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Exome Sequencing in Suspected Monogenic Dyslipidemias

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Abstract:

Background - Exome sequencing is a promising tool for gene mapping in Mendelian disorders. We utilized this technique in an attempt to identify novel genes underlying monogenic dyslipidemias.

Methods and Results - We performed exome sequencing on 213 selected family members from 41 kindreds with suspected Mendelian inheritance of extreme levels of low-density lipoprotein (LDL) cholesterol (after candidate gene sequencing excluded known genetic causes for high LDL cholesterol families) or high-density lipoprotein (HDL) cholesterol. We used standard analytic approaches to identify candidate variants and also assigned a polygenic score to each individual in order to account for their burden of common genetic variants known to influence lipid levels. In nine families, we identified likely pathogenic variants in known lipid genes (*ABCA1*, *APOB*, *APOE*, *LDLR, LIPA,* and *PCSK9)*; however, we were unable to identify obvious genetic etiologies in the remaining 32 families despite follow-up analyses. We identified three factors that limited novel gene discovery: (1) imperfect sequencing coverage across the exome hid potentially causal variants; (2) large numbers of shared rare alleles within families obfuscated causal variant identification; and (3) individuals from 15% of families carried a significant burden of common lipid-related alleles, suggesting complex inheritance can masquerade as monogenic disease.

Conclusions - We identified the genetic basis of disease in nine of 41 families; however, none of these represented novel gene discoveries. Our results highlight the promise and limitations of exome sequencing as a discovery technique in suspected monogenic dyslipidemias. Considering the confounders identified may inform the design of future exome sequencing studies.

Key words: genetics, human, DNA sequencing, exome, lipids, exome sequencing, mendelian genetics

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Introduction

"Exome" sequencing refers to the use of next generation sequencing (NGS) technology[1] to sequence all protein-coding regions of the genome. This approach has emerged as a promising tool for gene discovery in families with suspected monogenic disorders[2] with some reports suggesting a success rate in excess of 50%[3]. Identifying the genetic basis underlying monogenic forms of dyslipidemia has revealed insights into human biology[4] and spurred the development of novel therapeutics[5]. In an attempt to map novel dyslipidemia genes, we performed exome sequencing on 213 selected family members from 41 kindreds with suspected Mendelian inheritance of extreme levels of low-density lipoprotein cholesterol (LDL-C) or high-density lipoprotein cholesterol (HDL-C). To enrich for novel gene discoveries, we excluded probands from high LDL-C families that had mutations in genes known to cause monogenic hypercholesterolemia.

Methods

Subject Recruitment

Forty-one families of European ancestry with suspected Mendelian inheritance of extreme LDL-C or HDL-C levels were recruited from eight different centers across North America and Europe. The pedigrees of these 41 families are shown in Supplementary Figure 1. Families A1-A9 were recruited as part of the French National Research Network on Hypercholesterolemia that includes clinicians from 11 different cities in France. Probands were selected if they met the following criteria: total and LDL-C levels above the 95th percentile when compared with a sex- and age-matched French population[6], triglyceride level below 1.5 mmol/L, and presumed autosomal dominant transmission of hypercholesterolemia in the family. Family A10 was recruited from the Preventive Cardiology/Lipid Clinic of the McGill University Health Centre.

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Affected individuals had LDL-C concentration exceeding the 95th percentile for age- and gender-matched subjects, a plasma triglyceride concentration less than 1.0 mmol/L, and no known secondary causes of hypercholesterolemia. Families A11-A14 were recruited from the Lipid Clinic at the Academic Medical Center, University of Amsterdam, the Netherlands based on a clinical diagnosis of familial hypercholesterolemia in the proband. LDL-C levels exceeding the 95th percentile when adjusted for age and gender defined affected family members. Families A15-A20 were recruited from the Lipid Clinic of the University Hospital of Palermo. The Simon-Broome Register criteria were used to clinically diagnose heterozygous autosomal dominant hypercholesterolemia after excluding secondary hypercholesterolemia. In family A20, a pathogenic mutation in *LDLR* (c.2390-1G/A) was discovered previously but displayed incomplete penetrance (Supplementary Figure 1; A20, individuals shaded in black) and was not present in the individual with the highest level of LDL-C (the proband III:1 did not carry the LDLR mutation and had LDL-C = 455 mg/dL in addition to a history of myocardial infarction at the age of 35). Two other subjects (Supplementary Figure 1; A20, individuals shaded in blue) also showed high LDL-C and did not have the *LDLR* mutation. Based on a review of the pedigrees (Supplementary Figure 1) an autosomal dominant mode of inheritance was presumed for Families A1-A20 with the exception of A13 in which an autosomal recessive mode of inheritance was presumed.

Families B1 and B2 were recruited from the University Hospital of Palermo and Modena-Reggio Emilia. Families B3-B12 were recruited from the Washington University Lipid Research Clinic. Affected individuals in these families were identified due to an LDL-C level corresponding to the bottom 5th percentile when adjusted for age, ethnicity, and gender. The proband (subject III:A) in family B13 was referred to the MGH Lipid Metabolism Unit due to a

LDL-C value of 25 mg/dL. She was noted to have 4 family members with LDL-C values less than 47 mg/dL. An autosomal recessive mode of inheritance was presumed for family B1 while an autosomal dominant mode of inheritance was presumed for B2-B13 based on the pedigrees (Supplementary Figure 1).

Family C1 was recruited as part of the Genomic Resource in Arteriosclerosis and Metabolic Disease at the Cardiovascular Research Institute of the University of California, San Francisco. The clinical diagnosis of familial hypoalphalipoproteinemia was based on levels of HDL-C below the 5th percentile for five individuals, and below the 10th percentile for one individual, when adjusted for age, sex, and the known inverse relationship between TG and HDL-C. Family C2 was recruited from the Preventive Cardiology/Lipid Clinic of the McGill University Health Centre. Families C3 and C4 were recruited from the outpatient clinic for Vascular Medicine at the Academic Medical Center, University of Amsterdam, the Netherlands. Affected individuals from families C2-C4 had HDL-C concentration below the 5th percentile for age- and gender-matched subjects, a plasma triglyceride concentration less than 1 mmol/L, and no known secondary causes of hypoalphalipoproteinemia. An autosomal dominant mode of inheritance was presumed for families C1-C4 based on the pedigrees (Supplementary Figure 1).

The probands in families D1 and D2 were ascertained from a general patient population in the center of The Netherlands and were selected based on having HDL-C levels above the 99th percentile after adjusting for age and gender[7]. Family members with HDL-C levels above the 95th percentile for age- and gender-matched subjects were considered affected. Families D3 and D4 were recruited at the Perelman School of Medicine at the University of Pennsylvania as part of a study enrolling individuals with HDL-C levels above the 75th percentile for age-, race-, and gender-matched subjects. Spouses and blood relatives of affected individuals were also recruited.

An autosomal dominant mode of inheritance was presumed for families D1-D4 based on the pedigrees (Supplementary Figure 1).

Causal mutations in *LDLR*, *APOB*, and *PCSK9* were excluded in the probands of families A1-A20 as previously described[8, 9]. In addition, causal mutations in *LDLRAP1* were excluded in the proband from family A13 in which an autosomal recessive mode of inheritance was presumed. Candidate gene sequencing was not performed in the other families.

Replication in Japanese Families

The families shown in Supplementary Figure 6 (Families A-D) were recruited from Kanazawa University Hospital in Kanazawa, Japan. The probands in Families A, C, and D were identified due to high LDL-C values and tendinous xanthomas. An off-treatment LDL-C value was not available for the proband in Family B; her LDL-C value was normal, however she was on intensive lipid-lowering therapy and was noted to have tendinous xanthomas. Affected relatives in Families A-D had LDL-C values exceeding 200mg/dL. An autosomal dominant mode of inheritance was presumed for all four families based on the pedigrees (Supplementary Figure 6). All individuals in Families A-D were of self-described Japanese ancestry.

Exome Sequencing

Subsets of samples from each family were selected for exome sequencing based on DNA availability, presence of informed consent allowing for genetic studies, and prioritization of phenotypic extremes. These selected samples underwent exome sequencing at the Broad Institute. The IRB at the Broad Institute and all participating sites approved the study protocols and all individuals who were selected for sequencing provided informed consent. Randomly sheared genomic DNA was used as input for library construction and in-solution hybrid selection to enrich for exomic DNA as previously described[10]. In all samples except seven, 33Mb of

genomic sequence was defined as the "exome" and targeted using the Whole Exome Agilent 1.1 plus boosters preparation kit (Agilent Technologies, Santa Clara, CA, USA). The remaining seven samples underwent hybrid selection using a prior version of the Agilent whole exome preparation kit that targeted 28.6 Mb of genomic sequence. Exome-enriched DNA for each sample was then sequenced on an Illumina GA-II sequencer using 75-base pair paired-end reads. Samples were sequenced with a goal of achieving at least 20-fold coverage in at least 80% of targeted bases. Samples with less than 80% of targeted bases covered by ≥ 20 sequencing reads were not used for the primary analysis.

The Burroughs-Wheeler Alignment algorithm[11] was used to map raw sequence reads to the human reference sequence (UCSC build HG19). The Genome Analysis Toolkit (GATK version 2)[12] and SAMtools[13] were used to locally realign reads, recalibrate individual base qualities, and flag duplicate sequencing reads for removal. The GATK UnifiedGenotyper (UG) was then used to identify single nucleotide variants and small insertions and deletions in the exome target definition specific for each sample along with up to 50 flanking intronic bases. The UG was used in multisample mode and samples were grouped into three batches keeping related samples together when possible. The GATK Variant Score Recalibration tool was used to update the quality score of the identified variants and SnpEff[14] was used to predict the functional consequences of each variant. The population allele frequency of each variant was estimated using the National Heart Lung and Blood Institute's Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS).

Analysis of Exome Sequencing Data

To exclude genetic variation unlikely to be causal for the extreme phenotype in the affected individuals from the families, we employed a heuristic analytical process commonly used in the

analysis of exome sequencing studies[15]. Starting with the total number of variants shared by individuals from the family, we excluded variation that did not fit the expected pattern of inheritance based on examining the pedigree. Next, we excluded common genetic variation, defined as ≥ 1% frequency in the population (given the extreme excess of very rare alleles in the human population[16], the exact choice of this threshold – i.e. 1% or 0.01% – has little practical impact since most rare alleles within families are well below this threshold). On the assumption that a specific causal variant could not be responsible for both high and low lipid levels, we excluded variation present in the affected individuals of the opposite extreme. Finally, we excluded silent and non-genic variation as most Mendelian syndromes are caused by coding or splice site mutations that alter the protein sequence[17]. The remaining single nucleotide variants and short insertions or deletions were considered candidates. When possible, from this list of candidates we attempted to identify variants demonstrating co-segregation with the phenotype in the extended kindred. We considered candidate mutations as causal if (1) the mutation was identified in prior publications as causal for the same phenotype; (2) if the mutation was novel but in a gene known to cause the phenotype and functionally similar to causal mutations in that gene (i.e. a novel nonsense mutation occurring in a gene in which other nonsense mutations have been shown to be causal), or (3) if the mutation was novel and occurred in a novel gene but demonstrated co-segregation with the phenotype in the extended kindred. The 95% confidence intervals (CI) surrounding the success estimates were estimated from the binomial distribution.

Polygenic score analysis

To determine the likelihood that polygenic inheritance could explain the extreme lipid phenotype in some families, individuals with sufficient DNA (n=130) were genotyped on the Illumina HumanExome Beadchip v1.0 according to the manufacturer's recommended protocol. This

genotyping array includes the SNPs reported in the Global Lipids Genetic Consortium (GLGC) meta-analysis of genome-wide association studies of plasma lipid levels[18]. Of the 102 SNPs reported in GLGC Table 1, we successfully genotyped 87 SNPs plus 4 proxies ($r^2 > 0.9$ with the GLGC SNP). Using all 91 SNPs (all SNPs were used for each lipid trait since some of the SNPs are associated with more than one lipid fraction), we built baseline polygenic models for the LDL-C and HDL-C phenotypes in 9,134 subjects not taking lipid-lowering medications from the Ottawa Heart Study[19], PROCARDIS[20] and the Malmo Diet and Cancer Study[21] to obtain estimated regression coefficients. Next, we used the estimated coefficients to obtain a predicted lipid level for each individual in our study based on these 91 SNPs. This predicted lipid level was the population mean plus the sum of the individual's observed genotypes weighted by the estimated coefficients. We calculated a residualized phenotype for each individual by subtracting the observed lipid level from the predicted lipid level based on the common SNPs. The observed lipid levels were either obtained off treatment or adjusted for lipid-lowering treatment by dividing the observed value by 0.7. Externally standardized residuals were created to assess the statistical significance of each individual's residualized phenotype. We used a threshold of ≤ 0.01 (a Bonferroni correction for the average number of individuals sequenced in each family) to define a significant residualized score.

Results

We performed exome sequencing on 213 selected individuals from the 41 families with suspected monogenic inheritance of extreme lipid levels, with a median of 4 individuals selected per kindred. On average, the mean coverage of targeted bases for each individual was 103. We identified an average of 12,544 nonsynonymous single nucleotide variants and 802 insertion/deletions per individual. Within each kindred, we used standard approaches to identify

candidate variants[15] (Supplementary Figure 2). In five families (12%), we identified likely pathogenic variants (Table 1) in genes previously proven to cause monogenic dyslipidemias ("known lipid genes", Supplementary Table 1). We also identified the genetic etiology in three families after follow-up analysis of their candidate variants (Supplementary Figure 1, Families A1[26], A10[30], and A13[27]) and one after considering the effect of common genetic variants (described below), bringing the total to nine (22%; 95% CI [9.3%,34.7%]) (see Table 1 and Supplementary Table 2 for details).

In the remaining 32 families however, the number of candidate variants ranged from 0-287, without obvious genetic etiologies despite follow-up analyses. We sought to understand potential reasons for the lack of novel gene discovery and identified three main confounders: 1) an inability to identify potentially causal variants due to imperfect sequencing coverage; 2) an inability to identify the causal variant among hundreds of shared variants within families; and 3) an inability to identify the effect of complex genetics using exome sequencing.

To successfully discover a causal variant, the variant must first be identified. We find that despite high average coverage across the exome (on average 89% of targeted bases are covered with ≥ 20 reads; see Figure 1A), a small but substantial portion of the exome is poorly covered across all affected individuals. Across the known lipid genes we find affected individuals have, on average, 3.7% of targeted bases covered with ≤ 10 sequencing reads (Figure 1a), a sequencing depth that provides 99% confidence of observing a rare allele at least twice. At these positions there is a chance we would fail to identify a variant in the affected individual with shallow sequencing coverage and these positions would then be removed from consideration under the assumption of complete penetrance without phenocopies.

Family A1 (Supplementary Figure 1) illustrates this problem. In this family, affected

individuals were identified to harbor a pathogenic *APOE* deletion[26]. Initially, the pathogenic deletion (p.Leu167del) was only identified in two of three affected individuals using exome sequencing and was thus removed from further consideration. When orthogonal methods (linkage analysis and sequencing under linked peaks) identified p.Leu167del as a candidate, we performed Sanger sequencing to confirm the presence of the deletion in all affected individuals. This mutation occurs in the last exon of *APOE* which is difficult to capture and sequence with NGS[31] and has the lowest coverage and highest GC content of the known lipid genes (Supplementary Tables 3 and 4). The individual in Family A1 initially misclassified by NGS only had one sequencing read at that position of the genome. Across the remainder of the exome, we find affected individuals have, on average, 6.4% of targeted bases covered with ≤ 10 sequencing reads (Figure 1a). This effect appears to be independent of overall sequencing depth (Supplemental Figure 3) suggesting it cannot be solved simply by sequencing to deeper overall coverage. It has been previously suggested that exome sequencing fails to identify the genetic basis of some strongly inherited conditions due to causal non-coding mutations[32]; another explanation could be that the causal variant is present in the coding region but hitherto unidentified.

Second, we find a confounding effect from the many rare alleles within families that also segregate with the phenotype by chance. This is highlighted by examining the total number of candidate variants even in families harboring pathogenic variants in a known lipid gene. In these families, between 2-346 additional variants remain candidates at the end of the analysis and would be considered potentially causal if a pathogenic variant had not been identified. This is similar to the number of variants remaining in families without known genetic causes (range 0-287; see Figure 1b), highlighting the vast amount of very rare variation "private" to families that

segregates with the disease phenotype merely by chance.

Third, we also find that the effect of complex genetics in families with suspected monogenic dyslipidemias can be substantial. Both LDL-C and HDL-C levels are influenced by multiple common genetic loci[18]; we[18] and others[33] have previously demonstrated that polygenic inheritance may be sufficient to explain extreme lipid phenotypes. To address this possibility in the families sequenced in the present study, we genotyped a set of common genetic variants robustly associated with lipid traits in genome-wide association studies[18]. Using these common variants, we created a polygenic score and calculated a residualized phenotypic z-score, effectively assigning a level of statistical significance to each individual's lipid level after correcting for that individual's burden of common lipid-related alleles (Supplementary Figure 4).

As a proof-of-principle, we find highly significant scores for individuals from families A9 and B2, in which pathogenic *PCSK9* and *APOB* mutations, respectively, perfectly segregate with disease status, indicating that common genetic factors are not sufficient to explain the phenotype in these families (Supplementary Table 5). In contrast, of the families without a readily apparent genetic answer, we find six (15% of all families) where either all affected individuals have non-significant scores or only one affected individual retains a significant score, suggesting that the burden of common alleles is sufficient to largely explain the extreme phenotype in these families (Supplementary Table 6 and Supplementary Figure 5).

We also find this approach can help refine phenotypic definitions within families. In Family A7, an initial analysis using clinically-defined affection status (Figure 2a) yielded 17 candidate variants; additional analyses were unable to identify a causal variant from this list. Using the polygenic score, we found individuals III-1 and III-2 had LDL-C levels that were largely explained by a burden of common variants whereas individuals II-3 and III-5 had highly

significant residualized scores (Supplementary Table 7). An analysis using these updated phenotypes identified a candidate variant in *LDLR* (p.E228K, also known as FH Modena, previously shown to be pathogenic[28]) that was subsequently confirmed to perfectly segregate with extreme LDL-C levels in the extended kindred (Figure 2b).

We attempted to extend these findings to a non-European population and found similar results in families of Japanese descent with extreme LDL-C levels (Supplementary Figure 6). In this analysis a pathogenic variant in *LDLR* was found in one family (Supplementary Table 8) while there was no clear molecular etiology in the remaining three, resulting in a 25% success rate. We found similar levels of imperfect sequence coverage and numbers of rare variants segregating within the family (Supplementary Table 9) compared to the families of European descent.

Discussion

The present study summarizes our experience using exome sequencing to map novel genes in families with a suspected Mendelian dyslipdemia. After sequencing the exome in 213 individuals across 41 kindreds, we find this technique identifies a likely causal variant in 22% of cases. Three of these families harbor causal mutations in known lipid genes that were excluded by candidate sequencing prior to entry into this study, reconfirming previous results that candidate gene sequencing can fail to identify causal mutations in candidate genes that are subsequently identified via NGS[34]. Notably, we did not identify any novel monogenic dyslipidemia genes. From the remainder of the families in our study, we identify evidence of polygenic inheritance in 15%. We are, however, currently unable to define the genetic basis in the remaining 63% of families (Figure 3).

Several conclusions emerge from these results. First, from this empiric evaluation, we

find the yield of exome sequencing as a tool for novel gene mapping to be modest. Multiple reports detailing the success of this technique have been published since 2009[2]; however, these reports may be susceptible to the well-known bias to publish positive results. Overall, we are unaware of reports detailing the overall success rate of exome sequencing. Our study highlights the "real-world" challenges in using this technique for mapping novel genes in Mendelian disorders and appears to reflect the collective experience in monogenic dyslipidemias as evidenced by the current literature. We are unaware of a Mendelian dyslipidemia gene other than *ANGPTL3*[35] that has been identified using exome sequencing.

Second, we identified several technical issues that have not been previously highlighted that adversely impacted our ability to discover novel genes. An underlying assumption when mapping genes with NGS is that all potentially causal variants will be identified. Our study reveals some of the limitations of exome sequencing which may be useful to address in future design of research or clinical sequencing studies.

Third, we find a substantial concerted effect of common lipid-related alleles that appears to result in extreme phenotypes in some individuals and families. A similar effect has been shown previously for samples drawn from the extremes of the population distribution of plasma lipids[18] and in a significant proportion of mutation-negative probands who underwent clinical genetic testing for familial hypercholesterolemia[33]. While only approximately 10% of the total phenotypic variance in lipid traits is explained by the common variants genotyped in our study[18], by removing this variance we were able to create a more refined phenotype and enrich for genetic factors not present in the set of common variants. We show that incorporating this information may inform genetic mapping as we have demonstrated above in Family A7.

Our study has several limitations. Exome sequencing has limited reliability to identify

large structural variants and while we did consider small insertions and deletions, this technique is less reliable in identifying these compared with single nucleotide substitutions. Reliable incorporation of these forms of genetic variation might identify additional candidates[36]. We also did not consider genome-wide linkage data due to the small size of the pedigrees and incorporating such information has the potential to reduce the number of candidates[37]. From a technical perspective, it is possible that other exome capture reagents or sequencing platforms (including longer reads) may result in more complete coverage. Finally, it is important to note this experience may not necessarily generalize to other phenotypes. For example, one might expect the yield to be higher for studying extreme syndromic phenotypes less susceptible to the influences of polygenic inheritance and environmental factors.

Addressing the three problematic areas outlined above has the potential to improve the success of gene mapping. Whole genome sequencing (WGS) could be used to remove the bias of target definition and hybrid selection inherent in exome sequencing, although we recognize certain portions of the genome will remain recalcitrant to NGS technology[38]. The process for identifying causal rare alleles within families can be improved as larger population-based sequencing studies are performed and as better high-throughput functional assays are developed. Sequencing more distantly related relatives can decrease the total number of shared alleles; however, investigators typically resort to exome sequencing in small pedigrees for which linkage analysis has been intractable. Finally, as we identify additional alleles contributing to complex phenotypic traits, we can use these findings to inform family-based genetic studies by both selecting families without significant polygenic inheritance and refining phenotypic definitions within families.

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Conflict of Interest Disclosures: None

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Table 1: Genetic etiologies identified from exome sequencing

Family (Trait)	Gene	Genomic position*	Reference allele	Alternate allele	Effect †	Notes
Genetic etiology discovered during initial exome sequencing analysis						
A4 (high LDL)	*APOB*	2:21229554	C	T	p.A3396T	‡
A9 (high LDL)	*PCSK9*	1:55509689	T	A	p.S127R	§
B2 (low LDL)	*APOB*	2:21233022	T	A	p.K2240*	‖
B13 (low LDL)	*APOB*	2:21229005	-	G	p.T3579Hfs*34	#
C1 (low HDL)	*ABCA1*	9:107553287	T	C	p.N1948S	**
Genetic etiology discovered from follow-up analysis						
A1 (high LDL)	*APOE*	19:45412048	CTC	-	p.L167del	††
A10 (high LDL)	*APOE*	19:45412048	CTC	-	p.L167del	††
A13 (high LDL)	*LIPA*	10:90982268	C	T	Disruption of donor splice site	‡‡
Genetic etiology discovered based on phenotypic refinement within the family						
A7 (high LDL)	*LDLR*	19:11216264	G	A	p.E228K	§§

*Genomic position lists chromosome and position in hg19 coordinates.
†Effect refers to the predicted protein change using proposed nomenclature[22] based on the cDNA sequence with the ATG initiation codon numbered p.1. The following reference sequences were used: *ABCA1*: NM_005502.3, *APOB*: NM_000384.2, *APOE*: NM_000041.2, *LDLR*: NM_000527.4, *PCSK9*: NM_174936.3, *LIPA*: NM_000235.2.
‡To our knowledge this mutation has not been previously identified as causing autosomal dominant hypercholesterolemia (ADH). However, this mutation occurs at a highly conserved position within the highly conserved LDLR-binding domain of ApoB where other missense mutations causing ADH have been identified. A threonine for alanine substitution at this position is computationally predicted to be damaging.
§This mutation has been previously identified as causing ADH in other families[5].
‖A report detailing this mutation has been previously published[23].
#To our knowledge this mutation has not been previously identified as causing familial hypobetalipoproteinemia (FHBL) however this is expected type of causal mutation for FHBL, inducing a premature truncation of ApoB.
**To our knowledge this mutation has not been previously identified as causing Tangier disease. However, this mutation is in the second conserved nucleotide-binding domain (ATP binding cassette) within the Walker A/P-loop of *ABCA1*. It affects the amino acid residue position corresponding to the equivalent Asparagine in the 1st nucleotide binding domain which is known to be causal in Tangier disease[24,25]. The sequences are GHNGAGKTTTM (domain 1) and GVNGAGKSSTF (domain 2) (the mutated residue is underlined).
††A report of this mutation causing ADH has been previously published[26].
‡‡A report detailing this mutation has been previously published[27].
§§This mutation, also known as FH-Jerusalem, has been previously identified as causing familial hypercholesterolemia[28,29].

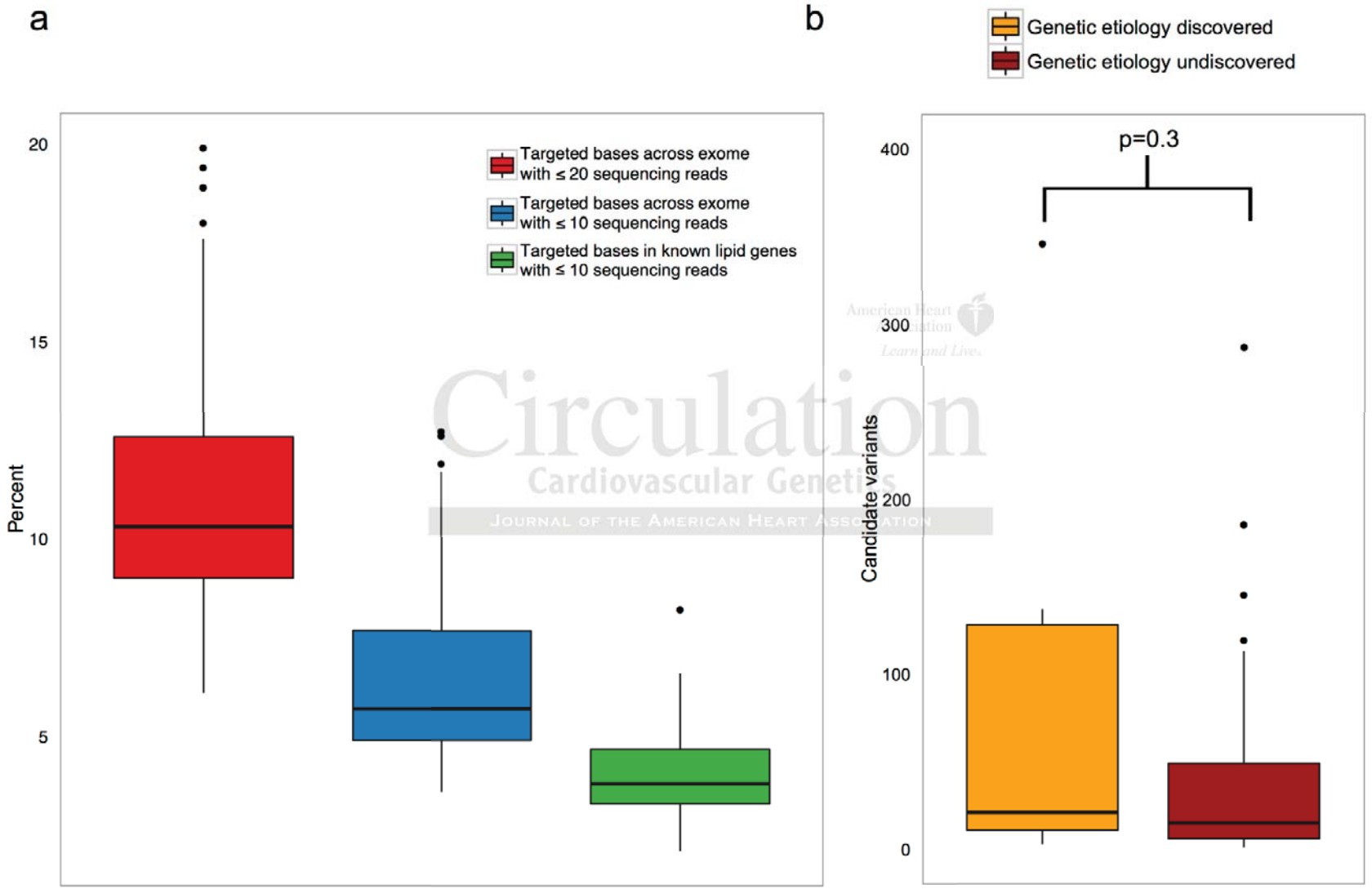
DOI: 10.1161/CIRCGENETICS.114.000776

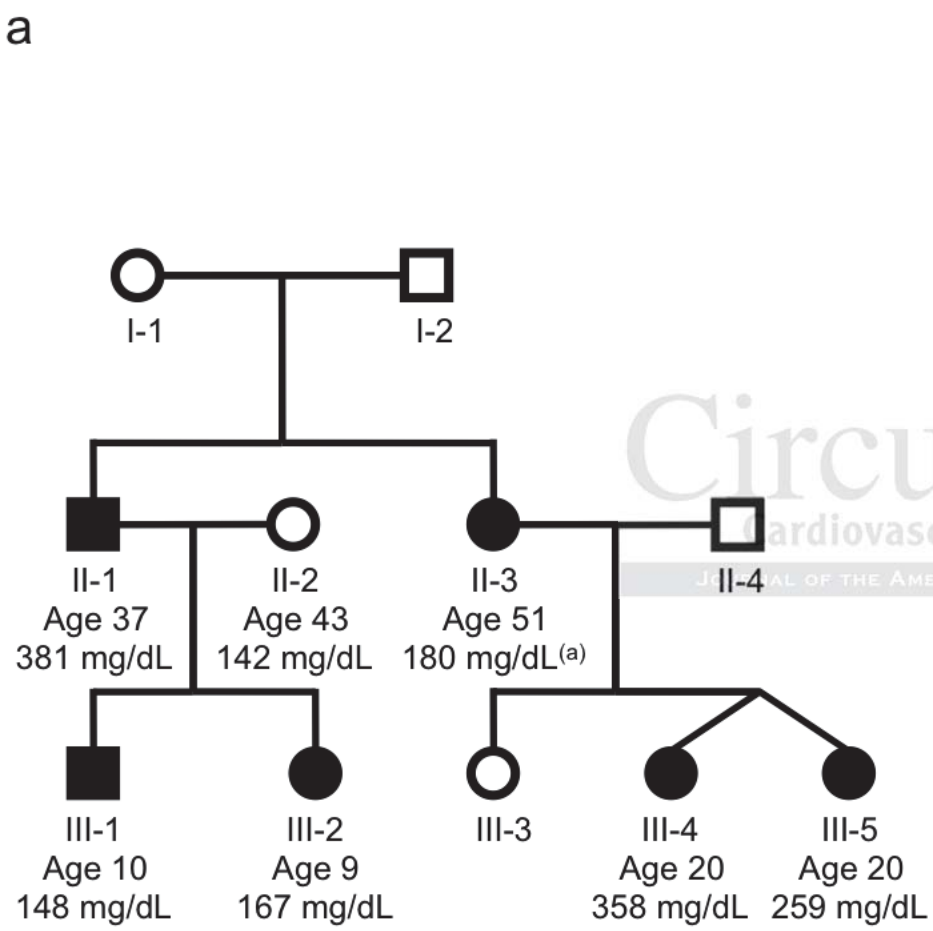
Figure Legends:

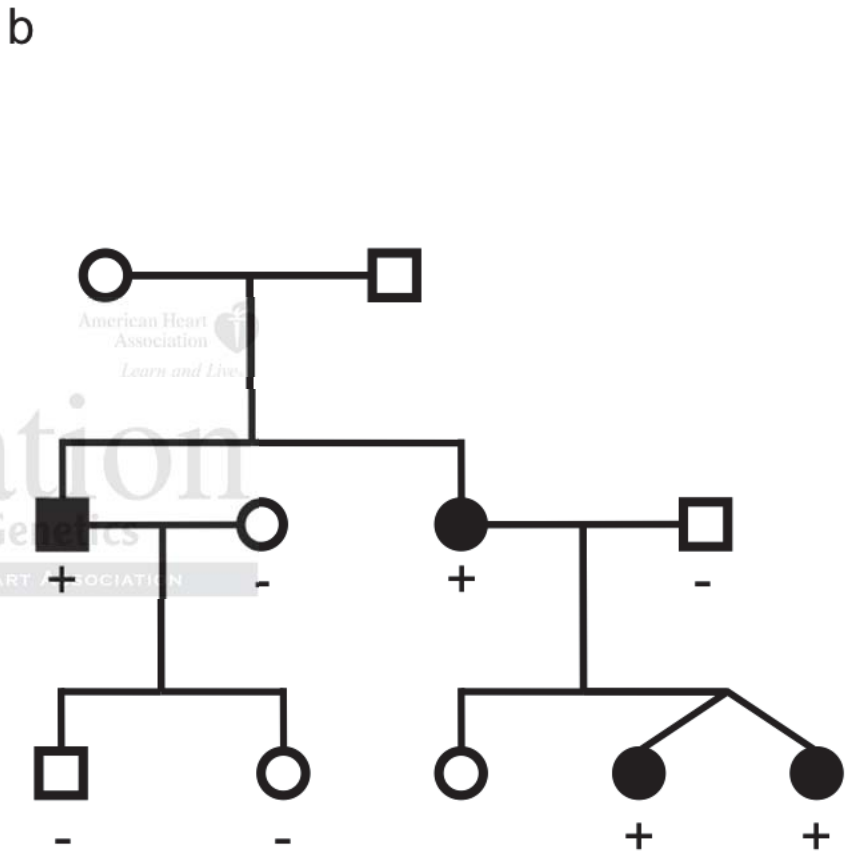
Figure 1: Selected metrics from exome sequencing analysis. (a) Percent targeted bases across the exome definition supported by ≤ 20 sequencing reads (Red) or ≤ 10 sequencing reads (Blue) or across genes previously identified to cause monogenic dyslipidemia supported by ≤ 10 sequencing reads (Green). (b) Number of candidate variants after analysis for families with a suspected pathogenic variant in a gene known to cause monogenic dyslipidemia (orange) compared with families without known cause (brown). P refers to the p-value from the Kolmogorov–Smirnov test in testing for differences between the distributions.

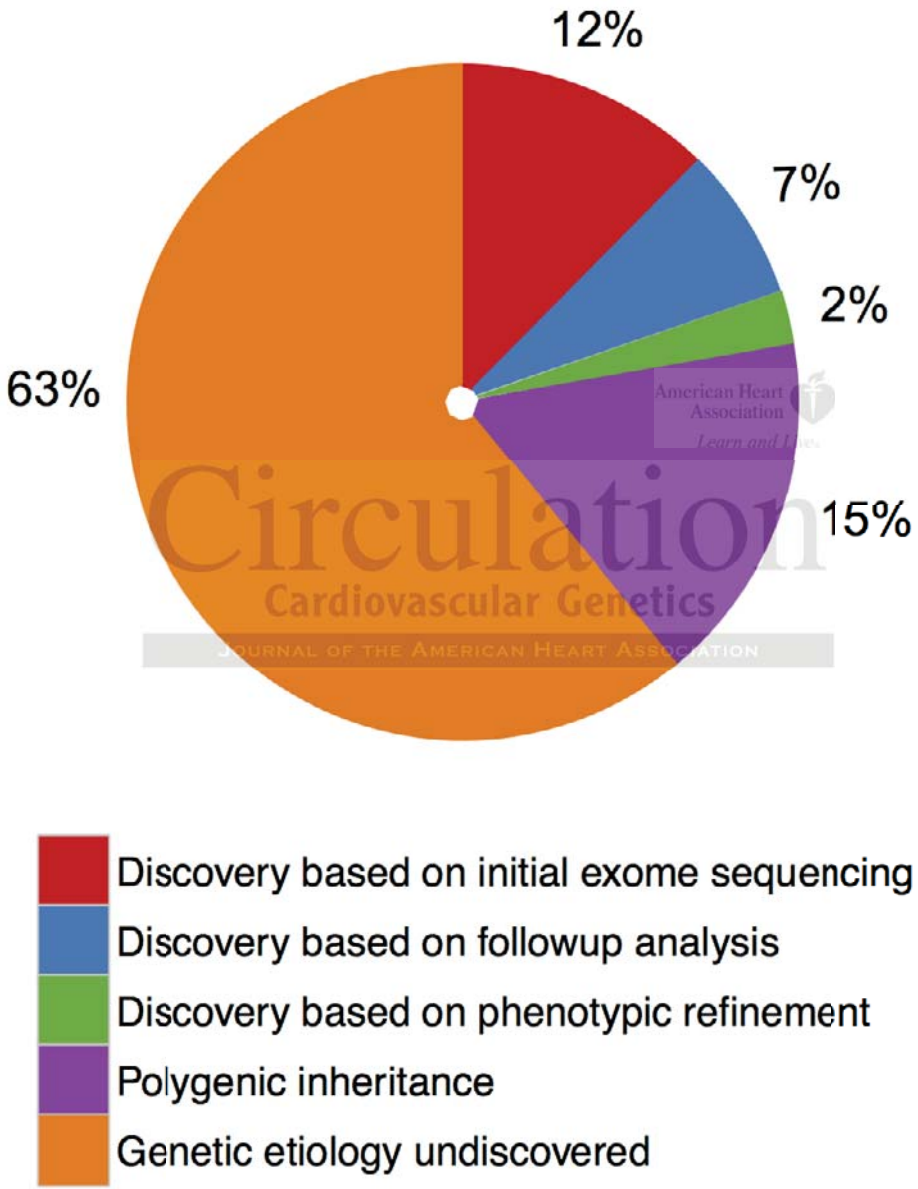
Figure 2: Pedigree of Family A7, demonstrating the utility of refining phenotypes based on burden of common alleles. (A) Initial pedigree defining affected individuals (shaded) by LDL-C level adjusted for age and gender. (B) Updated pedigree based on residualized phenotype score (see text) where individuals III-1 and III-2 are classified as unaffected. *LDLR* p.E228K carrier status is indicated with + (heterozygous) or - (wild type). The superscript (a) indicates the LDL-C level was obtained while the individual was on lipid-lowering medication therapy.

Figure 3: Discovery rates from exome sequencing. The distribution of final discovery status for the 41 families with suspected monogenic dyslipidemias that underwent exome sequencing is shown with approximate percentages.





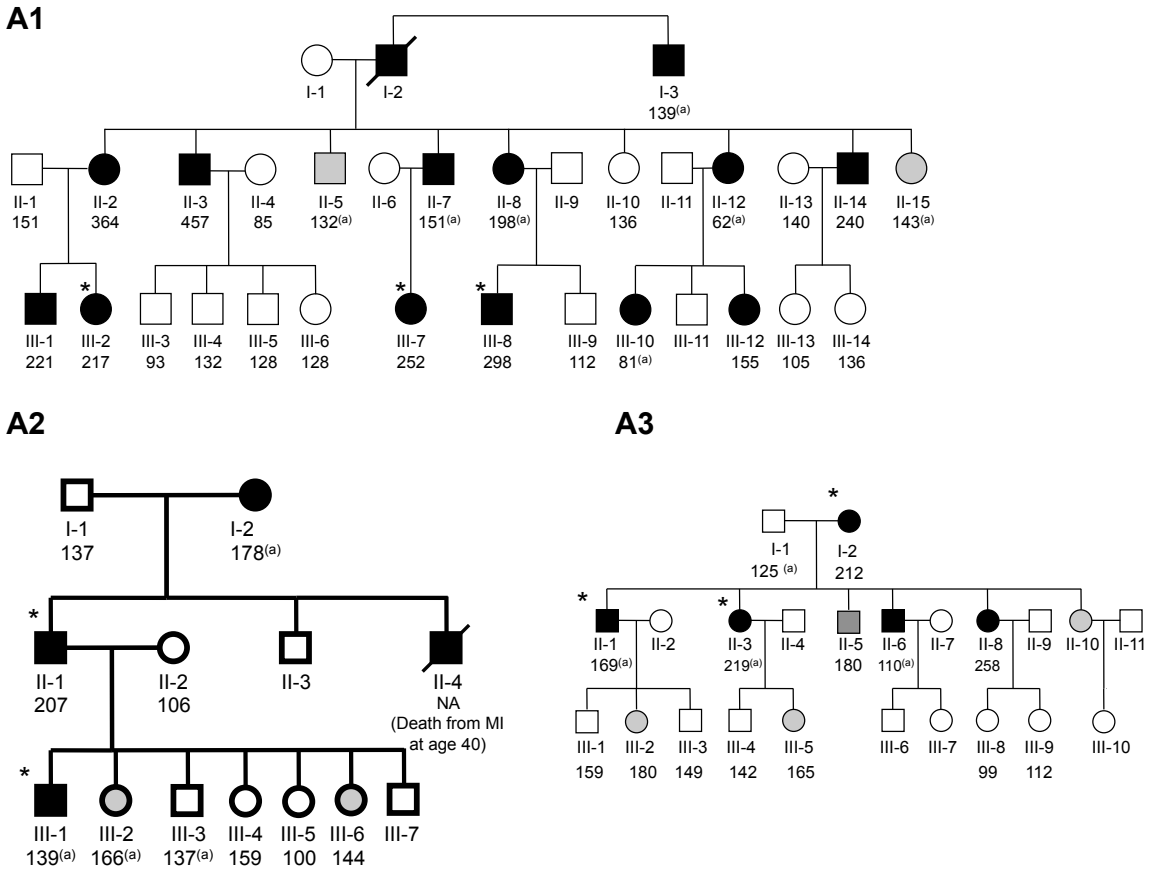


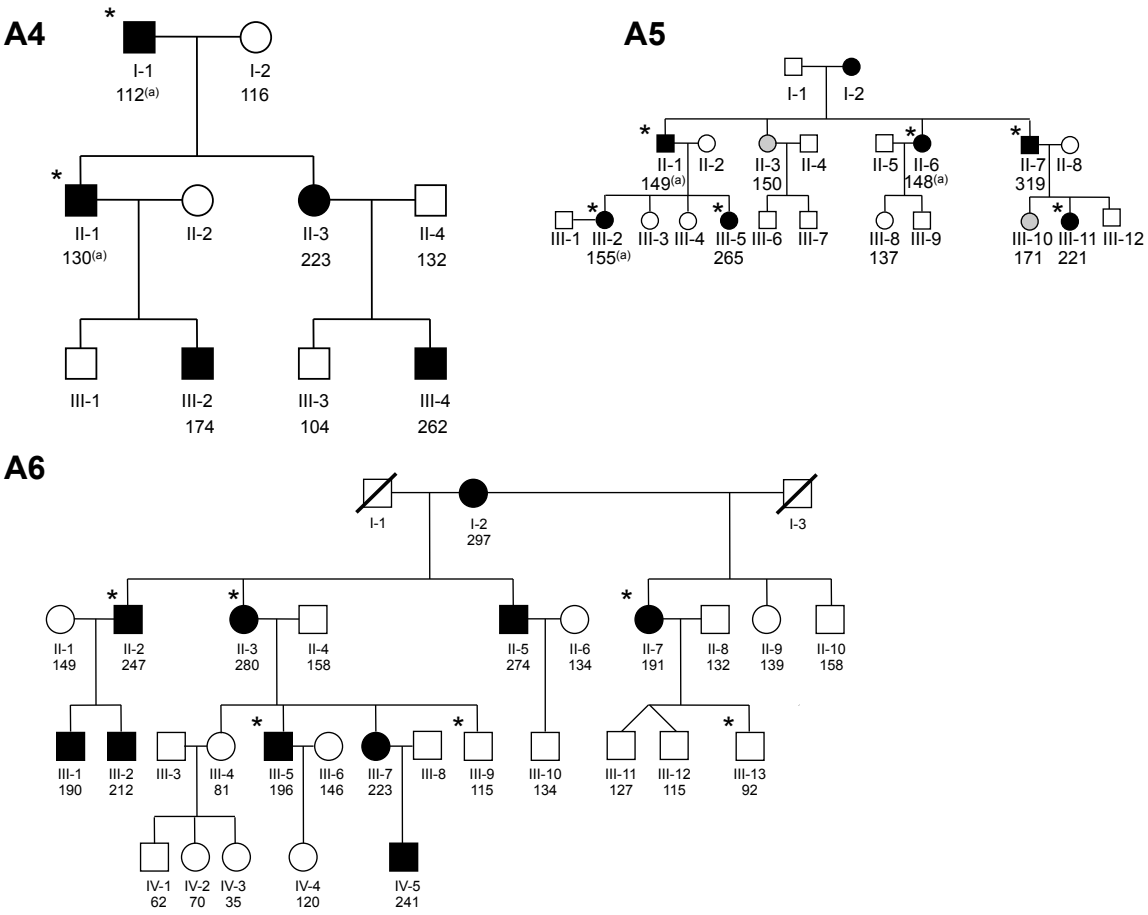


SUPPLEMENTAL MATERIAL

Supplementary Figure 1. Pedigrees of families with suspected monogenic dyslipidemias included in the study. Individuals selected for exome sequencing are indicated with asterisks (*). Black shading indicates clinically affected individuals; grey shading indicates an uncertain clinical diagnosis.

A) Families with suspected monogenic inheritance of high low-density lipoprotein cholesterol (LDL-C) levels. LDL-C levels in mg/dL are displayed below individual identifiers. Individuals with LDL-C values obtained while taking lipid-lowering medications are noted with (a). For individuals noted with (b), total cholesterol is reported in mg/dL instead of LDL-C.




A4
I-1
112(a)
I-2
116
II-1
130(a)
II-2
II-3
223
II-4
132
III-1
III-2
174
III-3
104
III-4
262
A5
I-1
I-2
II-1
149(a)
II-2
II-3
150
II-4
II-5
II-6
148(a)
II-7
319
II-8
III-1
III-2
155(a)
III-3
III-4
III-5
265
III-6
III-7
III-8
137
III-9
III-10
171
III-11
221
III-12
A6
I-1
I-2
297
I-3
II-1
149
II-2
247
II-3
280
II-4
158
II-5
274
II-6
134
II-7
191
II-8
132
II-9
139
II-10
158
III-1
190
III-2
212
III-3
III-4
81
III-5
196
III-6
146
III-7
223
III-8
III-9
115
III-10
134
III-11
127
III-12
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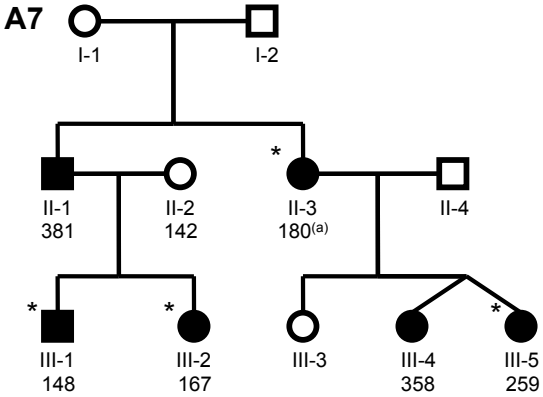
III-13
92
IV-1
62
IV-2
70
IV-3
35
IV-4
120
IV-5
241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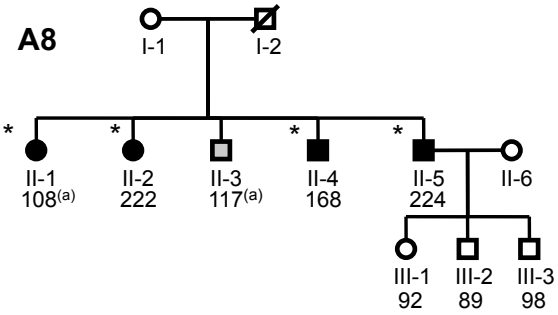
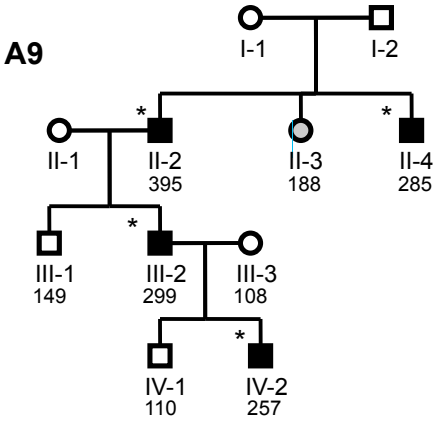
I-1
I-2
II-1
381
II-2
142
II-3
180(a)
II-4
III-1
148
III-2
167
III-3
III-4
358
III-5
259


A8
I-1
I-2
II-1
108(a)
II-2
222
II-3
117(a)
II-4
168
II-5
224
II-6
III-1
92
III-2
89
III-3
98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I-1
I-2
II-1
II-2
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II-3
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II-4
285
III-1
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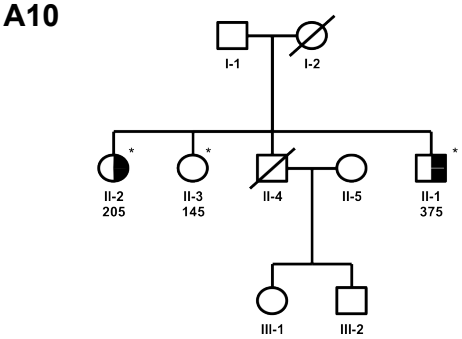
III-2
299
III-3
108
IV-1
110
IV-2
257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I-1
I-2
II-2
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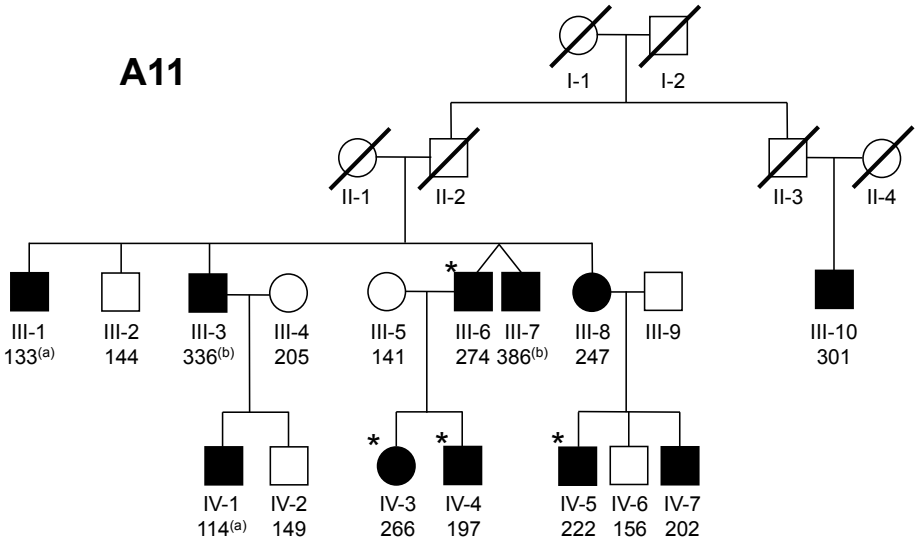
II-3
145
II-4
II-5
II-1
375
III-1
III-2

A11



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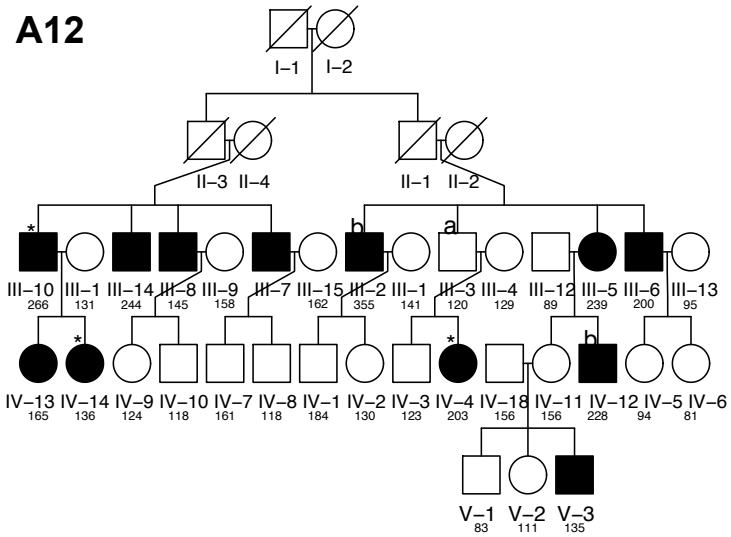

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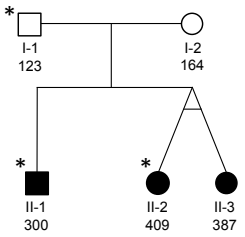
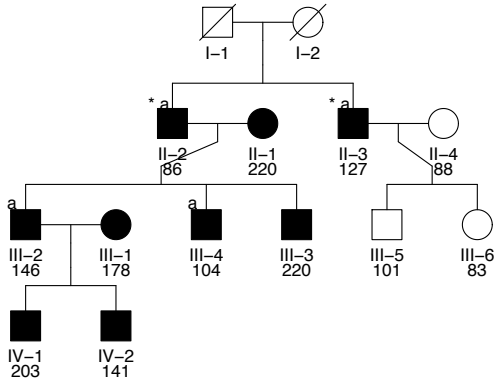


A14



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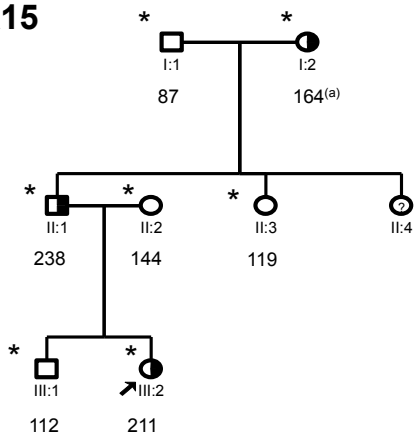
I:1
I:2
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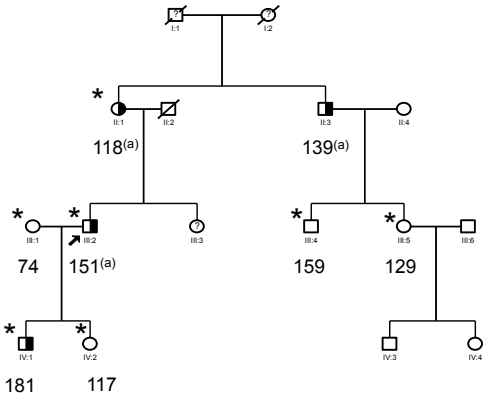
164(a)
II:1
II:2
II:3
II:4
238
144
119
III:1
III:2
112
211

A16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I:2
II:1
II:2
II:3
II:4
118(a)
139(a)
III:1
III:2
III:3
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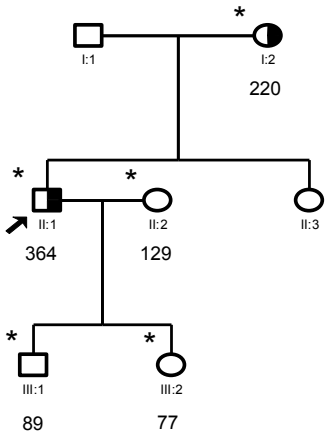
151(a)
159
129
IV:1
IV:2
IV:3
IV:4
181
117

A17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I:2
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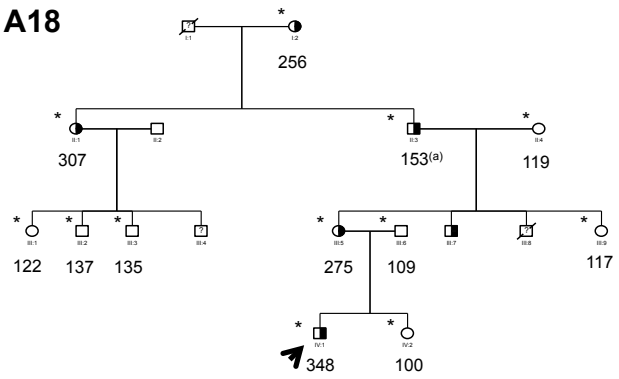
II:1
II:2
II:3
364
129
III:1
III:2
89
77

A18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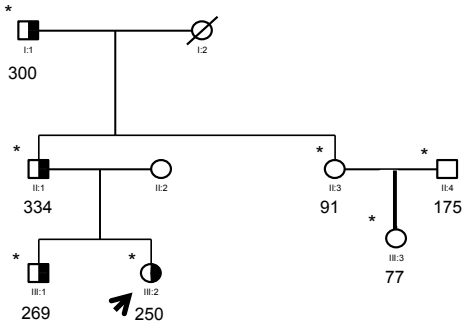
307
153(a)
119
122
137
135
275
109
117
348
100

A19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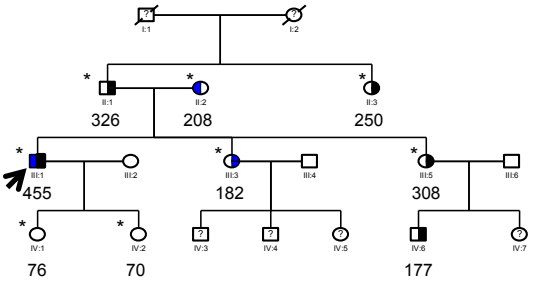
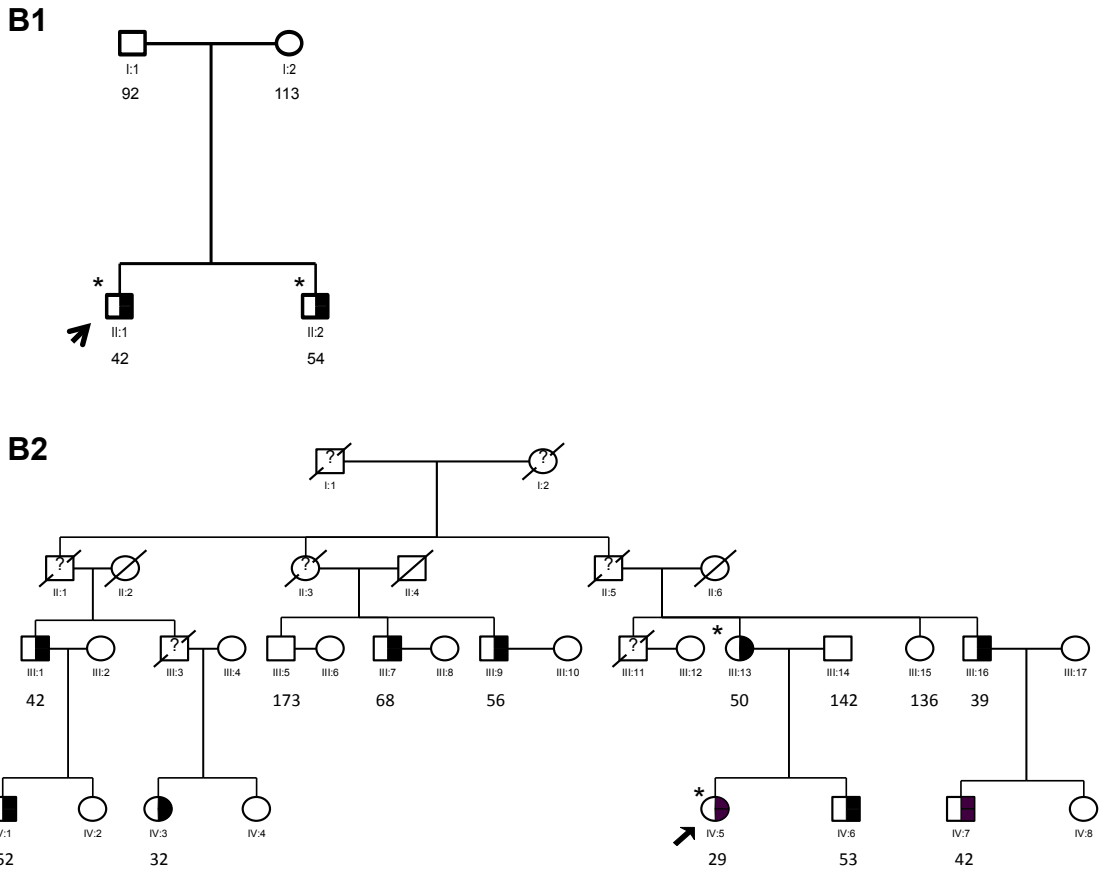
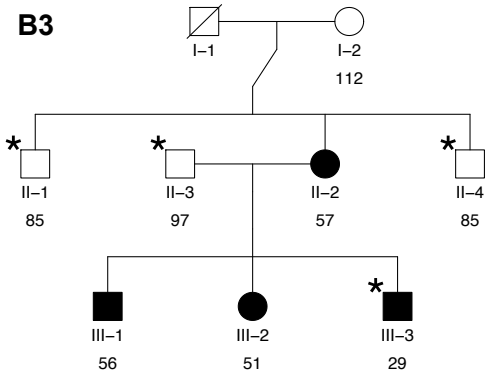
334
91
175
77
269
250

A20


326
208
250
455
182
308
76
70
177

B) Families with suspected monogenic inheritance of low LDL-C levels. LDL levels in mg/dL are displayed below individual identifiers. LDL-C value not available in individual II-3 from Family B13; TC refers to Total Cholesterol.




B3
I–1
I–2
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II–1
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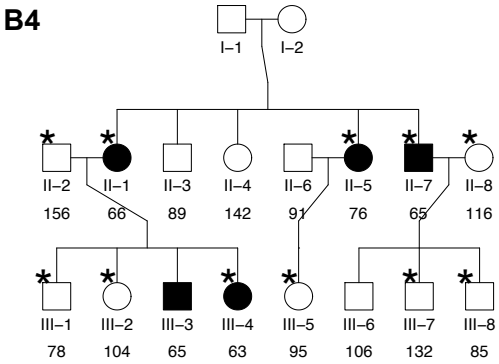
II–3
97
II–2
57
II–4
85
III–1
56
III–2
51
III–3
29


B4
I–1
I–2
II–2
156
II–1
66
II–3
89
II–4
142
II–6
91
II–5
76
II–7
65
II–8
116
III–1
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III–2
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III–3
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III–6
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III–7
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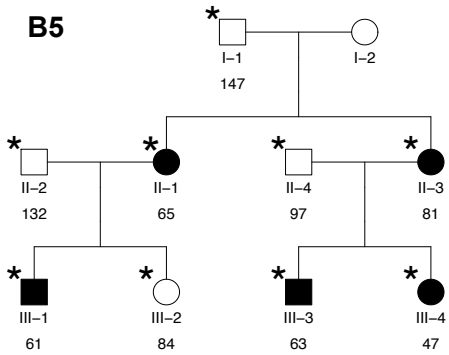
III–8
85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I–1
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I–2
II–2
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II–1
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II–3
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III–1
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III–2
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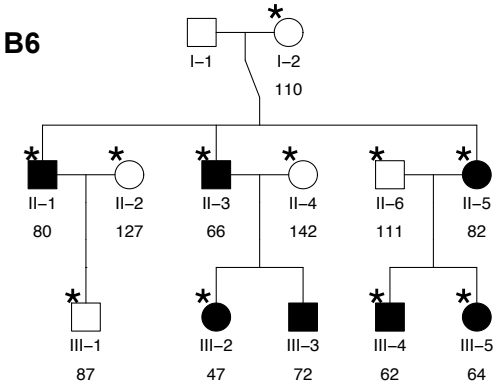
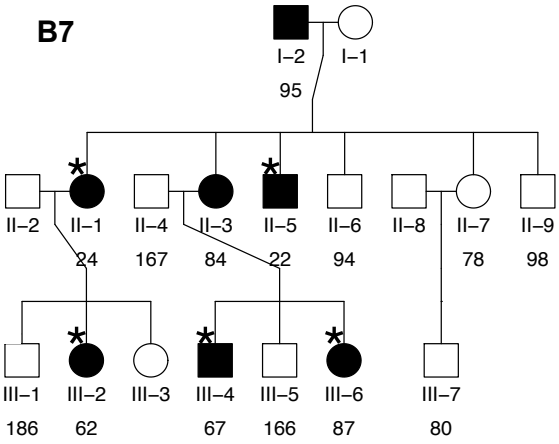
III–3
63
III–4
47


B6
I–1
I–2
110
II–1
80
II–2
127
II–3
66
II–4
142
II–6
111
II–5
82
III–1
87
III–2
47
III–3
72
III–4
62
III–5
64


B7
I–2
I–1
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II–2
II–1
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II–4
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II–3
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II–5
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II–6
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II–8
II–7
78
II–9
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III–1
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III–2
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III–3
III–4
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III–5
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III–6
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III–7
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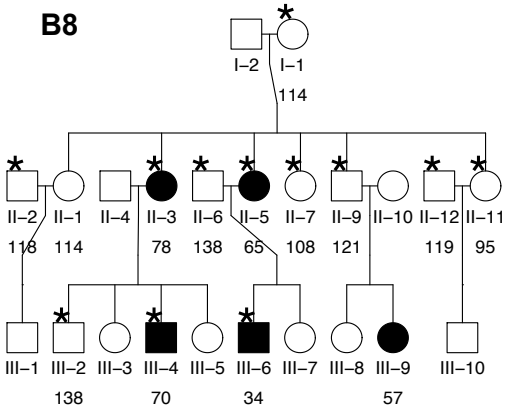
B8
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I–1
114
II–2
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II–1
114
II–4
II–3
78
II–6
138
II–5
65
II–7
108
II–9
121
II–10
II–12
119
II–11
95
III–1
III–2
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III–3
III–4
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III–5
III–6
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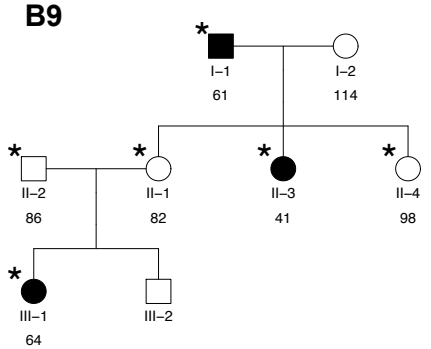
III–7
III–8
III–9
57
III–10


B9
I–1
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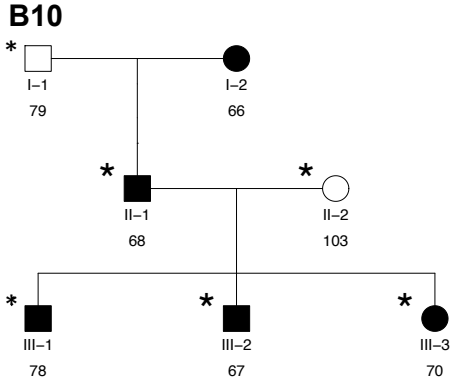
I–2
114
II–2
86
II–1
82
II–3
41
II–4
98
III–1
64
III–2


B10
I–1
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I–2
66
II–1
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II–2
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III–1
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III–2
67
III–3
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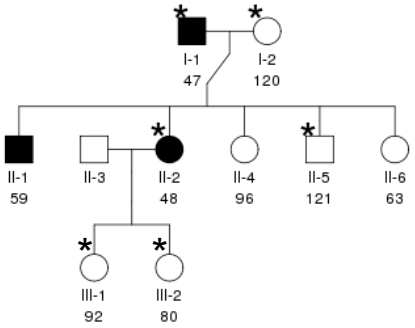
B11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I-2
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II-1
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II-2
48
II-4
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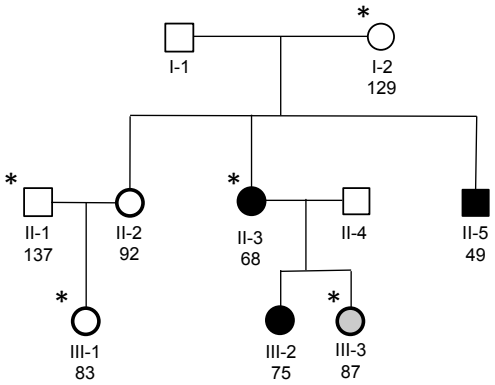
II-5
121
II-6
63
III-1
92
III-2
80

B12


I-1
I-2
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II-1
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II-2
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II-3
68
II-4
II-5
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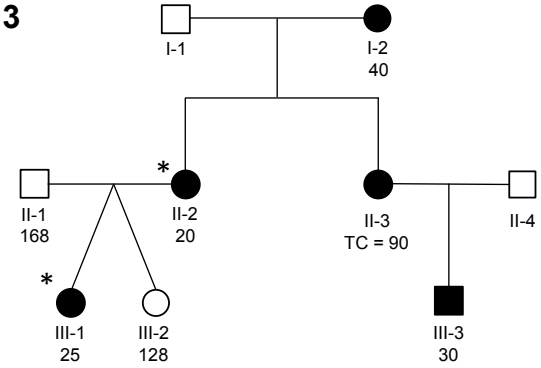
III-1
83
III-2
75
III-3
87

B13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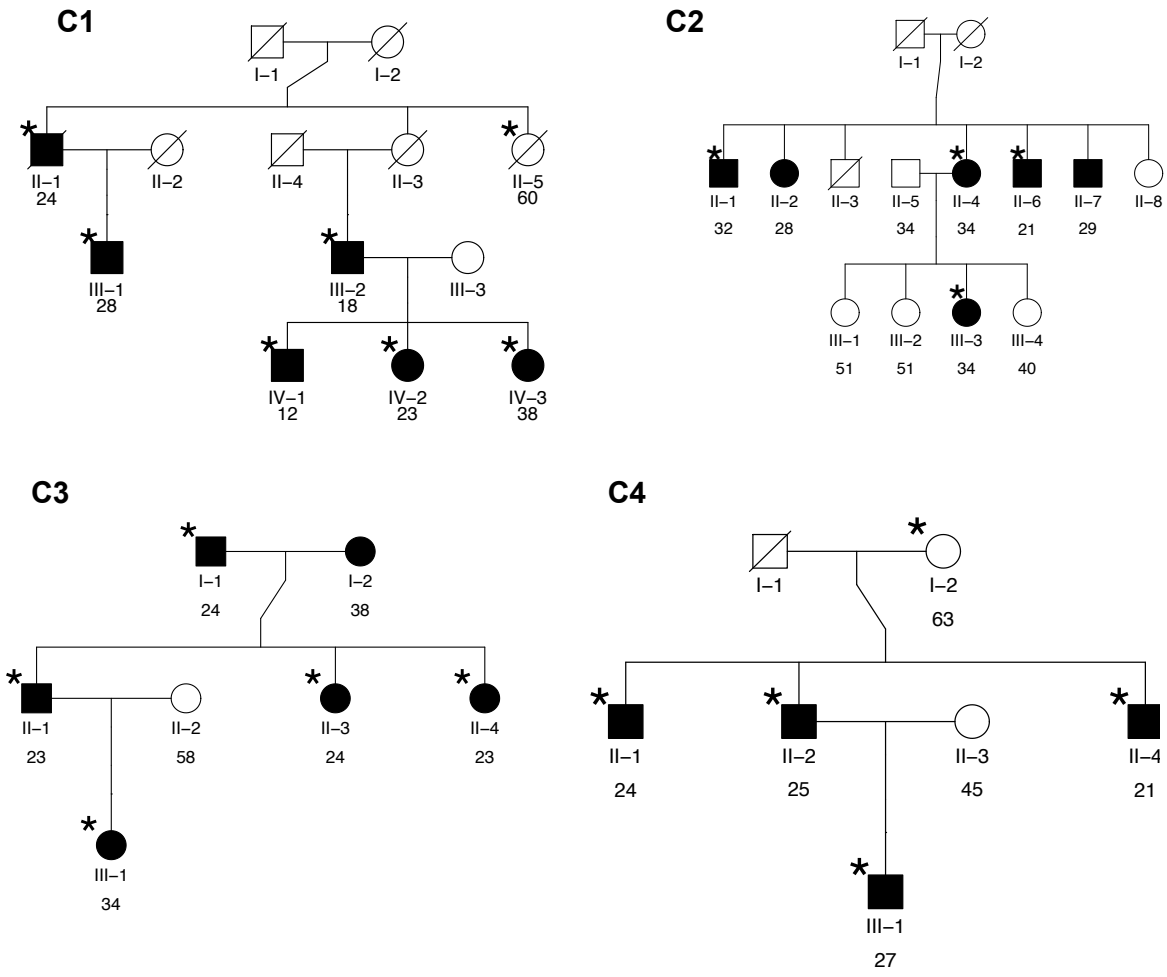
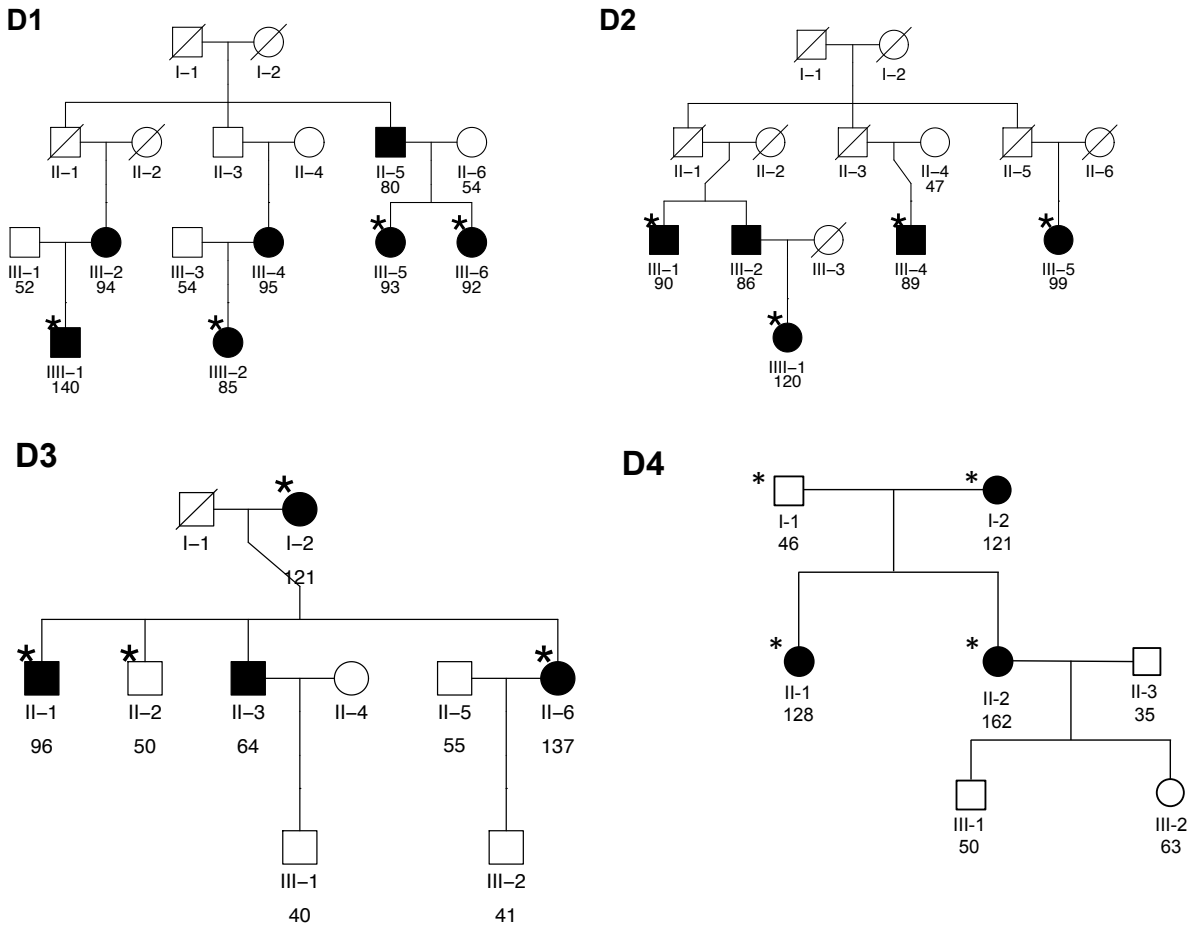
I-2
40
II-1
168
II-2
20
II-3
TC = 90
II-4
III-1
25
III-2
128
III-3
30

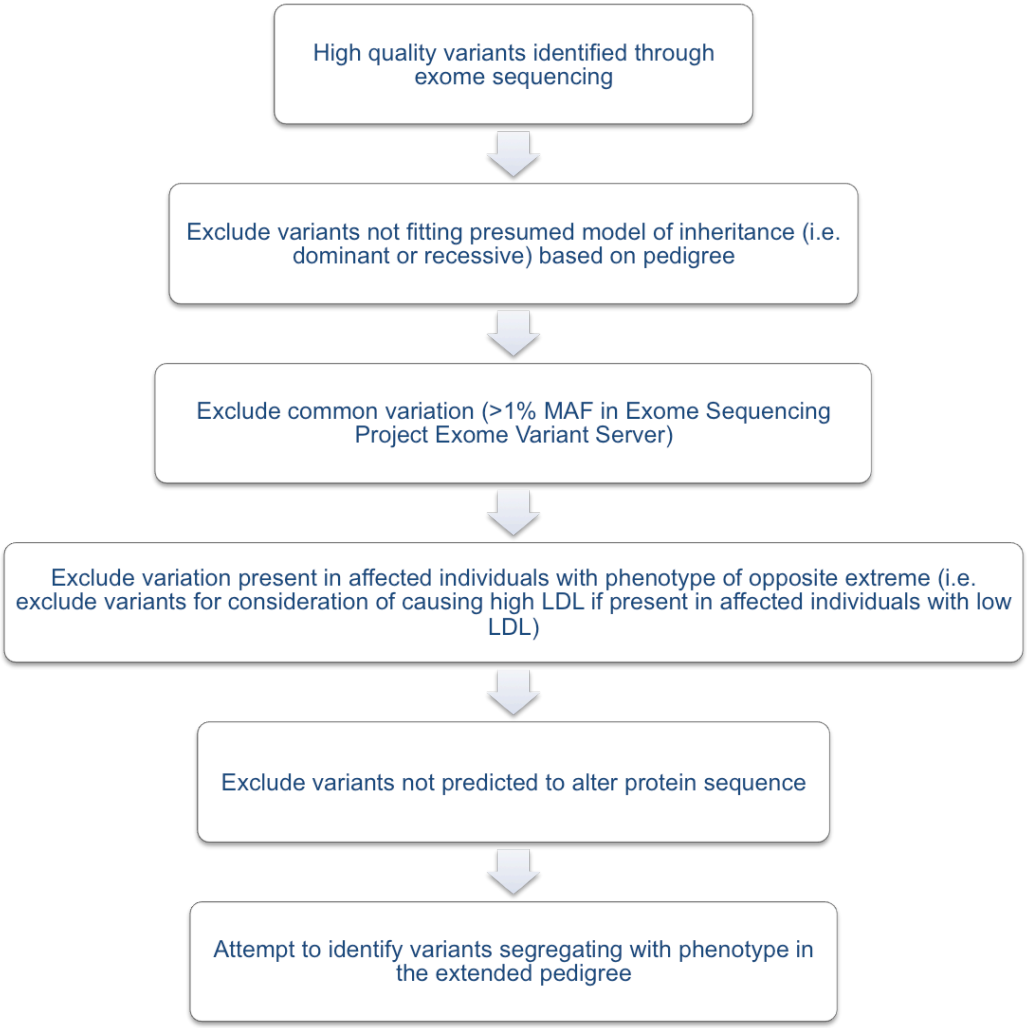
C) Families with suspected monogenic inheritance of low high density lipoprotein cholesterol (HDL-C) levels. HDL-C levels in mg/dL are displayed below individual identifiers.



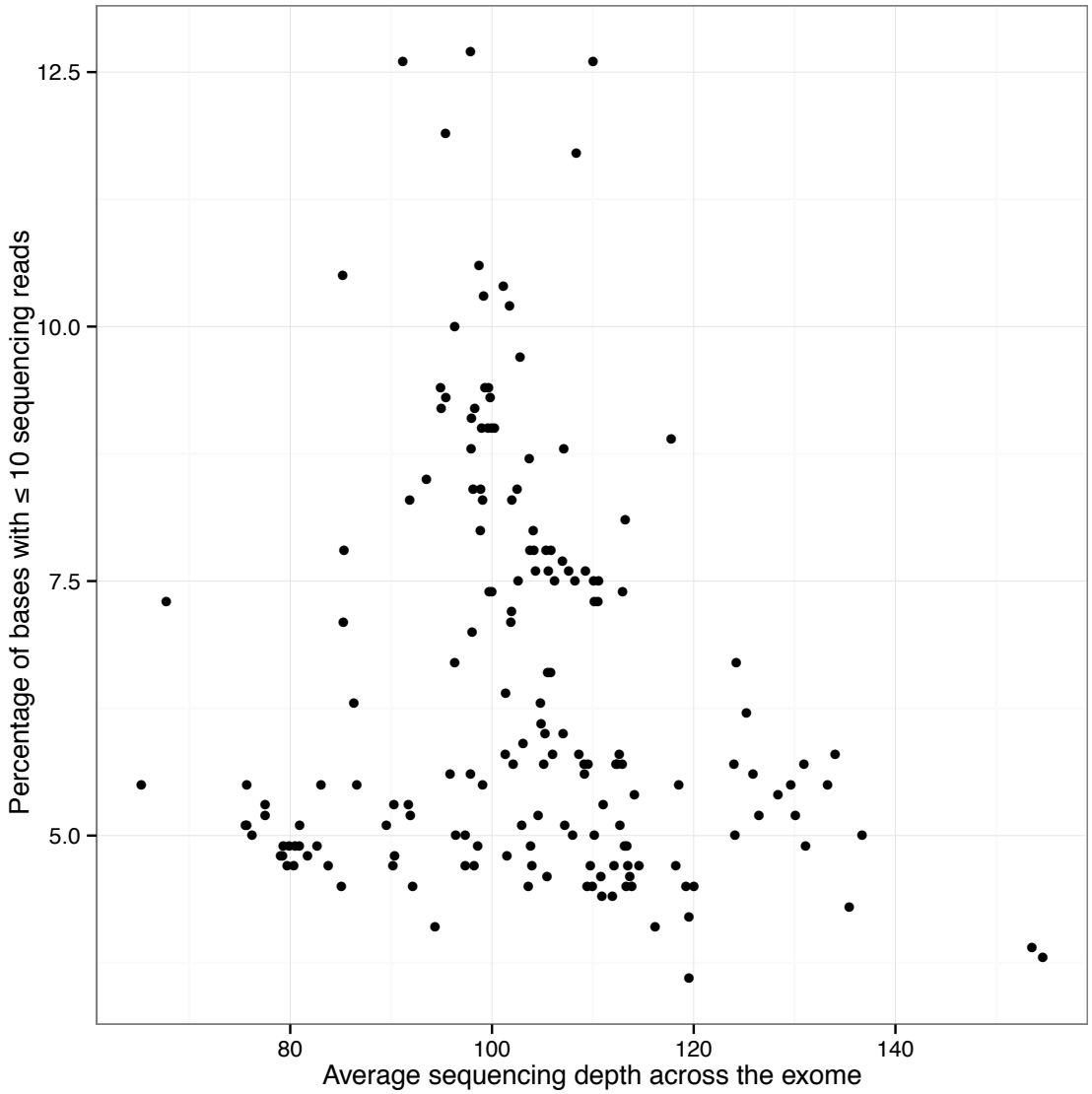
D) Pedigrees of families with suspected monogenic inheritance of high HDL-C levels. HDL-C levels in mg/dL are displayed below individual identifiers.



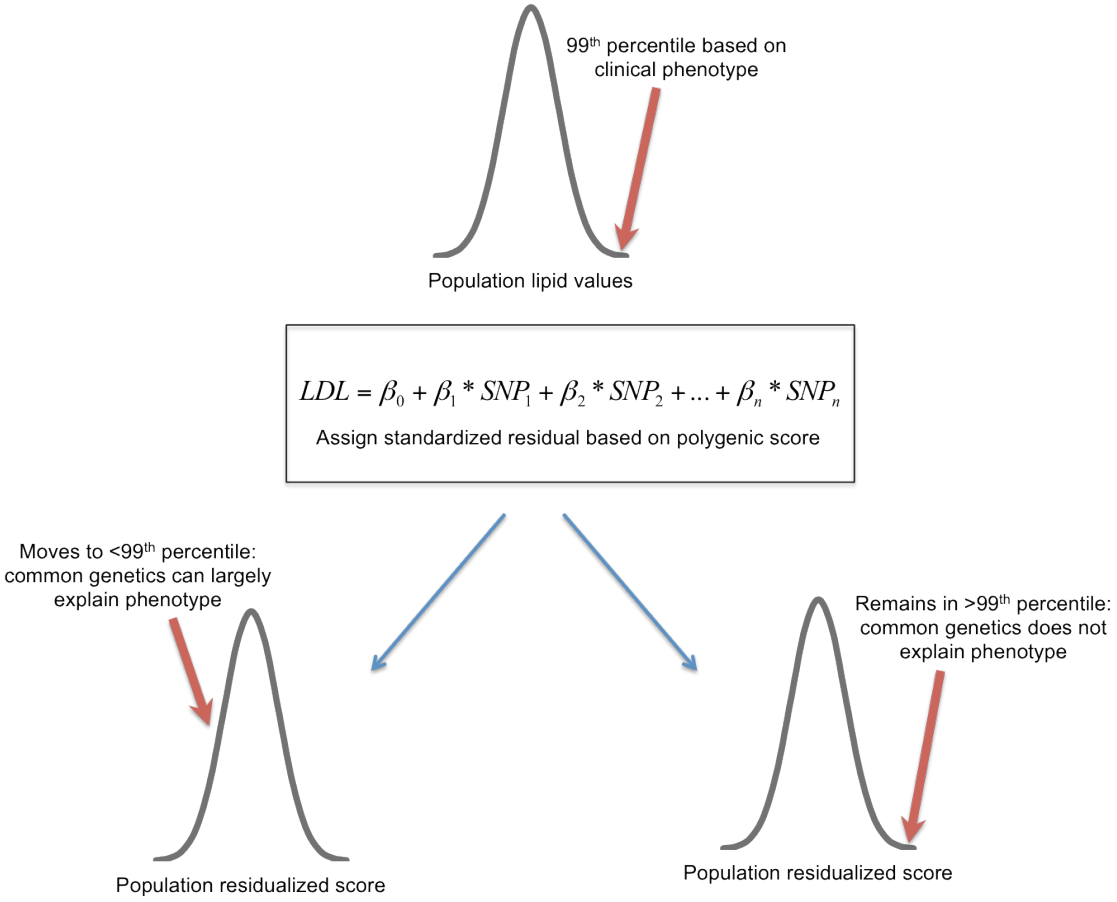
Supplementary Figure 2. Flowchart describing analytical process for removing variants unlikely to explain the phenotype within each family.



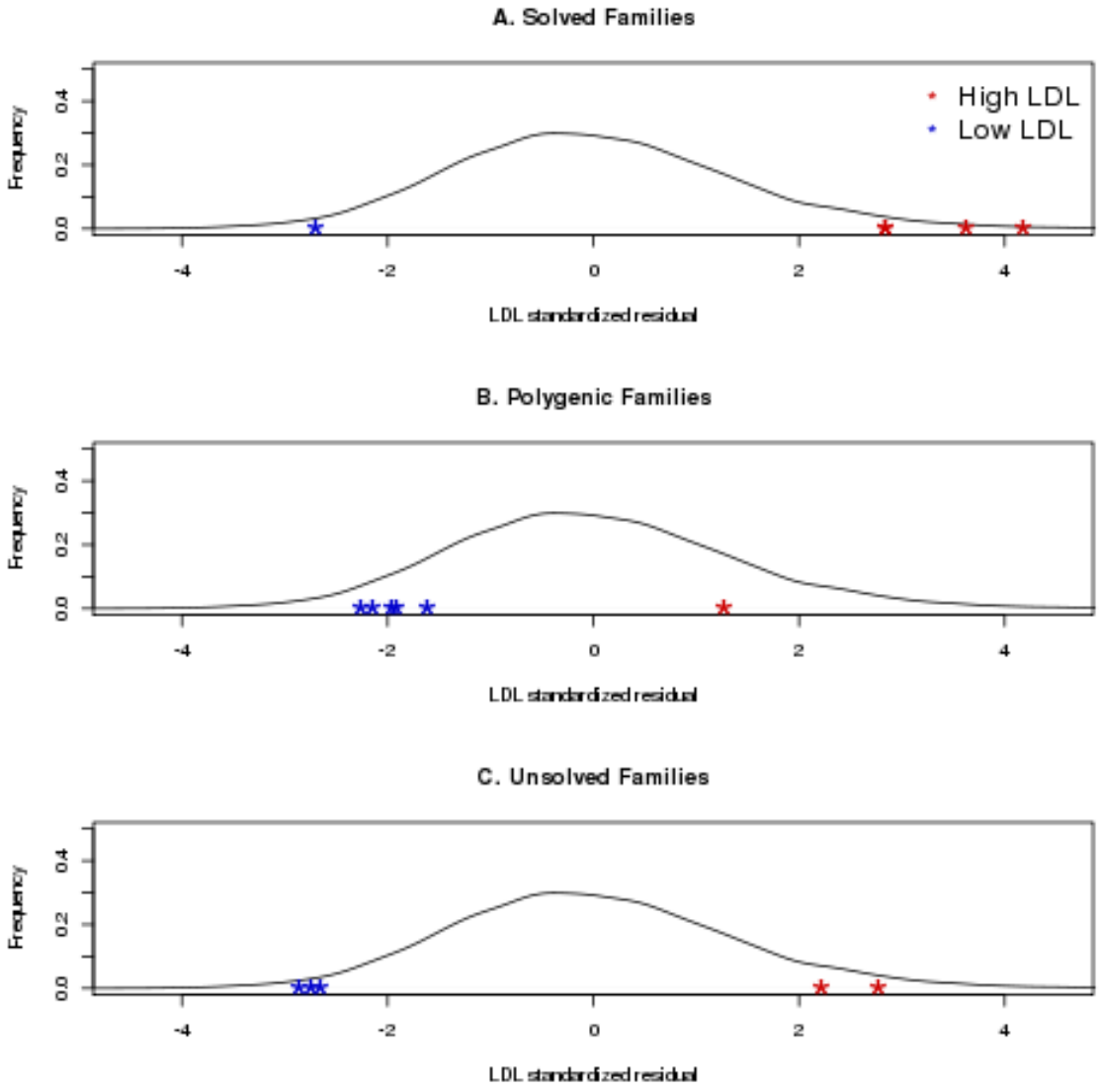
Supplementary Figure 3. Missing coverage in individuals across the exome as a function of overall sequencing depth.



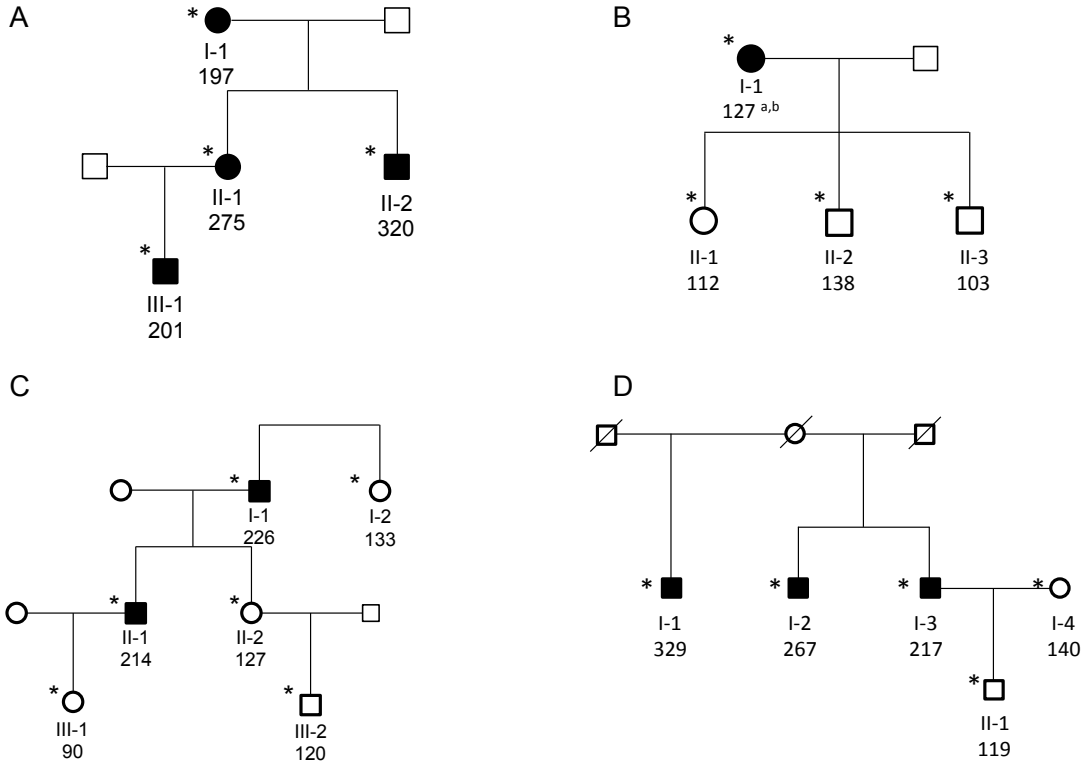
Supplementary Figure 4. Overview of method for determining likelihood of polygenic inheritance of lipid levels.



Supplementary Figure 5. Distribution of residualized phenotypes in families after removing phenotypic variance due to common lipid-related alleles. In each panel, the median standardized residual LDL of affected individuals is plotted for each family having a polygenic score available in more than one individual. The black line is the standardized residual in the training population as described in the Methods. (A) Families with causal mutations discovered. (B) Families thought to have significant polygenic inheritance. (C) Families without causal mutations.



Supplementary Figure 6. Pedigrees of families of Japanese descent with suspected monogenic inheritance of high LDL-C levels. LDL-C levels in mg/dL are displayed below individual identifiers. Individuals selected for exome sequencing are indicated with asterisks (*). Individuals with LDL-C values obtained while taking lipid-lowering medications are noted with (a) and those noted to have tendinous xanthomas on clinical examination are noted with (b).



Supplementary Table 1. Genes previously identified to cause monogenic dyslipidemia

Gene	Locus	Disorder and lipid phenotype
ABCA1	9q31.1	Tangier disease: low HDL
ABCG5	2p21	Sitosterolemia: high LDL
ABCG8	2p21	Sitosterolemia: high LDL
ANGPTL3	1p31	Combined familial hypolipidemia: low LDL, HDL, and VLDL
APOA1	11q23-q24	ApoA-I deficiency: low HDL
APOA5	11q23	ApoA-V deficiency: high VLDL and chylomicrons
APOB	2p24	Familial hypobetalipoproteinemia: low LDL
		Familial defective ApoB-100: high LDL
APOC2	19q13	Familial ApoC-II deficiency: high chylomicrons
APOE	19q13	Familial dysbetalipoproteinemia: high VLDL remnants and chylomicrons
CETP	16q13	Cholesteryl ester transfer protein deficiency: high HDL
LCAT	16q22	Lecithin-cholesterol acyltransferase deficiency (fish-eye disease): low HDL
LDLR	19p13	Familial hypercholesterolemia: high LDL
LDLRAP1	1p36-p35	Autosomal recessive hypercholesterolemia: high LDL
LIPC	15q22	Familial hepatic lipase deficiency: high VLDL remnants
LPL	8p21	Lipoprotein lipase deficiency: high chylomicrons
MTTP	4q24	Abetalipoproteinemia: low LDL
PCSK9	1p32	Autosomal-dominant hypercholesterolemia: high LDL
		PCSK9 deficiency: low LDL

Supplementary Table 2. Number of families with successful identification of causal mutations by trait.

Trait	Total families sequenced	Families with causal mutations
Low LDL	13	2 (15%)
High LDL	20	6 (30%)
Low HDL	4	1 (25%)
High HDL	4	0 (0%)

Supplementary Table 3. Average sequencing coverage across targeted bases in known lipid genes. “Percent GC content” refers to the percentage of targeted bases in the respective gene that are guanine or cytosine.

Gene	Average sequencing coverage	Average percent targeted bases with ≤ 10-fold coverage	Percent GC content
ANGPTL3	130	0	36%
APOB	99	1.8	43%
CETP	115	0.1	43%
APOA5	62	2.9	44%
MTTP	100	1.1	45%
LPL	99	1.9	49%
ABCA1	100	1.6	50%
ABCG5	91	9.9	52%
LIPC	98	3.6	52%
ABCG8	97	4.5	57%
APOC2	110	0.01	58%
LDLR	100	1.5	58%
LDLRAP1	89	12.2	60%
LCAT	88	13.2	61%
APOA1	94	7.0	63%
PCSK9	79	21.9	65%
APOE	56	44.9	70%

Supplementary Table 4. Average sequencing coverage for targeted bases in the three targeted exons of *APOE*. The first exon of *APOE* encodes the 5'-untranslated region and was not targeted by the Agilent exome capture reagent. "Percent GC content" refers to the percentage of targeted bases in the respective exon that are guanine or cytosine.

Gene	Average sequencing coverage	Percent GC content
APOE Exon 2	158	55%
APOE Exon 3	51	65%
APOE Exon 4	13	72%

Supplementary Table 5. Polygenic score analysis results for Families A9 and B2. LDL-C value refers to each individual's baseline LDL-C value. LDL-Corr represents each individual's expected LDL-C value after correcting for that individual's burden of common lipid-related alleles. P-value refers to the statistical significance of the difference between LDL-C and LDL-Corr.

Family	ID	Mutation carrier status	LDL-C	LDL-Corr	P-value
A9	II-4	Heterozygous *PCSK9* p.S127R	285 mg/dL	159 mg/dL	0.0007
A9	IV-2	Heterozygous *PCSK9* p.S127R	257 mg/dL	157 mg/dL	0.007
B2	III-13	Heterozygous *APOB* p.K2240Ter	50 mg/dL	141 mg/dL	0.01
B2	IV-5	Heterozygous *APOB* p.K2240Ter	29 mg/dL	137 mg/dL	0.003

Supplementary Table 6. Polygenic score analysis results for six families exhibiting polygenic inheritance. LDL-C value refers to each individual's baseline LDL-C value. LDL-Corr represents each individual's expected LDL-C value after correcting for that individual's burden of common lipid-related alleles. P-value refers to the statistical significance of the difference between LDL-C and LDL-Corr.

Family	ID	LDL-C	LDL-Corr	P-value
A8	II-1	108 mg/dL (154 mg/dL correcting for medications)	146 mg/dL	0.82
A8	II-2	222 mg/dL	140 mg/dL	0.03
A8	II-4	168 mg/dL	147 mg/dL	0.57
A8	II-5	224 mg/dL	150 mg/dL	0.04
B4	II-1	66 mg/dL	136 mg/dL	0.06
B4	II-2	156 mg/dL	145 mg/dL	0.76
B4	II-5	76 mg/dL	136 mg/dL	0.11
B4	II-8	116 mg/dL	145 mg/dL	0.41
B4	III-1	78 mg/dL	135 mg/dL	0.12
B4	III-2	104 mg/dL	150 mg/dL	0.21
B4	III-4	63 mg/dL	145 mg/dL	0.03
B4	III-5	95 mg/dL	140 mg/dL	0.22
B4	III-8	85 mg/dL	146 mg/dL	0.10
B5	I-1	147 mg/dL	149 mg/dL	0.95
B5	II-2	132 mg/dL	149 mg/dL	0.64
B5	II-3	81 mg/dL	126 mg/dL	0.23
B5	II-4	97 mg/dL	129 mg/dL	0.38
B5	III-1	61 mg/dL	126 mg/dL	0.08
B5	III-3	63 mg/dL	148 mg/dL	0.02
B5	III-4	47 mg/dL	140 mg/dL	0.01
B6	I-2	110 mg/dL	147 mg/dL	0.32
B6	II-1	80 mg/dL	152 mg/dL	0.05
B6	II-2	127 mg/dL	146 mg/dL	0.61

Family	ID	LDL-C	LDL-Corr	P-value
B6	II-3	66 mg/dL	140 mg/dL	0.04
B6	II-4	142 mg/dL	158 mg/dL	0.67
B6	II-5	82 mg/dL	145 mg/dL	0.09
B6	II-6	111 mg/dL	133 mg/dL	0.56
B6	III-1	87 mg/dL	156 mg/dL	0.06
B6	III-2	47 mg/dL	153 mg/dL	0.004
B6	III-4	62 mg/dL	142 mg/dL	0.03
B6	III-5	64 mg/dL	144 mg/dL	0.03
B8	I-1	114 mg/dL	154 mg/dL	0.27
B8	II-3	78 mg/dL	152 mg/dL	0.05
B8	II-5	65 mg/dL	131 mg/dL	0.08
B8	II-6	138 mg/dL	160 mg/dL	0.56
B8	II-7	108 mg/dL	141 mg/dL	0.37
B8	II-9	121 mg/dL	145 mg/dL	0.51
B8	II-11	95 mg/dL	154 mg/dL	0.11
B8	II-12	119 mg/dL	154 mg/dL	0.34
B8	III-4	70 mg/dL	155 mg/dL	0.02
B8	III-6	34 mg/dL	144 mg/dL	0.003
B10	I-1	79 mg/dL	118 mg/dL	0.29
B10	II-1	68 mg/dL	119 mg/dL	0.16
B10	II-2	103 mg/dL	165 mg/dL	0.09
B10	III-1	78 mg/dL	141 mg/dL	0.09
B10	III-2	67 mg/dL	138 mg/dL	0.05
B10	III-3	70 mg/dL	148 mg/dL	0.03

Supplementary Table 7. Polygenic score analysis results for Family A7. LDL-C value refers to each individual's baseline LDL-C value. LDL-Corr represents each individual's expected LDL-C value after correcting for that individual's burden of common lipid-related alleles. P-value refers to the statistical significance of the difference between LDL-C and LDL-Corr.

Family	ID	Mutation carrier status	LDL-C	LDL-Corr	P-value
A7	II-3	Heterozygous *LDLR* p.E187K	180 mg/dL (257 mg/dL correcting for medication)	149 mg/dL	0.004
A7	III-1	Wild type	148 mg/dL	173 mg/dL	0.49
A7	III-2	Wild type	167 mg/dL	164 mg/dL	0.93
A7	III-5	Heterozygous *LDLR* p.E187K	259 mg/dL	157 mg/dL	0.006

Supplementary Table 8. Likely pathogenic variant identified from exome sequencing data in families of Japanese descent

Family (Trait)	Gene	Genomic position*	Reference allele	Alternate allele	Effect †	Notes
Genetic etiology discovered during initial exome sequencing analysis						
2A (high LDL)	*LDLR*	19:11238761	G	A	p.V797M	‡

*Genomic position lists chromosome and position in hg19 coordinates.

†Effect refers to the predicted protein change using proposed nomenclature[1] based on the cDNA reference sequence for *LDLR* (NM_000527.4) with the ATG initiation codon numbered p.1.

‡This mutation has been identified in multiple individuals with familial hypercholesterolemia[2].

Supplementary Table 9. Selected metrics from exome sequencing analysis in families of Japanese descent

Metric	Average	Range
Percent targeted bases across the exome supported by ≤ 20 sequencing reads	15.9%	9.1% – 18.7%
Percent targeted bases across the exome supported by ≤ 10 sequencing reads	7.6%	4.7% – 8.9%
Percent targeted bases across "lipid genes" supported by ≤ 10 sequencing reads	4.2%	2.7% – 4.8%
Number of candidate variants remaining after analysis in Family 2A discovered to have a pathogenic mutation in *LDLR*	53	Not applicable
Number of candidate variants remaining after analysis in families without an obvious genetic etiology	52.6	0 – 103

Supplementary References

1. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. *Human mutation*. 2000;15:7-12
2. Leigh SE, Foster AH, Whittall RA, Hubbart CS, Humphries SE. Update and analysis of the university college london low density lipoprotein receptor familial hypercholesterolemia database. *Ann Hum Genet*. 2008;72:485-498

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