# Autosomal dominant hypercholesterolemia in Catalonia: correspondence between clinical-biochemical and genetic diagnostics in 967 patients studied in a multicentric clinical setting

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Running title: Molecular diagnosis of FH in Catalonia

**ABSTRACT**

**BACKGROUND:** Autosomal dominant hypercholesterolemia (ADH) is associated with mutations in LDL receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin 9 (*PCSK9*) genes and is estimated to be greatly underdiagnosed. The most cost-effective strategy to increase ADH diagnosis is a cascade screening from mutation-positive probands.

**OBJECTIVE:** To evaluate the results of ADH genetic analysis in our clinical laboratory between 2008 and 2016, giving service to most lipid units of Catalonia, an autonomous region of Spain with approximately 7.5 millions of inhabitants.

**METHODS:** After the application of the Dutch Lipid Clinic Network (DLCN) clinical diagnostic score for ADH, this information together with blood or saliva was referred to our laboratory from 23 different Lipids Clinic Units. DNA were screened for mutations in *LDLR*, *APOB* and *PCSK9*, by the DNA-array Lipochip®, the next-generation sequencing SEQPRO LIPO RS® platform, and multiplex ligation-dependent probe amplification (MLPA). Simon Broome FH (SBRG) criteria was calculated in our laboratory and also analyzed for comparative purposes.

**RESULTS:** A total of 967 unrelated samples with biochemical and/or clinical traits of ADH were analyzed. One hundred fifty-eight potential pathogenic variants were detected in 356 patients. The main components of the DLCN criteria associated with the presence of mutation were plasma LDLc, age and the presence of tendinous xanthomata. The contribution of family history to diagnosis was lower than in other studies. DLCN and SBRG were similarly useful for predicting the presence of mutation,

**CONCLUSION**: In a real clinical practice, multicentric setting in Catalonia, the percentage of positive genetic diagnosis in patients potentially affected by ADH was 38.6%. The DLCN showed a relatively low capacity to predict mutation detection but a higher one for mutation rule out.

**KEYWORDS:** (5 to 10): Familial hypercholesterolemia; polygenic hypercholesterolemia; Dutch Lipid Clinic Network score; cardiovascular risk; molecular diagnosis.

## INTRODUCTION

Autosomal dominant hypercholesterolemia (ADH) is one common cause of dyslipidemia, i.e., classical hyperlipoproteinemia type 2A phenotype (HLP2A) [1]. It is characterized by elevated plasma low-density lipoprotein cholesterol (LDLc) mainly due to defective cellular LDL receptor (LDLR) function, referred to as familial hypercholesterolemia (FH, OMIM # 143890). In a recent exome sequencing study of 9,793 cases with early myocardial infarction, *LDLR* rare variants were identified as the most common genetic defect [2].ADH includes variants in other genes encoding proteins that interact with LDLR, such as the LDLR ligand, apolipoprotein B-100 (APOB) gene [1], referred to as familial ligand-defective hypercholesterolemia (OMIM #144010), and the LDLR catabolic regulator, proprotein convertase subtilisin/kexin type 9 (PCSK9) gene, referred to as FH3 (OMIM #603776). A mutation in the *APOE* gene has also been found to be associated with ADH [3]. There are also recessive forms of HLP2A, mainly due to variants in the low-density lipoprotein receptor adaptor-protein 1 gene (*LDLRAP1*) and referred to as autosomal recessive hypercholesterolemia (ARH, OMIM #603813).

Heterozygous ADH was generally believed to affect 1 in 500 individuals, although it was found to affect 1 in 250 in Denmark [4] and Catalonia [5], data that could actually reflect the actual worldwide ADH prevalence [6]. Cholesterol-lowering treatment with statins has been shown to dramatically reduce CHD risk in patients with ADH [7] . Therefore, early detection of subjects carrying pathogenic variants in LDLR, APOB and/or PCSK9, combined with a cholesterol-lowering therapy, which is also cost-effective from a socioeconomic point of view, should be used to decrease CHD at a population level [1]. A recent study showed that patients with FH and confirmed mutations in *LDLR* had an increased risk of premature CHD compared with women with genetically unexplained FH [8]. Thus, the genetic confirmation of ADH may be

important to identify patients at higher risk of CHD [9]. Characterizing *LDLR* pathogenic variants is thus a key point to provide an accurate diagnosis of ADH. In addition, it could help to predict patients' statin response, depending on the LDLR class mutation [10], and be of help to estimate their cardiovascular disease risk [11] In general, patients homozygous or compound heterozygous for LDLR variants, or double heterozygous for LDLR and APOB variants present a more severe phenotype than simple heterozygous [12].

The clinical diagnosis of ADH includes an increase in plasma LDL cholesterol (>4.9 mmol/L or 190 mg/dl), a family history of hypercholesterolemia, a personal and/or first-degree family history of premature CHD, the presence of tendinous xanthomata (TX), and/or premature arcus cornealis (prior to 45 years). These symptoms are often scored clinically by applying the Dutch Lipid Clinic Network (DLCN) modification of the Make Early Diagnosis to Prevent Early Death (MedPed) criteria [1, 13]. Other countries, such as the U.K., use the Simon Broome Register Group (SBRG) criteria for this purpose [14].

A major—if not the main—reason for the genetic analysis of ADH is to perform a direct genetic cascade with the aim of detecting the greatest number of affected individuals. This is particularly relevant since ADH is underdiagnosed and undertreated [1]. Our study is an example of a targeting approach in a clinical, multicentric setting, prior to developing a thorough cascade screening based on the genetic analysis of first-degree relatives of mutation-positive ADH probands.

## MATERIALS AND METHODS

### Patients

A total of 967 citrate blood or saliva samples from unrelated patients with biochemical and/or clinical traits of ADH referred to our laboratory (Biochemistry Service, Hospital Santa Creu i Sant Pau, Barcelona) from 23 lipid clinic units around Catalonia, from July

2008 to December 2016, were studied. Catalonia is an autonomous region of Spain with approximately 7.5 millions inhabitants. In our country, lipid clinic are located in hospitals and include one or more specialists of Internal Medicine, Endocrinology, Cardiology, Pediatrics or Clinical Laboratory. Most Catalan lipidologists belong to and participate in the Catalan Network of Lipids and Atherosclerosis, XULA, a non-profit organization created to improve the assistance and clinical research in preventive cardiovascular medicine. A concrete XULA mission is to coordinate efforts in preventive cardiovascular medicine with specialists in Family Medicine. The clinical diagnosis of ADH was assessed after the application of the DLCN criteria [1, 13], which are based on the presence of the typical physical symptoms of xanthomas, xanthelasmas and arcus cornealis; a family and personal history of cardiovascular disease; and LDL-cholesterol levels (in all cases, obtained before treatment). Although genetic analysis of ADH index cases was recommended only when DLNC ≥ 6, all samples that arrived at our clinical laboratory were processed, since in some specific cases the suspicion of ADH was not based only on the DLCN score. Only 80 of the patients studied were followed at our hospital. Thus, clinical and/or biochemical data were provided, in a summarized DLNC score form, by the medical center of origin, dispersed throughout the Catalonia territory. The SBRG criteria was calculated in our laboratory in case of a detailed DLCN score was provided. The Ethics Committee of the Hospital de la Santa Creu i Sant Pau reviewed and approved the study protocol. Only individuals who provided their written informed consent were included in this study.

**DNA analysis**

Genomic DNA was extracted from the leukocytes of peripheral whole blood samples, obtained after 12 hours of fasting, or saliva collected in Oragene® DNA sample collection kit (DNAGenotek) using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. From July 2008 to June 2012, 515 DNA samples were

analyzed using a DNA-array (LIPOchip®, Progenika Biopharma, Derio, Spain) following a procedure described elsewhere [15, 16]. We used versions 7 to 9 of this microarray, which detect the presence of the approximately 250 most frequent Spanish point mutations in the *LDLR*, the *APOB* exon 26, and the *PCSK9* p.Ser127Arg, p.Phe216Leu, p.Arg218Ser, and p.Asp374Tyr variants, as well as the copy number variations (CNV) of the *LDLR*. From July 2012 to December 2016, 452 samples were analyzed using the next-generation sequencing (NGS) kit SEQPRO LIPO RS® (Progenika Biopharma Grifols, Derio, Spain)[17]. This kit detected mutations in *LDLR*, *APOB*, *PCSK9*, and LDLR adapter protein 1 (*LDLRAP1*) genes, and CNV in *LDLR*. To correct discrepancies between the two methodologies, samples negative by Lipochip® and with DLCN ≥ 8 (n=181) were sequenced by NGS using an Ion AmpliSeq custom panel (Thermofisher, Waltham, MA, USA) and also tested for CNV in the *LDLR* by the multiplex ligation-dependent probe amplification (MLPA) method, using the SALSA MLPA P062 LDLR probe mix kit (MRC-Holland, Amsterdam, Nederland) according to the manufacturer's instructions.

**Variant characterization and bioinformatics analysis**

The nomenclature of the allelic variants follows the recommendations of the Human Genome Variation Society (http://www.hgvs.org). Point mutations causing premature stop codons, small insertions or deletions, causing a frameshift and a premature stop codon, large rearrangements, and mutations affecting intron donor or acceptor splice sites (positions +1, +2, -2 or -1) were considered directly as pathogenic. The rest of the variants (synonymous, missense, in frame small insertions and deletions, mutations affecting promoter, 5'UTR or 3'UTR, and intronic variants) were considered pathogenic depending on the existence of functional analysis previously reported in the literature, the justification as pathogenic or likely pathogenic in databases, like Exome Aggregation Consortium (exac.broadinstitute.org), 1000 Genomes Project (browser.1000genomes.org), Exome Variant Server (evs.gs.washington.edu/EVS),

ClinVar (www.ncbi.nlm.nih.gov/clinvar), UCL-LDLR (www.ucl.ac.uk/ldlr/LOVDv.1.1.0/), and Human Gene Mutation Database (www.hgmd.org). In the absence of the previous information, a variant was considered pathogenic, or likely pathogenic, when most of the programs used for bioinformatics analysis concluded a probable alteration in gene regulation, protein function, or protein expression.

For bioinformatic analysis, the impact of point mutations on the protein structure and function were assessed with SIFT (sift.bii.a-star.edu.sg)[18], PolyPhen2 (genetics.bwh.harvard.edu/pph2/index.shtml)[19], Panther (www.pantherdb.org)[20], Provean (provean.jcvi.org)[21], i-Mutant (gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0)[22], SNPs3D (www.snps3d.org)[23], PMut (mmb.pcb.ub.es/pmut2017)[24] Mutation Taster (www.mutationtaster.org)[25], and Mutation Assessor software (mutationassessor.org)[26]. Variants affecting introns were analyzed using Human Splicing Finder v.3.0 software (www.umd.be/HSF3/HSF.html)[27].

**Statistical analyses**

Data are presented as means (standard deviation) for continuous variables, and frequencies for categorical variables. The plasma LDLc cholesterol of patients without treatment was used in logistic regression in categorized form, according to DLCN criteria [1], i.e. 1) <155 mg/dL, 2) 155–189 md/dL, 3) 190–249 mg/dL, 4) 250–329 mg/dL, and 5) >330 mg/dL. Differences in the mean values between groups were assessed by Student's t-test, and categorical variables were compared using the chi-square test. Statistical calculations were performed using SPSS v13.0 for Windows (SPSS Inc). A value of $P<0.05$ was considered statistically significant.

**RESULTS**

Nine hundred sixty-seven unrelated samples from independent patients were included in this study, 46.2% males and 53.8% females, with a mean (SD) age and DLCN score

of 44 (14.3) years, and 7.82 (3.4), respectively **(Table 1)**. A total of 23 hospitals around Catalonia participated in the study and provided clinical and/or biochemical (DLCN) data. However, the clinical and biochemical information provided was sometimes incomplete: 30 of the samples were sent without a DLCN score and 89 were sent without a detailed DLCN score.

A putative pathogenic variant was detected in 386 (39.9%) subjects. Of them, 361 (93.5%) were heterozygous, 23 (6%) compound heterozygous, one (0.26%) double heterozygous *LDLR-PCSK9* and one (0.26%) homozygous.

A total of 176 putative pathogenic variants were identified (**Supplementary Table 1**): 172 (97.7%) in *LDLR*, two (1.14%) in *APOB* and two (1.14%) in *PCSK9*. Ninety-five (55.2%) of the *LDLR* variants were missense or in-frame mutations, nine (5.2%) affect promoter or 5'UTR, 35 (20.3%) were nonsense or frameshift mutations, 18 (10.5%) were splicing mutations, 12 (7.0%) were large rearrangements and three (1.7%) were synonymous variants with a potential functional effect. The two variants in *APOB* were missense mutations affecting the same residue (p.Arg3527Trp and p.Arg3527Gln) in exon 26. One of the *PCSK9* variants was a missense mutation (p.Arg496Trp) and the other was an in-frame insertion (p.Leu22_leu23dup). Twenty-one of the variants were novel, all in the *LDLR* gene.

A database search and bioinformatic analysis of the variants resulted in 152 (86.4%) probably pathogenic, six (3.4%) possibly pathogenic, three (1.7%) conflicting interpretation and 15 (8.5%) probably benign variants.

It is noteworthy that not all patients with two *LDLR* variant alleles present with a severe homozygous phenotype[28]. Four of the variants were always associated with another one: c.1690A>C (p.Asn564His) with c.2397_2405del (p.Lys799_Phe801del) in eight

subjects, and c.313+1G>C with c.274C>G (p.Gln92Glu) in five subjects. In the first case, each of these isolated variants had little effect on receptor function, but together reduced the receptor function by 80% [29, 30]. In the second case, only one of the variants (c.313+1G>C) was likely pathogenic; therefore, patients were considered single heterozygotes. Of the remaining 10 compound heterozygotes, only one presented a combination of pathogenic variants (p.Gly335Ser and p.Ala540Thr), two a combination of probably benign variants, and the rest, likely a pathogenic/benign combination.

Of the three synonymous variants, c.48C>A (p.Leu16Leu) was identified once, in one patient carrying the nonsense variant c.2001T>A (p.Cys667*), a three-year-old girl, with no reported DLCN. The *in silico* analysis shown the potential deleterious effect of this variant on splicing, but it was considered a silent variant in ClinVar and LOVD-*LDLR* databases[31]. An *in vitro* analysis of another synonymous variant, c.621C>G (p-Gly207Gly), supports an alteration of splicing leading to an in-frame deletion of 75 bp [32]. The third synonymous variant, c.1503G>A, lacks functional studies that demonstrate splicing impairment; our *in silico* analysis was inconclusive, so we considered it probably nonpathogenic, in accordance with UCL-LDLR database [31].

The most frequent pathogenic variant was the missense *APOB* c.10580G>A (22 patients), followed by the frameshift c.1045del (13 patients), the nonsense c.1342C>T (11 patients) and the splicing c.1845+1G>C (11 patients) variants, all in the *LDLR* (**Supplementary Table 1**).

Regarding the type of mutation in *LDLR*, large rearrangements presented the highest DCLN score (**Supplementary Table 2**), followed by nonsense + frameshift, missense + inframe, splicing + intronic and promoter + 5'UTR, in descending order, although the differences were not statistically significant ($p = 0.482$). The DCLN score, however,

was significantly different between missense + frameshift *LDLR* (mean 9.64) and *APOB* variants (mean 7.09) ($p$ = 0.024).

The patients with a positive genetic diagnosis were younger than those without genetic defects ($p$ =1.5E-06) and presented a higher DLCN score (9.1) than negative patients (7.1) ($p$ = 7.95E-16; **Table 1**). These differences were mainly due to the LDLc levels: patients with a positive genetic diagnosis presented a family history with a higher frequency of hypercholesterolemic relatives, both adults and children, and a personal history of higher LDLc (Table 1). As shown in **Figure 1**, the distribution profile of LDLc classes was significantly different between patients with or without a pathogenic variant ($p$ = 1.36E-16). In addition, the personal history of TX was more frequent in mutation-positive than in mutation-negative patients (**Table 1**). It is noteworthy that there were no differences between groups between family and personal histories of premature coronary, cerebral or peripheral disease (**Table 1**).

The variables independently associated with genetic diagnosis were plasma LDLc, a family history of hypercholesterolemia, both adult and children, a personal and family history of TX and a personal history of premature CAD, while age was inversely associated with the presence of a mutation (**Table 2**). Together, these variables explained up to 27% of the genetic diagnosis variation, and age alone accounted for 7.3% of the variation. Nevertheless, it is of note that only 37%, 50% and 48% of patients with a first-degree family history of hypercholesterolemia, children (aged <18 years) with hypercholesterolemia, and patients with first-degree relatives with TX, were positive for genetic diagnosis, respectively. The frequency of mutation carriers in the group with a personal history of TX and premature CAD were 45% and 39%, respectively. In fact, of the factors that contribute to the DLCN score calculation, only subjects with plasma LDLc above 330 mg/dL exceeded 50% of positives, reaching up to 75%.

As some of the most frequently used clinical diagnostic criteria, DLNC and SBRG give priority to positive genetic testing, in this study we considered the presence of a pathogenic variant as the reference diagnostic method. The proportion of mutation positives increases as the DLCN score increases (**Figure 2**), reaching a value of 50.7% when the clinic diagnosis was definite (DLCN > 8). Therefore, it is noteworthy that distribution of groups reflects degrees of diagnostic suspicion of FH rather than representing a population-based sampling. The predictive values of the presence of a mutation were determined for the DLCN and SBRG criteria (**Table 3**). The “probable + definite” (DLCN ≥ 6) diagnosis of FH showed a good diagnostic sensitivity (89.4%) but low diagnostic specificity (24.5%), while the “definite” diagnosis of ADH showed limited sensitivity (45.5%) and moderate specificity (74.4%). In our sample, the cutoff DLCN score that maximized the AUC was DLCN ≥ 8 (60.1% sensitivity, 62.3% specificity). With respect to the predictive values, positive predictive values (PPVs) were lower than negative predictive values (NPVs) in all DLCN classes. Moreover, for DLCN ≥ 6 the accuracy, defined as the probability of a correct diagnosis, i.e. the number of correctly diagnosed divided by all subjects, was 48.6 %, increasing to a moderate 63.3% for DLCN > 8 (**Table 3**).

The SBRG criteria showed PPV, NPV and accuracy values slightly lower than DLCN. In case of “definite” SBRG clinical diagnosis, the sensitivity was lower than DLCN, only 26.4%, but specificity was higher, 82.4%. The level of agreement between DLCN and SBRG diagnostic criteria, measured by kappa test, were k = 0.31, meaning a fair agreement between both methods.

The predictive values for the different diagnostic components from Table 1 were also calculated (**Supplementary Table 3**). The cutoff point for LDLc with the best balance of sensitivity and specificity was LDLc ≥ 250 mg/dL (6.5 mmol/L, Youden index 0.22)

Except for an extreme LDLc (> 330 mg/dL), PPV were lower than NPV, as DLCN and SBRG. Nevertheless, the accuracy was, in general, better for the different components than for DLCN ≥ 6 or "possible + definite" SBRG criteria, and similar for "definite" diagnostic of both criteria.

## DISCUSSION

We report here our nine-year experience in the molecular diagnosis of ADH in a clinical setting, as a reference laboratory for a region of 7.5 million inhabitants. This study is the first part of a project aimed at increasing the detection of ADH patients by a genetic cascade screening in relatives of mutation-positive probands. Since 2005, the Catalan Health Department (SCS) has prioritized the genetic screening of ADH at a selected network of lipid and atherosclerosis units, which is currently composed of 23 nodes. However, and despite this approach, it is noteworthy that current recommendations advice the use of the clinical/biochemical FH diagnosis for cascade screening in addition to the genetic one[33].

We achieved a genetic confirmation in 36.8% of subjects, a percentage of positives slightly lower than 41.4% and 41.5% reported in Spanish [15] and Portuguese [34] populations, respectively, but similar to other European [35-38] and non-European [39, 40] populations. Since these studies used similar genetic diagnostic methods, mainly sequencing and MLPA analysis, potential explanations of the low number of mutation-positive probands in our sample could be differences in the recruitment of patients. Regarding this, and considering only lipid units that sent more than 30 patients, the differences among centers in the percentage of mutation-positive patients were statistically significant ($p < 0.0001$), ranging from 18.1% to 56.1%.

Considering the price of each ADH genetic case index study, 1,358 euros were needed for each positive genetic diagnosis. It is noteworthy that, in our hands, a much lower expense is needed for the molecular diagnosis of first-degree relatives of genetically-positive index cases. This is explained by a 6-fold lower price of the Sanger mutation-specific sequencing used and, also, to the higher percentage of genetically-positives obtained in these familial studies (data not shown). Therefore, we are currently trying to fully implement this genetic ADH cascade approach.

In our sample the contribution of family history is low compared with another study in a Spanish population [41], where 98% of subjects reported a family history of hipercholesterolemia, 75% children aged <18 years hypercholesterolemia, and 50.4% first-degree relatives TX and/or arcus cornealis, versus 59.1%, 26.1%, and 6.5% in our sample, respectively. It is possible that, at least in part, this means that our study included fewer patients from registries with a previous and extensive follow up, and more relatively new patients with a clinical suspicion of ADH. Indeed, in our case, the reported DLCN score was based mainly on personal history and, to a lesser extent, on family history. This is also facilitated by social factors, like increased divorces, and geographical mobility, which makes it difficult to keep in contact with other family members and to recall health information, such as TX in relatives or young members with hypercholesterolemia.

With respect to clinical diagnostic methods, DLCN and SBRG seemed to be equally useful for predicting the presence of mutation, presenting a fair agreement between methods . Nevertheless, the predictive values obtained indicate that DLCN and SBRG were better at predicting a negative value than a positive one, with slightly better values for DLCN than SBRG, as in other similar studies[41-43]. In our case, including DLCN scores equal to 8 in the definite DLCN diagnostic gave the best balance of sensitivity and specificity. In the case of “probable + definite” (DLCN ≥ 6), only 41.1% were

mutation positive, while close to 80% of DLCN < 6 were mutation negative. It is well known that the predictive values are influenced by the proportion of mutation positives in the sample, i.e., the prevalence of genetically confirmed ADH, so this result could be due to a low number of mutation positives with DLCN in our sample (36.8%). However, in another study with a similar number of positives (38.4%), and similar sensitivity (89.1%), PPV and NPV for DLCN ≥ 6 were higher than ours: 53.5% and 88.4%, respectively [39]. In this context, it is noteworthy that the difference in DLCN scores between mutation-positive and mutation-negative patients was similar to scores obtained in studies from a Spanish population [15]. One possible explanation could be errors in the application of the diagnostic criteria or because genetic analysis was used in cases that were unlikely to have the disease (such as to try to exclude ADH when DLNC was low, or to try to diagnose the patient as affected by ADH so he or she could qualify for lower prices in statins and/or ezetimibe, as would have been the case of Catalan patients with genetically confirmed ADH). On the other hand, potential limitations of the conducted genetic diagnostic analyses, NGS and DNA-array based techniques, include the lack of consistent detection of large rearrangements. However, in our case, the analysis of a subsample of 181 mutation-negative probands by MLPA showed only two carriers (1.1%) of a previously undetected large deletion. Further, the percentage of large rearrangement carriers in the total sample (8.4%) was similar to other studies in a Spanish population [15, 44].

In our sample, the main component of the DLCN associated with positive genetic diagnosis was the plasma LDLc level, which explains up to 10.6% of variation in genetic diagnosis. Approximately 50% of the interindividual variation in the LDLc plasma level is attributable to genetic variation [45], mainly due to the cumulative effects of multiple sequence variants in an individual. The overlap in the LDLc plasma level between heterozygous carriers and non-carriers is to a large extent due to the high prevalence of modestly severe *LDLR* mutations, at least in The Netherlands [46]. Only

very high plasma levels correspond to monogenic forms of hypercholesterolemia, i.e., functional variants in *LDLR*, *APOB* or *PCSK9* genes. Other very rare gene mutations causing recessive hypercholesterolemia are also known, including the LDL receptor adaptor protein 1 (*LDLRAP1*) and cholesterol 7α-hydroxylase deficiency [47]. Sitosterolemia can also be confounded with FH [48, 49]. However, these alternative possibilities are expected to explain only a very minor part of the mutation-negative ADH patients, and it is unlikely that pathogenic variations in other genes affecting LDL metabolism could explain a significant number of cases. These observations raise the possibility of polygenic hypercholesterolemia in patients until now considered to have monogenic ADH. Further, a recent study proposed the diagnosis of polygenic hypercholesterolemia in three out of every four ADH mutation-negative patients by analyzing six SNPs of the following five genes: *CELSR2*, *APOB*, *ABCG5/G8*, *LDLR*, and *APOE* [50]. The potential existence of a polygenic hypercholesterolemia in patients who, until now, were considered to have monogenic ADH has initiated a debate on whether the cascade is suitable in mutation-negative, potentially polygenic ADH [50, 51]. In any case, our ADH diagnosed patients, in which no monogenic causes were found, present with an increased burden of common risk variants that increase LDL cholesterol, compared to the control population [52]. Polygenic forms of disease are usually characterized by a late-onset expression. As in most published studies, mutation carriers were younger than non-carriers[39, 53-58], and in fact the number of positive genetic diagnoses decreased with age (**Supplementary Figure 1**). As the logistic regression analysis indicates (Table 2), age was inversely related to the presence of a mutation and explains the 7.3% of genetic diagnosis variability. Some studies exclude patients under age 18 years, but in our sample, in the age range of 0-21 years (74 subjects), we obtained 51.4% mutation positives (**Supplementary Figure 1**). Moreover, the mean DLCN score increases with age in mutation carriers, but not in non-carriers (**Supplementary Figure 2**). Some authors have suggested that different clinical criteria score thresholds depending on age should be considered [57]. Efforts to

further differentiate monogenic and polygenic forms of hypercholesterolemia with clinical, biochemical, and familial data could help to improve mutation detection, which is a critical step from which to develop genetic cascade screening. In a recent study, close to 30% of mutation-negative hypercholesterolemic patients presented an extreme LDL weighted genetic risk score (wGRS), compared with 11.8% in the 1000 Genomes Project [59]. However, the difference between patients with extreme wGRS and those without, did not seem to be reflected in the LDLc plasma level. Therefore, other phenotypic traits must be studied.

Possible explanations for the relatively low number of genetic confirmation could relate to limitations of the molecular detection methods employed, and the possibility of polygenic forms of FH. With the recent introduction into the clinic of high-throughput sequencing and CNV detection methods, the possibility of undetected mutations in candidate genes has been reduced. Also, pathologic variants in unidentified genes are expected to explain only a minor part of mutation-negative patients. Given the complexity of FH genetics, clinical and biochemical diagnosis will certainly still be the major diagnostic tool in many patients. In our case, as in other studies with patients referred to lipid clinics [40-42, 60], DLCN and SBRG were both useful tools, with slightly better results for DLCN > 5 (definite + probable). The best balance between sensibility and specificity were obtained including DLCN = 8 in the definite category, and including possible (DLCN 3-5) identified most mutation carriers (37.3 %), However, as a difference with other studies [42], this was obtained with low specificity (24.5 %). The presence of xanthomata was not as predictive of the presence of mutation as LDLC, as a difference with other studies [40, 41, 60]. In some cases, the difference could be related with the severity of the clinical phenotype of the studied population [41]. Some authors proposed the use of plasma pre-treatment LDLc as an alternative clinical diagnostic criteria [40, 41, 57]. In our case LDLc was the best parameter related to the presence of mutation, and LDLc ≥ 250 mg/dL showed a Youden index of 0.22, equal to DLCN > 8,

so in the case of difficulties in obtain personal and family history data, LDLc cutoff value of 250 mg/dL could be a good alternative. Finally, the presence of mutation-positive cases in patients with “possible” and “unlikely” categories was 20.2 and 23.1%, respectively, with a decreasing positive rate as the age increases, thus supporting the convenience of the screening for FH at a young age [61].

**Limitations**

Our study has several limitations. The DLCN score was calculated in the lipid unit of origin by different physicians, and we did not have access to raw data like personal and family histories or the lipid profile, or serum or plasma samples before pharmacological treatment. Therefore, we can neither test the uniformity in the application of DLCN criteria nor perform genotype-phenotype association studies. As pointed out before, our sample consists mainly of recently diagnosed patients, not of a cohort with an extensive follow-up. Finally, a small percentage of mutations could have been undetected in 206 patients with DLNC < 8 studied between 2008 and 2012 and mutation negative with LIPOchip®, which detected the 250 most frequent mutations in Spain, as these patients (unlike those with DLNC ≥ 8) were not subjected to NGS and MLPA.

**Conclusions**

In a real clinical practice setting in Catalonia, and in our hands, the percentage of pathogenic variants detected in patients who were considered likely to have FH is 38.6%. The relatively low number of mutation-positive probands in our sample could be due to differences in patient recruitment which would –at least in part- explain up to a 3.1-fold difference in the ratio of mutation detection from different centers. In this context, DLNC scores of ≥ 6 are expected to yield only around 40% of potential

pathogenic variants, with only 50.7% in DLNC >8, whereas about 80% of DLNC scores < 6 are expected to be mutation negative.

This study has comprehensively evaluated the results of the effort performed so far to genetically diagnose FH in our region. The effort should be continued with an emphasis in familial-cascade diagnosis of the disease, which should include not only genetic diagnosis but also the clinical and biochemical diagnosis, which will be especially needed in cases in which no mutations are identified.

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Contribution statement: J.M.M-C. compiled data, performed statistical analysis, interpreted the data and prepared the manuscript. N.P., R.F., D.I., A.C., E.E., A.P., M.B., M.M., X.P., L.M. and the rest of members of the XULA recruited subjects and performed clinical evaluations. R.R. and S.M. performed the genetic analysis. J.J. was involved in data collection, analysis and interpretation. F.B-V. designed the study, interpreted the data, and had the final approval of the article. All authors revised the article critically and approved the final version of this manuscript.

**Disclosures**

The authors declare that there is no duality of interest associated with this manuscript.

**Conflicts of interest**

All authors have read the journal's policy on the disclosure of potential conflicts of interest. The authors declare that there is no duality of interest associated with this article.

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**Table 1**: Characteristics of the population studied according to the absence (FHM-) or presence (FHM+) of a mutation.

| | Total | FHM- | FHM+ | p-value[1] |
|---|---|---|---|---|
| N (%) | 967 | 611 (63.2%) | 356 (36.8%) | |
| **Method** | | | | |
| LIPOchip®, n (%) | 515 (53.3%) | 330 (64.1%) | 185 (35.9%) | |
| SEQPRO LIPO RS®, n (%) | 452 (46.7%) | 281 (62.2%) | 171 (37.8%) | ns |
| **Sex** | | | | |
| Males (%) | 46.2% | 46.2% | 46.0% | |
| Females (%) | 53.8% | 53.8% | 54.0% | ns |
| **Age (yrs)[2]** | 44.0 (14.3) | 45.8 (13.6) | 41.0 (14.9) | 1.51E-06 |
| **DLCN[2]** | 7.82 (3.4) | 7.08 (2.8) | 9.07 (4.1) | 7.95E-16 |
| **Family history** | | | | |
| 1a. first degree realtive with premature[3] coronary and/or vascular disease | 42.4% | 44.8% | 38.3% | ns |
| 1b. first degree realtive with LDL-c > 210mg/dL | 59.1% | 54.1% | 67.5% | 3.50E-04 |
| 1a and/or 1b | 80.3% | 78.9% | 82.7% | ns |
| 2a. first degree realtive with tendinous xanthomata and/or arcus cornealis | 6.5% | 5.3% | 8.6% | ns |
| 2b. children aged <18 yrs with LDL-c >150 mg/dL | 26.1% | 20.6% | 35.6% | 3.27E-06 |
| 2a and/or 2b | 34.4% | 28.1% | 45.0% | 5.70E-07 |
| **Clinical history** | | | | |
| 3a. patients with premature[3] coronary artery disease | 10.3% | 10.0% | 10.7% | ns |
| 3b. patients with premature[3] cerebral or peripheral vascular disease | 5.1% | 5.5% | 4.4% | ns |
| **Physical examination** | | | | |
| 4a. tendinous xanthomata | 17.7% | 15.5% | 21.4% | 0.029 |
| 4b. arcus cornealis before 45 years of age | 18.2% | 17.5% | 19.2% | ns |
| **LDL-cholesterol** | | | | |
| 5a. LDL-c > 330 mg/dL | 11.1% | 4.5% | 22.0% | 4.10E-15 |
| 5b. LDL-c 250-329 mg/dL | 42.5% | 40.8% | 45.3% | ns |
| 5c. LDL-c 190-249 mg/dL | 40.8% | 47.5% | 29.6% | 2.50E-07 |
| 5d. LDL-c 155-189 mg/dL | 3.3% | 4.0% | 2.2% | *ns* |

[1]. Chi-square testing for frequency comparison, or indepenednt-samples T-test for age and DLCN score. ns = not significant.

[2]. mean (standard deviation)

[3]. premature: men aged <55 years, women aged <60 years.

**Table 2:** Variables independently associated with the presence of a pathogenic mutation in probands by logistic regression analysis.

| Variable | B | p Value | Odds Ratio (95% C.I.) | Adjusted $R^2$ |
|---|---|---|---|---|
| LDLc (mg/dL) | 1.085 | 9.7E-18 | 2.959 (2.31-3.792) | 0.106 |
| Age (yrs) | -0.043 | 1.9E-09 | 0.958 (0.945-0.972) | 0.179 |
| Children aged <18 and LDLc>150 mg/dL | 0.903 | 4.3E-06 | 2.467 (1.679-3.625) | 0.218 |
| Tendinous xanthomata | 0.847 | 0.00016 | 2.332 (1.503-3.62) | 0.233 |
| First degree relative with LDLc>210 mg/dL | 0.607 | 0.0014 | 1.835 (1.265-2.661) | 0.251 |
| First degree relative with tendinous xanthomata and/or arcus cornealis | 0.854 | 0.012 | 2.349 (1.21-4.56) | 0.260 |
| Personal history of premature CAD | 0.716 | 0.017 | 2.045 (1.134-3.689) | 0.269 |
| Constant | -3.514 | 2.1E-11 | 0.030 | |

**Table 3**: Distribution of the Duch Lipid Clinic Network (DCLN) and Simon Broome Research Group (SBRG) categories, according to the absence (FHM-) or presence (FHM+) of pathological variant, and predictive values of a genetic defect for DCLN and SBRG.

| | Total n (%) | FHM- % | FHM+ % | DLCN cutoff | Sensitivity %(CI) | Specificity %(CI) | PPV %(CI) | NPV %(CI) | Accuracy %(CI) |
|---|---|---|---|---|---|---|---|---|---|
| DLCN | | | | | | | | | |
| <3 | 13 (1.4%) | 1.7% | 0.9% | | | | | | |
| 3-5 (possible) | 168 (17.9%) | 22.8% | 9.8% | ≥3 | 99.1 (97.3 - 99.9) | 1.7 (0.9 - 3.2) | 37.3 (34.2 - 40.6) | 76.9 (46.0 - 95.6) | 37.9 (34.8 - 41.1) |
| 6-8 (probable) | 450 (48.0%) | 49.9% | 44.8% | ≥6 | 89.4 (85.5 - 92.4) | 24.5 (21.1 - 28.2) | 41.1 (37.6 - 44.8) | 79.6 (72.8 - 85.0) | 48.6 (45.3 - 51.8) |
| ÷8 (definite) | 306 (32.7%) | 25.6% | 44.5% | >8 | 44.5 (39.3 - 49.9) | 74.4 (70.6 - 77.8) | 50.7 (44.9 - 56.4) | 69.4 (65.6 - 73.0) | 63.3 (60.1 - 66.4) |
| SBRG | | | | | | | | | |
| unlikely | 126 (14.9%) | 18.1% | 9.4% | | | | | | |
| Possible | 544 (64.2%) | 64.3% | 64.2% | | | | | | |
| Definite + possible | 721 (85.1%) | 81.9% | 90.6% | | 90.6 (86.7 - 93.5) | 18.1 (15.0 - 21.7) | 39.9 (36.4 - 43.6) | 76.2 (67.6 - 83.2) | 45.3 (42 - 48.8) |
| Definite | 177 (20.9%) | 17.6% | 26.4% | | 26.4 (21.7 - 31.7) | 82.4 (78.8 - 85.5) | 47.5 (40.0 - 55.1) | 65.1 (61.3 - 68.7) | 61.4 (58 - 64.7) |

**FIGURE 1**
Distribution of LDL-c classes, as defined in the DLCN score, in patients carrying a pathogenic mutation (positives) and non-carriers (negatives).

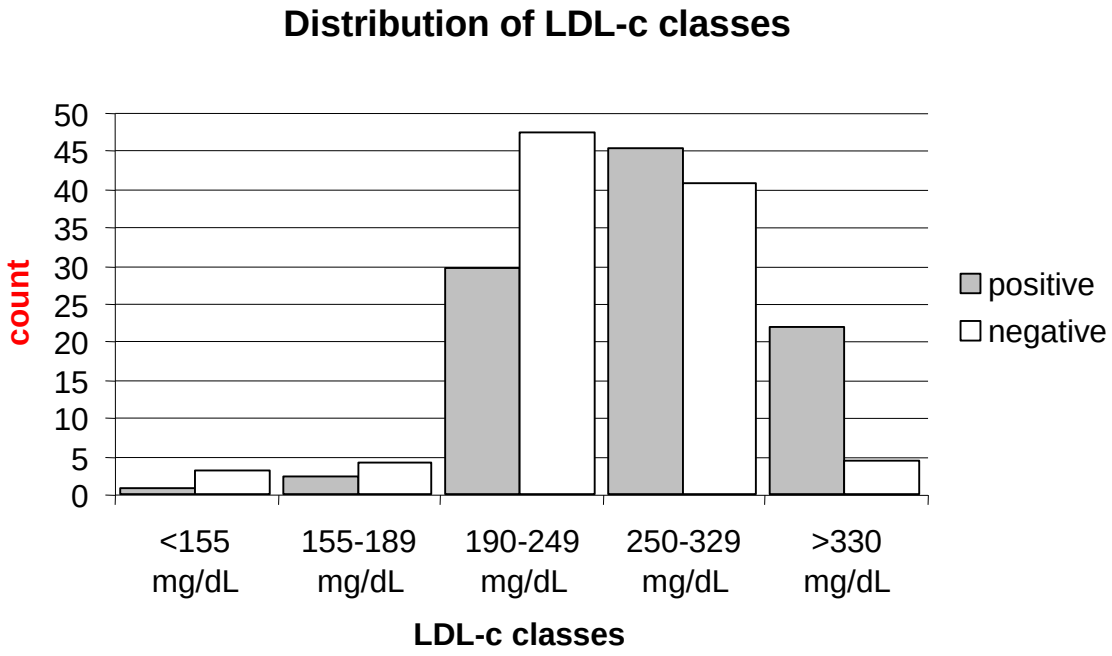
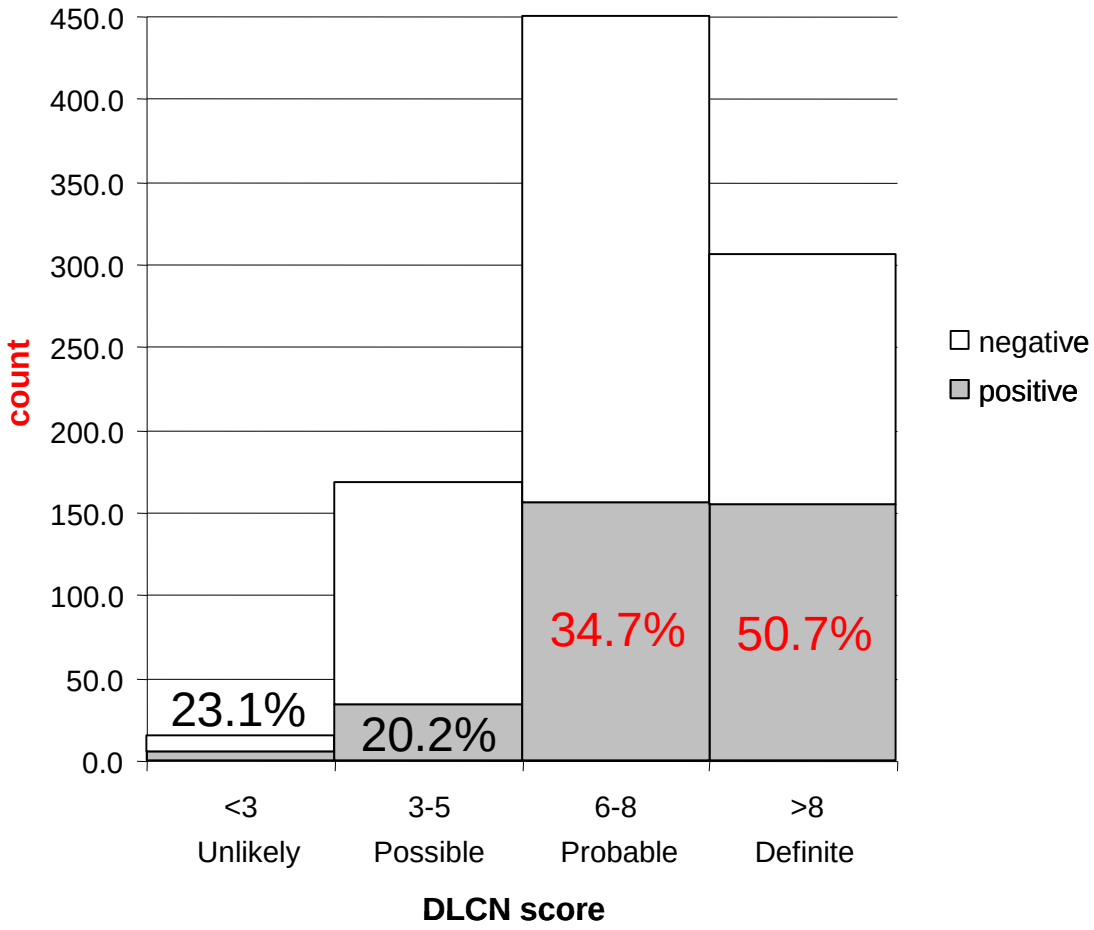


**FIGURE 2**
Percentage of probands where a mutation was found (positives), classified by the DLCN score.



**Supplementary Table 2**: DLCN scores for different mutation types

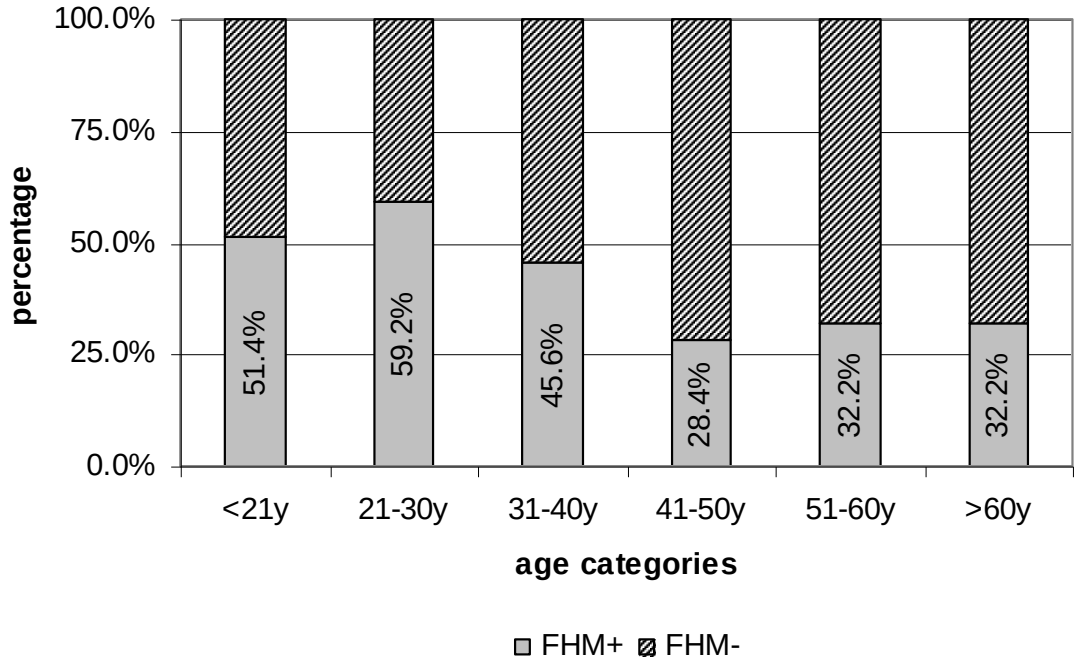
| | n | mean (SD) | CI (95%) |
|---|---|---|---|
| *LDLR* | | | |
| promoter + 5'UTR | 16 | 8.00 (3.16) | [6.31 - 9.69] |
| missense + in frame | 157 | 9.10 (4.08) | [8.46 - 9.75] |
| nonsense + frameshift | 76 | 9.64 (4.03) | [8.72 - 10.57] |
| splicing + intronic | 47 | 8.87 (3.76) | [7.77 - 9.98] |
| large rearrangements | 28 | 9.82 (4.13) | [8.22 - 11.42] |
| Total | 324 | 9.20 (3.99) | [8.77 - 9.64] |
| *APOB* | | | |
| missense + in frame | 23 | 7.09 (3.10) | [5.76 - 8.42] |

**Supplementary Table 3**: Predictive values for different components of the diagnostic criteria.

| Component | Sensitivity %(CI) | Specificity %(CI) | PPV %(CI) | NPV %(CI) | Accuracy %(CI) |
|---|---|---|---|---|---|
| **Family history** | | | | | |
| 1a First degree relative with premature[1] CVD | 38.3 (32.6 - 44.4) | 55.2 (50.5 - 59.8) | 34.0 (28.8 - 39.6) | 59.8 (54.9 - 64.5) | 48.8 (45.2 - 52.5) |
| 1b First degree relative with LDLc>210 mg/dL | 67.5 (61.6 - 73.0) | 45.9 (41.3 - 50.6) | 42.9 (38.2 - 47.8) | 70.1 (64.5 - 75.2) | 54.0 (50.3 - 57.7) |
| 1a and/or 1b | 82.7 (78.0 - 86.6) | 21.1 (17.8 - 24.9) | 38.6 (35.0 - 42.4) | 67.1 (59.3 - 74.0) | 44.2 (40.9 - 47.6) |
| 2a First degree xanthoma and/or arcus cornealis | 8.6 (5.7 - 12.5) | 94.7 (92.2 - 96.4) | 48.1 (34.2 - 62.2) | 64.2 (60.6 - 67.6) | 63.1 (59.6 - 66.5) |
| 2b Child <18 years with LDLc>150 mg/dL | 35.6 (30.2 - 41.4) | 79.4 (75.6 - 82.8) | 50.0 (43.0 - 57.0) | 68.1 (64.1 - 71.8) | 63.4 (59.9 - 66.7) |
| 2a and/or 2b | 45.0 (39.4 - 50.6) | 71.9 (67.8 - 75.6) | 49.0 (43.1 - 54.9) | 68.5 (64.5 - 72.3) | 61.8 (58.4 - 65.1) |
| **Clinical history** | | | | | |
| 3a Personal history premature[1] CAD | 10.7 (7.6 - 14.7) | 90.0 (87.0 - 92.4) | 39.1 (29.0 - 50.1) | 62.7 (59.1 - 66.1) | 60.3 (56.9 - 63.6) |
| 3b Personal history of premature[1] PVD | 4.4 (2.5 - 7.4) | 94.5 (92.1 - 96.3) | 32.6 (19.5 - 48.4) | 62.2 (58.8 - 65.6) | 60.7 (57.3 - 64.0) |
| **Physical examination** | | | | | |
| 4a Tendinous xanthomata | 21.4 (17.1 - 26.4) | 84.5 (81.1 - 87.5) | 45.3 (37.3 - 53.6) | 64.2 (60.5 - 67.7) | 60.8 (57.5 - 64.1) |
| 4b Arcus cornealis before 45 years | 19.2 (15.1 - 24.0) | 82.5 (78.9 - 85.6) | 39.6 (31.9 - 47.8) | 63.0 (59.2 - 66.6) | 58.7 (55.3 - 62.1) |
| Family with tendinous xanthomata (2a + 4a) | 28.0 (23.2 - 33.3) | 79.6 (75.9 - 82.9) | 45.2 (38.1 - 52.4) | 64.8 (61.0 - 68.5) | 60.3 (56.9 - 63.6) |
| **LDL-cholesterol** | | | | | |
| > 330 mg/dL | 22.0 (17.7 - 27.0) | 95.5 (93.2 - 97.0) | 74.5 (64.2 - 82.8) | 67.1 (63.6 - 70.4) | 67.9 (64.6 - 71.0) |
| = 250 mg/dL | 67.3 (61.8 - 72.4) | 54.7 (50.4 - 59.0) | 47.1 (42.5 - 51.8) | 73.6 (68.9 - 77.8) | 59.4 (56.0 - 62.7) |
| = 190 mg/dL | 96.9 (94.1 - 98.5) | 7.2 (5.2 - 9.8) | 38.5 (35.1 - 42.0) | 79.2 (64.6 - 89.4) | 40.8 (37.5 - 44.2) |
| = 155 mg/dL | 99.1 (97.0 - 99.9) | 3.2 (1.9 - 5.1) | 38.0 (34.7 - 41.5) | 85.0 (61.1 - 97.6) | 39.2 (35.9 - 42.5) |

[1]. premature: men aged <55 years, women aged <60 years.

**Supplementary Figure 1**: percentage of positives in genetic diagnostic test across age categories.



**Supplementary Figure 2**: mean DLCN score in different age categories.