, it may become both economical and efficacious to systematically use genome or exome sequencing as the backbone for genetic assessments.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

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Ethics Declaration

Institutional review board approval for activities involving human samples was received from WIRB-Copernicus Group (Protocol 20241866). Informed consent was not required.

Conflict of Interest

Laura M. Amendola, Alison J. Coffey, Josh Lowry, James Avecilla, Alka Malhotra, Aditi Chawla, Julie P. Taylor, Revathi Rajkumar, Carolyn M. Brown, Katie Golden-Grant, Rueben Hejja, Tasha Kalista, Phillip Medrano, Becky Milewski, Felipe Mullen, Andrew Walker, Adriana Huertez-Vasquez, Mauro Longoni, Keisha Robinson, Denise L. Perry, Damon Hostin, Subramanian S. Ajay, Akanchha Kesari, Samuel P. Strom, Elliott Margulies, and Ryan J. Taft were employees and/or shareholders of Illumina, Inc. when this work was completed. Ryan J. Taft was an employee of Tempus Inc. during manuscript preparation. David E. Lanfear is a consultant for Abbott Laboratories, ARMGO, AstraZeneca, Illumina, Janssen, and Martin Pharmaceuticals, and has participated in clinical research from Akros, AstraZeneca, Bayer, Illumina, Janssen, Lilly, and Pfizer, and has a patent (held by Henry Ford Health) for a beta blocker polygenic score.

Additional Information

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