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Analysis of publicly available *LDLR*, *APOB*, and *PCSK9* variants associated with familial hypercholesterolemia (FH): application of ACMG guidelines and implications for FH diagnosis

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Familial Hypercholesterolemia (FH)

- Lipid metabolism autosomal dominant condition
- Patients present elevated low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) values since birth → elevated cardiovascular risk

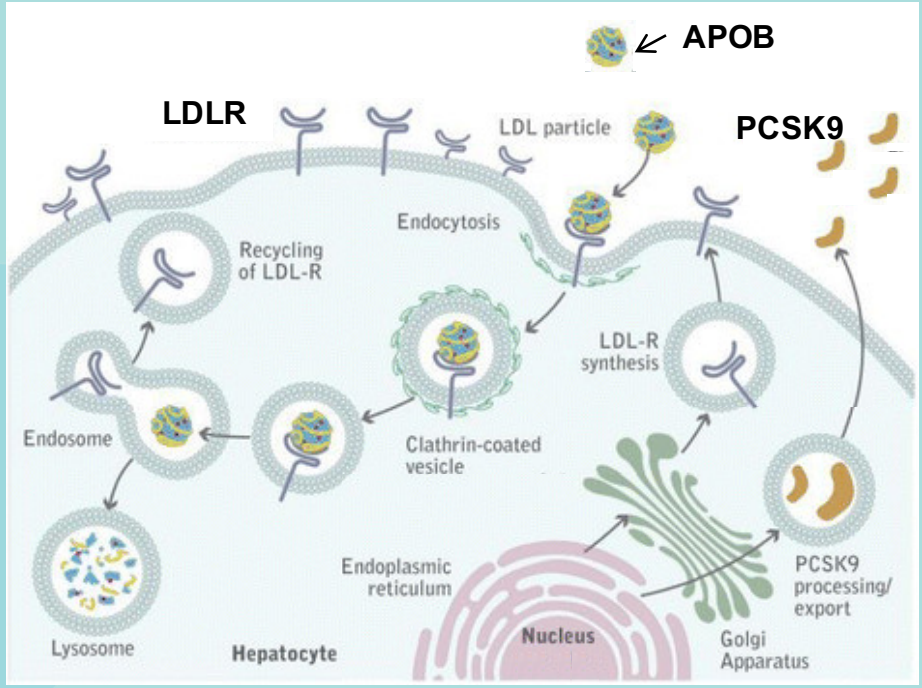
Possible FH	**a)** **Children (<16 years):** Total Cholesterol > 260mg/dL or LDL cholesterol > 155mg/dL **Adults:** Total Cholesterol > 290mg/dL or LDL cholesterol > 190mg/dL
	b) Family history of increased cholesterol levels in parents, brothers or sons; cholesterol > 290mg/dL in grandparents or uncles
	And/or (c) Family history of myocardial infarction before 50 years in grandparents, uncles or before 60 years in parents, brothers or sons
Definite FH	Patient or first degree relative with tendon xanthomas
	Or Evidence of a *LDLR*, *APOB*, *PCSK9* mutation

Adapted from Scientific Steering Committee on behalf of the Simon Broome Register Group 1991

Familial Hypercholesterolemia (FH)

- High heterozygote prevalence (1/250); Homozygous rare (1/300 000).
- Caused by pathogenic variants in *LDLR* (>90%), *APOB* (5-10%) and *PCSK9* (1-3%) genes.

>2,000 variants identified in FH patients



Adapted from Stoekenbroek *et al.*, 2015

Why does genetic diagnosis matter?

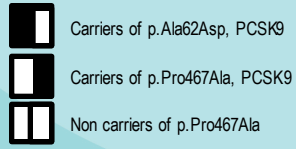
- **1) Familial hypercholesterolemia phenocopies**

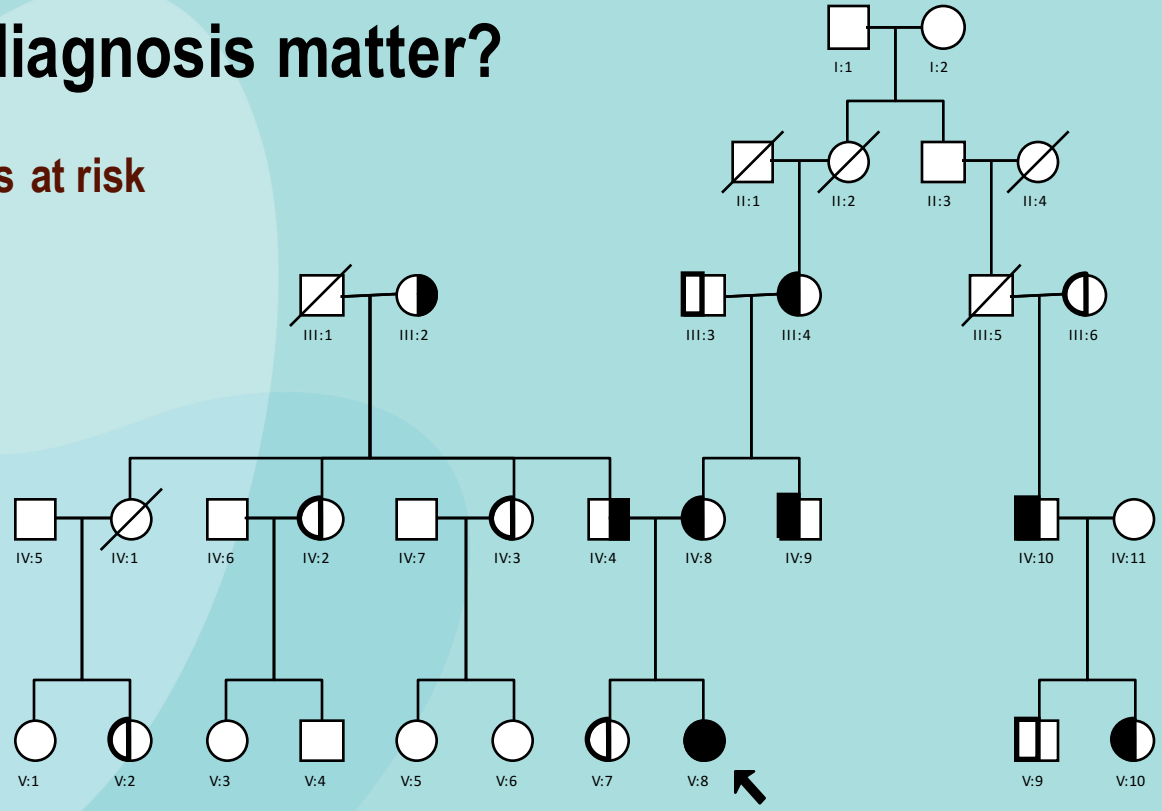
Phenotype	Genotype	Disorder	Treatment
Children (<16 years) Total cholesterol >260 mg/dL or LDL-C >155 mg/dL + family history of hypercholsterolemia **Adults** Total cholesterol >290 mg/dL or LDL >190 mg/dL + family history of hypercholsterolemia	LDLR, 92% APOB, 5% PCSK9 1%	FH	High-intensity statins + cholesterol absorption inhibitors Severe/homozygous patients: add PCSK9 inhibitors and/or LDL apheresis
	LDLRAP1	Autosomal recessive hypercholesterolemia	High-intensity statins + PCSK9 inhibitors and/or LDL apheresis
	APOE 2%	Dysbetalipoproteinemia	Statins + fibrates
	ABCG5, ABCG8	Sitosterolemia	**Low phytosterol** diet + cholesterol absorption inhibitors
	LIPA	Lisosomal acid lipase deficiency, Wolman disease	enzyme replacement therapy – Sebelipase Alfa **statins worsen the condition**

Why does genetic diagnosis matter?

- **2) Identification of relatives at risk**

Early diagnosis
↓
Early therapeutics
↓
substancial reduction of CVD risk!





Variant interpretation

© American College of Medical Genetics and Genomics **ACMG STANDARDS AND GUIDELINES** | Genetics inMedicine

Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology

Sue Richards, PhD[1], Nazneen Aziz, PhD[2,16], Sherri Bale, PhD[3], David Bick, MD[4], Soma Das, PhD[5], Julie Gastier-Foster, PhD[6,7,8], Wayne W. Grody, MD, PhD[9,10,11], Madhuri Hegde, PhD[12], Elaine Lyon, PhD[13], Elaine Spector, PhD[14], Karl Voelkerding, MD[13] and Heidi L. Rehm, PhD[15]; on behalf of the ACMG Laboratory Quality Assurance Committee

"(…) this recommendation describes a process for classifying variants into these five categories based on criteria using typical types of variant evidence (e.g., population data, computational data, functional data, segregation data)."

Pathogenic
Likely pathogenic
} **CONFIRMS DIAGNOSIS**

Uncertain significance
Likely benign
Benign
} **DOES NOT CONFIRM DIAGNOSIS**

Richards *et al.*, 2015

Aims

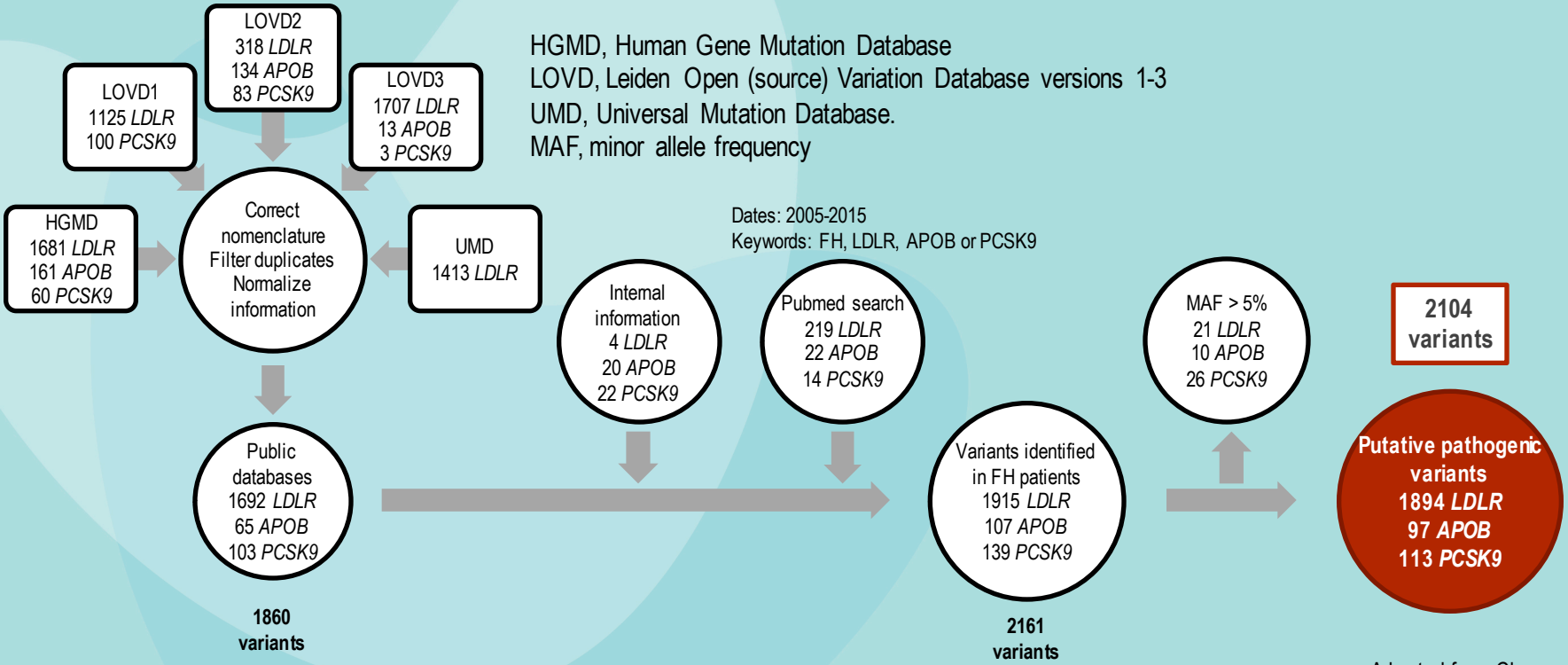
- Construction of a worldwide FH database to support diagnosis
  - *LDLR*, *APOB* and *PCSK9* published variants

Nucleotide & Protein change
Chromosomal & Genomic position
Protein domain
Alteration type – structural & functional
Functional study – type, description and result
In silico analysis – 8 different programs

References – first description & functional studies
Country & Continent origin
MAF % - 1000 genomes, ExAC, EVS
Normolipidaemic panel
Co-segregation

- Adaptation and application of ACMG guidelines to classify all FH variants

Collection of variants



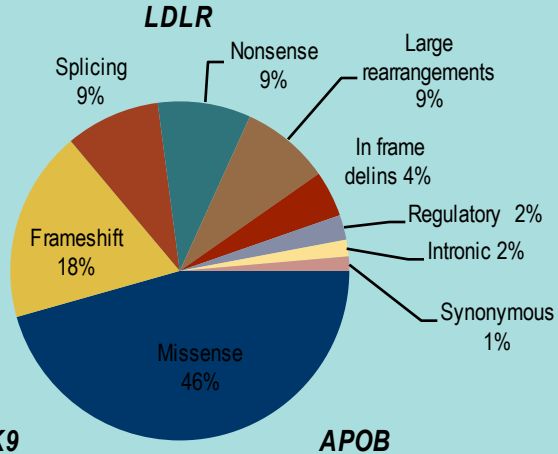
HGMD, Human Gene Mutation Database
LOVD, Leiden Open (source) Variation Database versions 1-3
UMD, Universal Mutation Database.
MAF, minor allele frequency

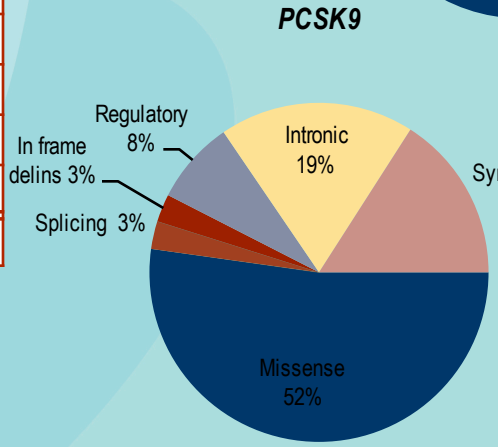
Dates: 2005-2015
Keywords: FH, LDLR, APOB or PCSK9

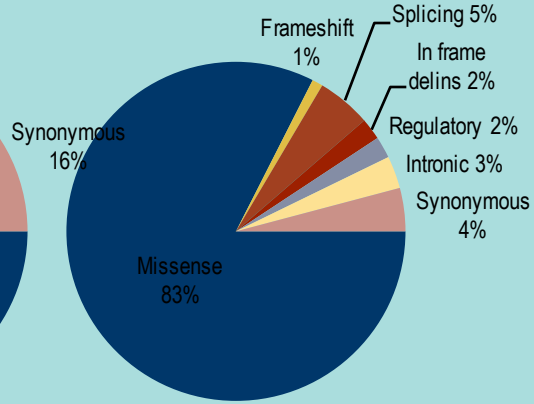
Adapted from Chora *et al.*, 2017

Variants by gene and type

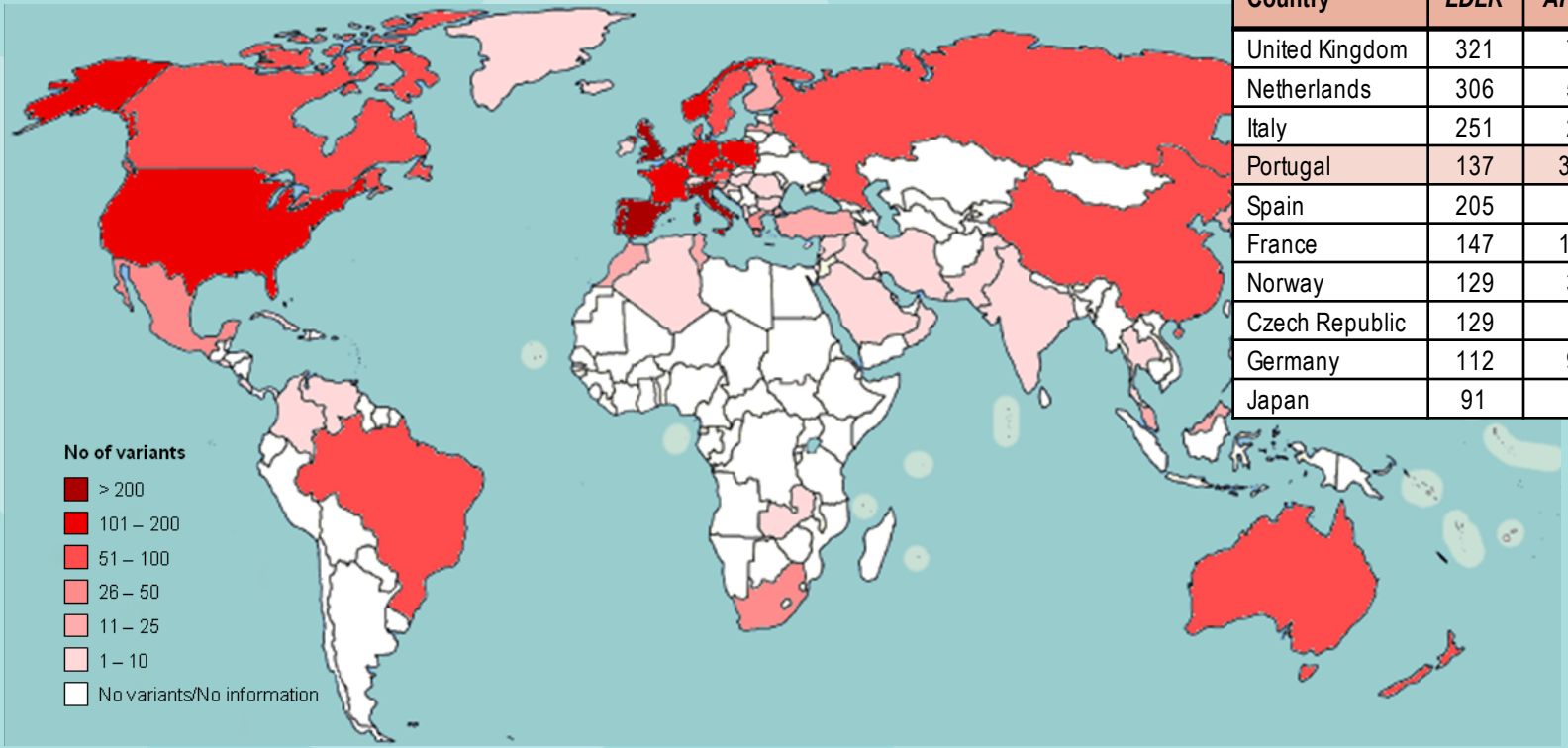
	LDLR	*APOB*	*PCSK9*	Total
Missense	864	80	59	1003
Frameshift	346	-	-	346
Splicing	170	5	3	178
Nonsense	168	-	-	168
Large rearrangements	161	-	-	161
In frame delins	83	2	3	88
Regulatory	45	2	9	56
Intronic	30	3	21	54
Synonymous	27	4	18	49
Total of variants	**1894**	**96**	**113**	**2104**







FH in the world



Country	*LDLR*	*APOB*	*PCSK9*	Total
United Kingdom	321	7	4	332
Netherlands	306	5	-	311
Italy	251	2	8	261
Portugal	137	39	39	215
Spain	205	1	5	211
France	147	12	-	159
Norway	129	3	14	146
Czech Republic	129	-	-	129
Germany	112	9	1	122
Japan	91	-	28	119

Variants with functional studies (FS)

	Type of FS	*LDLR*	*APOB*	*PCSK9*	Total
Level 1	Homozygous patients' cell studies	71	-	-	71
	RNA studies with transcript quantification	19	-	-	19
	Heterologous cell studies	65	-	-	65
	Luciferase studies	19	-	1	20
	APOB and PCSK9 - complete LDLR cycle studies	-	7	18	25
	Total level 1 FS	174	7	19	200
Level 2	Heterozygous patients' cell studies	40	-	-	40
	RNA studies without transcript quantification	34	-	-	34
	Other/Proliferation studies	11	11	2	24
	Total level 2 FS	85	11	2	98
	Total	259	18	21	298

298/2104
~14% of variants

ACMG guidelines adaptation for FH

Table 3 Criteria for classifying pathogenic variants

Evidence of pathogenicity	Category
Very strong	PVS1 null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease Caveats: • Beware of genes where LOF is not a known disease mechanism (e.g., *GFAP*, *MYH7*) • Use caution interpreting LOF variants at the extreme 3′ end of a gene • Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact • Use caution in the presence of multiple transcripts
Strong	PS1 Same amino acid change as a previously established pathogenic variant regardless of nucleotide change Example: Val→Leu caused by either G>C or G>T in the same codon Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level PS2 De novo (both maternity and paternity confirmed) in a patient with the disease and no family history Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to nonmaternity. PS3 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established. PS4 The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls Note 1: Relative risk or OR, as obtained from case–control studies, is >5.0, and the confidence interval around the estimate of relative risk or OR does not include 1.0. See the article for detailed guidance. Note 2: In instances of very rare variants where case–control studies may not reach statistical significance, the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence.
Moderate	PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation PM2 Absent from controls (or at extremely low frequency if recessive) (Table 6) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium Caveat: Population data for insertions/deletions may be poorly called by next-generation sequencing. PM3 For recessive disorders, detected in *trans* with a pathogenic variant Note: This requires testing of parents (or offspring) to determine phase. PM4 Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants PM5 Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before Example: Arg156His is pathogenic; now you observe Arg156Cys Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level. PM6 Assumed de novo, but without confirmation of paternity and maternity
Supporting	PP1 Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease Note: May be used as stronger evidence with increasing segregation data PP2 Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm should not be counted as an independent criterion. PP3 can be used only once in any evaluation of a variant. PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology PP5 Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

LOF, loss of function; OR, odds ratio.

PVS1	Nonsense, frameshift, canonical ± 1 or 2 splice sites, single or multiexon deletions.
PS1	Missense variant at the same codon than a variant proven pathogenic and leads to the same aa
PS3	functional *in vitro* studies (Level 1)
PM1	mutational hot spot: LDLR – exon 4; APOB – c.8458-c.9606, c.9727- c.10494, c.12541-c.13620
PM2	Absent on ESP AND 1KG AND ExAC
PM4	In frame deletions, duplications or delins, smaller than 1 whole exon. All except p.(Leu23dup)
PM5	Missense variant at the same codon than a variant proven pathogenic and leads to a different aa
PM7	functional *in vitro* studies (Level 2)
PM8	more than 1 country AND absent in 100 normolipidemics
PM9	co-segregation – minimum of 10/10;0/10
PP1	co-segregation – minimum of 2/2;0/2
PP2	All missense variants in LDLR
PP3	*in silico* prediction Pathogenic – either "missense" OR "splicing". Only in LDLR
PP4	All variants identified in FH patients

PATHOGENIC VARIANTS

1 PVS	+ 1 or more PS	
	+ 2 or more PM	
	+ 1 PM	+ 1 PP
	+ 2 or more PP	
2 or more PS		
1 PS	+ 3 or more PM	
	+ 2 PM	+ 2 or more PP
	+ 1 PM	+ 4 or more PP

LIKELY PATHOGENIC VARIANTS

1 PVS	+ 1 PM
1 PS	+ 2 PM
	+ 1 PM
	+ 2 or more PP
3 or more PM	
2 PM	+ 2 or more PP
1 PM	+ 4 or more PP

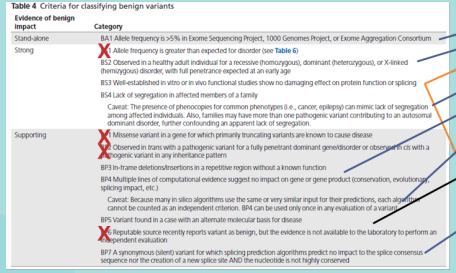


Table 4 Criteria for classifying benign variants

Evidence of benign impact	Category
Stand-alone	BA1 Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium
Strong	BS1 Allele frequency is greater than expected for disorder (see Table 6) BS2 Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age BS3 Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing BS4 Lack of segregation in affected members of a family Caveat: The presence of phenocopies for common phenotypes (i.e., cancer, epilepsy) can mimic lack of segregation among affected individuals. Also, families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation.
Supporting	BP1 Missense variant in a gene for which primarily truncating variants are known to cause disease BP2 Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern BP3 In-frame deletions/insertions in a repetitive region without a known function BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant. BP5 Variant found in a case with an alternate molecular basis for disease BP6 Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

BA1	Variants with MAF >5%
BS2	identified in a panel of normolipidemics
BS3	functional *in vitro* studies (Level 1)
BS4	Lack of co-segregation - 3/4;0/2 OR 4/4;1/2
BP3	In frame – only p.(Leu23dup)
BP4	*in silico* prediction Benign – both "missense" AND "splicing". Only in LDLR
BP5	Only applicable in diagnosis context. If the patient carries a proven pathogenic variant, any unknown variants identified in the same gene or other FH gene will receive a BP5 point
BP7	synonymous - only splicing *in silico* category. Only LDLR
BP8	functional *in vitro* studies (Level 2)

BENIGN VARIANTS

1 BA	
2 or more BS	

LIKELY BENIGN VARIANTS

1 BS	+ 1 BP
2 or more BP	

VARIANTS OF UNKNOWN SIGNIFICANCE (VUS)

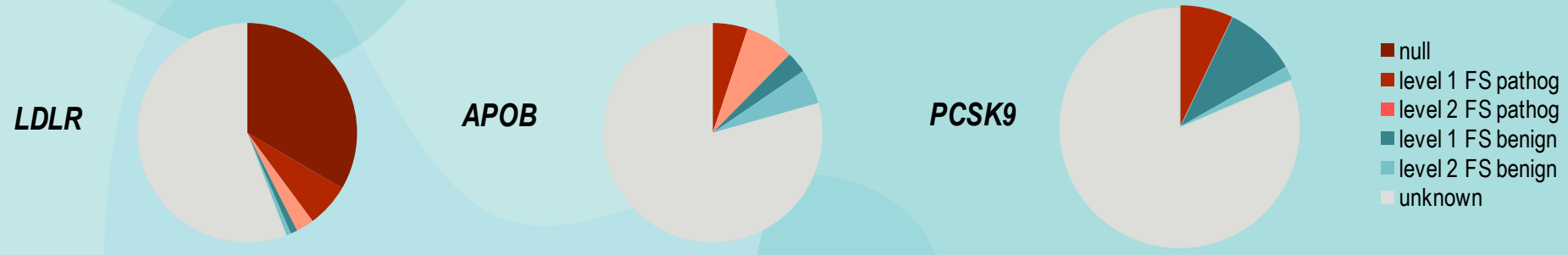
Contradictory classification – benign and pathogenic
Not enough points for classification in either category

ACMG classification

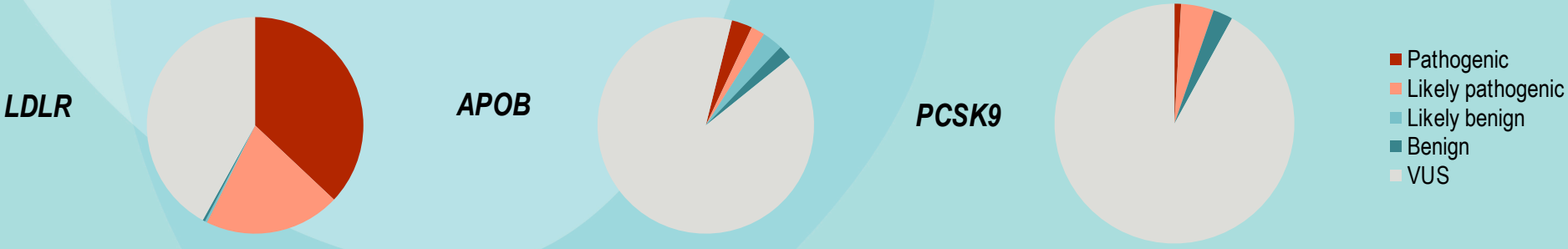
ACMG classification	*LDLR*	*APOB*	*PCSK9*	Total
Benign	7	2	3	12
Likely benign	6	3	0	9
Variants of unknown significance (VUS)	795	87	104	986
Likely pathogenic	385	2	5	392
Pathogenic	701	3	1	705
Total	**1894**	**97**	**113**	**2104**

Pathogenicity classification defined by:

Functional studies + null variants → ~42% variants classified



ACMG guidelines with FH adaptations → ~53% variants classified



Conclusions

- more than 2100 variants have been identified in clinical FH patients
- less than 15% of variants have been studied by in vitro functional assays.

- ACMG guidelines are a valid tool for variant classification
- several gaps in the algorithm have been identified:
  - large deletions are classified as VUS
  - functional studies do not have the weight they should have on variant classification

- Need to develop a more thorough adaptation of ACMG guidelines for FH covering these gaps and other specific criteria for FH.

ClinGen FH group
FH Variant Curation Committee

Conclusions

- more than 2100 variants have been identified in clinical FH patients
- less than 15% of variants have been studied by in vitro functional assays.
- ACMG guidelines valid tool for variant classification
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  - large deletions are cl
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Correct variant interpretation

A correct and timely FH diagnosis

Early and adequate therapy in FH patients

ClinGen FH group
FH Variant Curation Committee

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ORIGINAL RESEARCH ARTICLE | Genetics inMedicine

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