Comparison of the mutation spectrum and association with pre and post treatment lipid measures of children with heterozygous Familial Hypercholesterolaemia (FH) from eight European countries

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ABSTRACT

Background: Familial Hypercholesterolaemia (FH) is commonly caused by mutation in the *LDLR, APOB* or *PCSK9* genes, with untreated mean Low Density Lipoprotein-Cholesterol (LDL-C) concentrations being elevated in *APOB* mutation carriers, even higher in *LDLR* mutation and highest in those with a *PCSK9* mutation. Here we examine this in children with FH from Norway, UK, The Netherlands, Belgium, Czech Republic, Austria, Portugal and Greece.

Methods: Differences in characteristics and pre- and post-treatment lipid concentrations in those with different molecular causes were compared by standard statistical tests.

Results: Data were obtained from 2866 children, of whom 2531 (88%) carried a reported *LDLR/APOB/PCSK9* variant. In all countries, the most common cause of FH was an *LDLR* mutation (79% of children, 297 different), but the prevalence of the *APOB* p.(Arg3527Gln) mutation varied significantly (ranging from 0% in Greece to 39% in Czech Republic, $p<2.2\times10^{-16}$). The prevalence of a family history of premature CHD was significantly higher in children with an *LDLR* vs *APOB* mutation (16% vs 7% p=0.0005). Compared to the *LDLR* mutation group, mean (±SD) concentrations of pre-treatment LDL-C were significantly lower in those with an *APOB* mutation (n=2260 vs n=264, 4.96(1.08)mmol/l vs 5.88(1.41)mmol/l, $p<2.2\times10^{-16}$) and lowest in those with a *PCSK9* mutation (n=7, 4.71(1.22)mmol/l).

Conclusions: The most common cause of FH in children from eight European countries was an *LDLR* mutation, with the prevalence of the *APOB* p.(Arg3527Gln) mutation varying significantly across countries. In children, *LDLR*-FH is associated with higher concentrations of LDL-C and family history of CHD compared to those with *APOB*-FH.

Key words: Heterozygous Familial Hypercholesterolaemia; mutation spectrum; LDL-C concentrations; statin treatment.

INTRODUCTION

Familial hypercholesterolaemia (FH) is a monogenic autosomal dominant inherited disorder characterised by elevated low-density lipoprotein cholesterol (LDL-C) concentrations from birth and a very high risk of developing Coronary Heart Disease (CHD) at a young age (1), with a prevalence in many countries of around 1 in 250 (2). Mutations in one of four genes involved in clearance of LDL-C from the blood are known to cause FH, most commonly in the *LDLR* gene, which encodes the low-density lipoprotein receptor (LDL-R), but mutations in apolipoprotein B (*APOB*), and gain-of-function (GoF) mutations in proprotein convertase subtilisin/kexin type 9 (*PCSK9*), can produce the phenotype (3). Recently it has been reported that a single mutation in the gene for *APOE* can also cause the FH phenotype (4, 5).

Not all identified variants affect the gene-product and cause hypercholesterolemia. The ClinVar data base has used criteria published by the American College of Medical Genetics (ACMG) (6) to determine the likely pathogenicity of published variants in *LDLR/APOB/PCSK9* reported in patients with clinical FH (7). Classifications are "definitely not" and "likely not pathogenic", "variants of unknown significance" (VUS) and "likely" and "definitely pathogenic". While more than 70% of the 2314 published *LDLR* variants are likely or definitely pathogenic, only 10% of the *APOB* and 13% of *PCSK9* variants are classified as such (7). Mutations in the *LDLR* gene can also be grouped into 5 classes based on results of functional studies using patient-specific cell culture (8). Although there is a very large spectrum of different *LDLR* mutations causing FH (7), only one *APOB* mutation is common in Europeans, p.(Arg3527Gln), with a carrier frequency in gnomAD (https://gnomad.broadinstitute.org/) in non-Finnish Europeans of roughly 1/900. The frequency of this variant varies over Europe, being absent in Greece (9) and at a carrier frequency of roughly 1 in 200 in Switzerland (10). In clinical FH patients where no causative mutation can be found a polygenic cause of their hyperlipidaemia is most likely (11, 12).

In the last 10 years, many National and European guidelines have been published for the identification and management of children with FH (13-20), with lipid-lowering therapy using a statin as well as other agents being the key treatment recommendation. In the UK, the 2008/2017 NICE Guideline (CG71) recommends the diagnostic threshold for children under the age of 16 years should be a total cholesterol >6.7mmol/l and/or LDL-C >4.0mmol/l, and recommends statin therapy should be considered by the age of 10 years (13, 18), while the European Atherosclerosis Society 2015 consensus statement (19) use a diagnostic threshold of LDL-C ≥5mmol/l, or an LDL-C ≥4mmol/l with family history of premature CHD and/or high baseline cholesterol in one parent, to make the phenotypic diagnosis. If a parent has a genetic defect, the LDL-C cut-off for the child is ≥3.5mmol/l. This guideline recommends that statin use should be considered by the age of 8 years, and LDL-C be lowered below 3.5mmol/l, if possible (19). Both recommend use of Ezetimibe as an adjunct to statin therapy in those over the age of 10 years who are statin intolerant or who have not achieved the LDL-C target on a maximal tolerated statin dose. Children (and adults) with FH are also recommended to adopt a healthy life style to decrease their elevated cardiovascular risk (e.g. avoiding or stopping smoking, healthy eating, exercise).

In a study funded by the International Atherosclerosis Society (IAS) we have recently reported on the characteristics at diagnosis and the prevalence, age of initiation and the use of lipid-lowering treatment in FH children from eight countries across Europe (21). In the current paper we analyse the mutation spectrum in these children and examine the association between the gene mutation and predicted class of *LDLR* gene mutation and selected characteristics at diagnosis as recorded at registration as well as pre and post-treatment lipid concentrations. In adults with FH, compared to those with an *LDLR* mutation, those with the *APOB* mutation tend to have lower LDL-C concentrations and a better response to statin therapy (3). This is due to the fact that VLDL remnants can be cleared by their intact LDL-receptors using apoE as a ligand, and that their intact LDL-R will be upregulated by statin therapy (22, 23). We wished to examine if this difference was also seen in children with either an *LDLR* mutation or the *APOB* mutation. A recent study on adults with FH showed better statin response in patients with a monogenic cause of FH vs mutation negative FH patients (the polygenic cause), another example of a genotype-phenotype correlation in FH (24).

METHODS

Patient identification: The collection of data from 3064 children with FH from the eight countries has already been presented in detail (21), and methods used for DNA testing in the different countries are described in the respective references and summarised in **Supplementary Table 1**. In brief, **Norway:** Only children with a confirmed pathogenic mutation in the *LDLR* or *PCSK9* gene, or the p.(Arg3527Gln) mutation in *APOB* gene, or children with elevated LDL-C concentrations and a first or second degree relative with such a mutation, were included (25). **UK:** Children were diagnosed as having FH based on the UK Simon Broome criteria (13, 26), with the majority having been identified by family studies from an index case with a clinical diagnosis of FH. **The Netherlands:** The diagnosis of FH was based either on identification of a FH pathogenic variant in *LDLR/APOB/PCSK9* or Dutch Lipid Clinic Network criteria with definite FH score ≥8 (1). Most children were referred because they had a parent diagnosed with FH (27). **Belgium:** The majority of children were from a family with one parent with FH. A small proportion of were sent directly by their doctor, on suspicion of FH as a result of opportunistic cholesterol testing. **Czech Republic:** Approximately 50% of children were identified through cascade testing, offered to be done in a child when a disease-causing mutation is known in the family. The remainder were identified from a nationally adopted selective FH screening programme or, more frequently, from the other health care-related blood testing. When lipid concentrations in an index child case exceed age and gender specific values of 95th percentile of total and/or LDL-cholesterol distribution, they were referred to the regional paediatric FH centre for specialised counselling and confirmatory testing. All index cases sent to the diagnostic laboratory because of suspected FH were tested for the *APOB* mutation, while only those fulfilling clinical criteria (MEDPED) were tested for any possible *LDLR*/*PCSK9* mutation (28). **Austria:** Children were clinically diagnosed according to the Simon-Broome criteria. Also included were children

with 1st degree relatives with a FH causative mutation. **Portugal:** Children included in this study as index (>70% of total children) fulfilled Simon Broome FH clinical criteria. Also included were affected children that were relatives of adult patients with an FH-causative mutation (29). **Greece:** For Greece, the referring clinician requested that only those with an identified mutation should be included. Children were identified by measurement of cholesterol concentrations around the age of 3 years, and if concentrations were above the 97th centile for age and sex, they were referred to the Athens Metabolic Clinic. Children and their relatives who fulfilled clinical and biochemical criteria were screened as described (9).

Approvals of data collection and sharing were obtained in each country according to national regulations. Fully-anonymized data were sent as an excel sheet in a password protected file, with the password sent separately. Data were stored in the UCL Data Safe Haven, which is fully GDPR compliant.

Variant classification: For determination of pathogenicity of *LDLR* mutations, the LOVD *LDLR* database (https://databases.lovd.nl/shared/genes/LDLR), was used as published (30). This used the 2013 ACGS guidelines (31), which preceded the ACMG guidelines (6) but the differences between the two are minimal. Variants with ACGS scores 1 and 2 are "definitely not" and "likely not pathogenic", score 3 are "variants of unknown significance" (VUS) and scores 4 and 5 are "likely" and "definitely pathogenic". For the *APOB* and *PCSK9* genes ClinVar (7) was used to define pathogenicity. For *LDLR*, variants were also classified into "functional" classes as described by Hobbs et al(8). **Class 1** - variant fails to produce immunoprecipitable LDL receptor protein (null allele). The most frequent types of Class I mutations are nonsense, frameshift or splice site mutations. **Class 2A, 2B** - allele encode protein that are blocked, either completely (Class 2A) or partially (Class 2B) in transport between the endoplasmic reticulum and the Golgi apparatus (transport-defective allele). **Class 3** - variant encodes protein that is synthesized and transported to the cell surface but fails to bind LDL normally (binding-defective allele). **Class 4A, 4B** - variant encodes receptor that moves to the cell surface and binds LDL normally but is unable to cluster in clathrin-coated pits and thus does not internalize LDL (internalization-defective allele). The Class 4 variants have been subclassified into two groups: variants that alter the cytoplasmic domain alone (Class 4A) and variants that involve the cytoplasmic domain together with the adjacent membrane-spanning region (Class 4B). **Class 5** - variant encodes receptor that binds and internalizes ligand in coated pits, but fails to release the ligand in the endosome and thus does not recycle to the cell surface (recycling-defective allele). Where no published direct functional study was identified, the designated class was "context driven" from the type of mutation (eg frameshift, nonsense, splice site, large deletion considered as class 1 etc.). In addition, some variants were designated "No effect" where a published functional study had demonstrated this and some variants could not be classified since no published functional study was identified and "context driven" rules could not be applied.

Statistical methods: Only children with a DNA test for FH, positive or negative, were included. Results for continuous variables are presented as mean (± standard deviation (SD))

and median (with interquartile range (IQR)), and differences by sex and statin use are tested using Mann-Whitney U tests. Differences in the fall in LDL-C by statin use are adjusted for age using analysis of covariance. Changes in lipid concentrations are the difference between the baseline registration and follow-up of the patient. Categorical variables are presented as number and percentages, and tested using chi-squared tests or Fisher's exact test. For conversion to mg/dl, mmol/l concentrations of total and LDL-C should be multiplied by 38.67. In a proportion of Portuguese (6%) children the baseline untreated LDL-C was not available therefore the untreated concentrations were imputed from latest recorded LDL-C using the method as described (32), which adjusts for the type and dose of the lipid-lowering treatment.. All statistical analysis were performed using a language and environment for statistical computing, R version 3.5.1.

RESULTS

Mutation spectrum

Of the 3064 children in the database, information on DNA testing was available in 2866 (93.5%) children, of whom 2531 (88%) carried an *LDLR/APOB/PCSK9* variant. As shown in **Figure 1** (and **Supplementary Table 2**) the most common cause of FH was a mutation in *LDLR* in all countries, but the prevalence of an *APOB* mutation (mainly p.(Arg3527Gln), which accounted for 97% of reported *APOB* mutations) varied significantly across countries (ranging from 0% in Greece to 39% of all mutations in Czech Republic, (*LDLR* vs. *APOB*, Chi^2=601, 7 DoF, $p < 0.0001$)**.** In all countries the prevalence of GoF mutations in *PCSK9* was lowest (overall 0.3% of all mutations), ranging from 2% in Norway and 1% in Portugal, but not present in the rest of the studied countries.

Overall there were 297 different *LDLR* mutations reported (**Supplementary Table S3A**), with the most common mutations varying across countries (**Table 1**). The Czech Republic and the UK showed the highest degree of heterogeneity with 81 and 67 different mutations respectively, while Greece and Austria showed the lowest, with 16 and 17 different (although the sample size in Austria is small)**.** The three most common mutations differed across all countries except for the intron 3 c.313+1G>A mutation, which occurred commonly in Norway and the Netherlands, and p.(Trp44*) in the Netherlands and Czech Republic, demonstrating the extreme heterogeneity of *LDLR* mutations across these eight countries. When summing the contributions of the three most common mutations in each country the totals ranged from 63.9% in Greece to 13.6% in the UK. Overall, these most common mutations (**Table 1**) accounted for 50% (n=1123) of all *LDLR* mutations (n=2260) found in this study (**Supplementary Table S3A**).

For the *APOB* gene, 255 (97%) of the 264 mutation-positive patients carried the p.(Arg3527Gln), with seven other reported variants (**Supplementary Table S3B**), identified in the remaining nine individuals. For the subsequent analysis data from all reported *APOB*

variants were combined. For *PCSK9*, as shown in **Supplementary Table S3C,** two children from Norway carried the well-known pathogenic variant p.(Asp374Tyr), and three carried p.(Arg215His), while two children from Portugal carried p.(Ala62Asp). All of these variants are classified as “likely pathogenic” by ClinVar (7).

Genotype-Phenotype relationships

The baseline characteristics and pre- and post-treatment lipid concentrations by gene mutation are shown in **Table 2**. Since there were only seven children with a clear GoF *PCSK9* mutation, the sample is too small to give reliable estimates, so the majority of contrasts were performed omitting this group. The median age of diagnosis (IQR) was significantly different between groups, with the lowest age in the *LDLR* children and the highest in the *APOB* (5 (7) vs. 11 (7) years, $p < 2.2\times10^{-16}$), although when removing the Greek cohort from the comparison the difference was no longer statistically significant (**Supplementary Table S4**).The proportion of children with a family history of premature CHD was also significantly higher in those with an *LDLR* vs. *APOB* mutation (16% vs. 7%, $p = 0.0005$), with an intermediate value in the no-mutation group (14%).

As shown in **Figure 2A**, compared to those with no-mutation reported, where mean (±SD) concentrations of pre-treatment LDL-C were 5.18(1.30)mmol/l, those with an *LDLR* mutation had significantly higher concentrations (5.88(1.41)mmol/l, $p < 2.2\times10^{-16}$) while concentrations were lower in those with the *APOB* mutation (4.96(1.08)mmol/l), and lowest in those with a *PCSK9* mutation (4.71(1.22)mmol/l). The difference between the *LDLR* and *APOB* groups was still statistically significant after adjustment for age and gender ($p = 0.001$) and also after exclusion of the Greek cohort (5.61(1.48) vs 4.96 (1.08)mmol/l, $p = 9.5\times10^{-16}$, Supplementary Table S4). A similar trend was seen in concentrations of registration total cholesterol (TC). Mean concentrations of HDL-C were not significantly different between *LDLR* and *APOB* mutation carriers, but differed when *PCSK9* and mutation negative individuals were included ($p = 0.003$). Triglyceride (TGs) concentrations were the highest in the no-mutation children, whereas *APOB* mutation carriers had significantly lower TGs than *LDLR* mutation carriers (0.84(0.38)mmol/l vs. 0.93(0.48)mmol/l, $p = 6.2\times10^{-15}$). Compared to the *LDLR* mutation group, the proportion of children who had a registration LDL-C >4.0mmol/l (the Simon Broome diagnostic threshold) was significantly lower in the mutation negative and *APOB* group (92% vs. 86% vs 83%, $p = 4.8\times10^{-10}$). The untreated mean (±SD) LDL-C concentrations varied significantly between the 22 different most common mutations, from 4.53(0.93)mmol/l for p.(Tyr828Cys) to 7.25(1.33)mmol/l in p.(Val429Met) carriers (**Figure 2BA** and **Supplementary Table S5**).

When comparing mean baseline LDL-C between different *LDLR* mutation types according to their effect on the protein sequence (i.e. synonymous, missense, affecting splicing, nonsense, and large insertions/deletions), the highest mean (±SD) concentrations were observed in the large insertions/deletions mutation carriers (6.36(1.64)mmol/l) (**Figure 2C** and **Supplementary Table S6**, which were similar to nonsense mutation carriers

(6.14(1.49)mmol/l, *p* >0.05), but significantly higher than in promoter, splicing and missense mutation carriers (*p* =0.0002, *p* =0.003, *p* =0.001, respectively). The ranking did not change when children from the Greek cohort were excluded (**Supplementary Table S6**).

The *LDLR* variants were scored according to the ACGS criteria, and, as shown in **Supplementary Table S2**, 16 (0.7%) were classified as "likely benign" (score 2) and 44 (2%) as a VUS (score 3), with 1838 (81%) being "likely pathogenic" (score 4) and 362 (16%) "definitely pathogenic" (score 5). As shown in **Figure 2D** (and **Supplementary Table S7A**) there was a gradient in median baseline LDL-C concentrations from score 2 to score 5 (overall *p* value for trend $=3.7 \times 10^{-16}$). Excluding the Greek children did not alter the ranking, with overall effects still highly statistically significant (**Supplementary Table S7B**, overall $p = 9.6 \times 10^{-14}$)

When variants were classified according to the functional classes 1-5 (8), the difference in baseline LDL-C levels was statistically significant with or without the Greek cohort included in the analysis ($p=1.1 \times 10^{-5}$ and $p<2.2 \times 10^{-16}$, respectively), however the order of the classes by LDL-C varied. To remove any effects of the large number of children from the Greek cohort with class 4 and 5 mutations, data is presented in **Figure 2E** after excluding the Greek children (data in **Supplementary Table S8B**). Children carrying class 1 mutations, i.e. those with a null allele, had the highest baseline LDL-C (6.08 (1.53)mmol/l) followed by those with class 2A mutations (5.57 (1.17)mmol/l). As expected, the median LDL-C in the group of children carrying a variant predicted to have no effect were the lowest, with all others groups, including those of unknown function, having an intermediate median LDL-C concentration. Data with the Greek cohort included are shown in **Supplementary Table S8A.**

Genotype-statin response

In this group of children, we have previously reported (21) that overall the effect of statin therapy was to lower LDL-C by an average of 46%, but with the reduction in different countries ranging between 28-57%. This is due to potency of the different statins being used and the use of additional lipid lowering agents such as ezetimibe. Of note, in children aged >10 years, 23% of on-treatment children still had LDL-C >3.5mmol/l, which is above the EAS guideline recommended target. In order to examine whether the response to lipid-lowering therapy differed by the genetic cause of FH we determined the proportion of treated children over the 10 years of age who achieved this target by gene and by ACGS and functional class mutation carriers.

By Mutated Gene: As shown in **Table 2**, the proportion of over 10 years olds achieving the 3.5mmol/l target was higher in *LDLR* mutation carriers in comparison to *APOB* mutation carriers (74% vs. 42%, $p = 6.4 \times 10^{-7}$, **Table 2**). However this result was influenced by data from the Greek children, and after excluding the Greek cohort, 49% of the treated *LDLR* mutation carriers had LDL-C < 3.5mmol/l, with the difference vs the *APOB* group being no longer statistically significant (**Supplementary Table S4).**

By ACGS score: The same analysis between different *LDLR* mutation pathogenicity scores suggested that individuals with score 4 mutations were more likely to achieve the expected post-treatment LDL-C, than those with score 5 with 80% score 4 mutation carriers having post-treatment LDL-C below 3.5mmol/l vs. 49% in the score 5 group (**Supplementary Table 7A**). After excluding the Greek cohort (**Supplementary Table 7B**), however the difference between the ACGS scores 4 vs 5 was (51% vs 44%) and overall was no longer statistically significant

By Functional class: While data from all children is presented in **Supplementary Table 8A**, in order to ensure that interpretation of effects were not unduly influenced by the data from the Greek children, we excluded the Greek subjects from the analysis (**Supplementary Table 8B**). The 3.5mmol/l target was best achieved in children with class 4 mutations (80%), whereas carriers of class 2A mutations and class 1 mutations had the lowest proportion (30% and 45% respectively) of those with LDL-C lower than 3.5mmol/l. The difference in the proportions of children achieving the recommended LDL-C target was statistically significant between the different functional classes of *LDLR* mutations (p = 0.03).

Discussion

This analysis of one of the biggest sets of data of children with FH examined to date, with 2531 with a known mutation, has made several major findings. As expected, the spectrum of *LDLR* mutations across these eight countries is considerable, with more than 290 different mutations found. As described before (21), the children included here were registered by large tertiary referral centres in the different countries, who all received patients from large regions of their respective countries. As such are likely to be representative of children over the whole of the countries included. As previously reported in Holland (33), Greece (9) the Czech Republic (28) Norway (25) and Belgium (34), some *LDLR* mutations were common in particular countries. However, when examining the most frequent three mutations in each country, only two showed overlap, with the intron 3 c.313+1G>A mutation seen with high frequency in Norway and the Netherlands, and p.(Trp44*) in the Netherlands and Czech Republic. Overall, these most common mutations accounted for 50% of all *LDLR* mutations found in this study, but all countries showed considerable heterogeneity, with for example the Czech Republic with 81 different mutations and the UK with 67 different mutations. These data support the view that in all countries a comprehensive DNA diagnostic strategy should include sequencing of the entire *LDLR* gene, so that missing *LDLR* mutation carriers is avoided.

Again as expected, the prevalence of the *APOB* p.(Arg3527Gln) mutation varied significantly over the eight countries, but clearly testing for this mutation should also be carried out in all countries as part of the laboratory diagnostic work. In the gnomAD database, while the occurrence of this variant varies considerably in different populations, the allele frequency in the European non-Finnish group is roughly 1/1700, making this the most common FH-causing mutation known. The prevalence estimates in the child cohorts from the different countries

included here may have been influenced to some extent by the recruitment criteria and by the laboratory diagnostic approaches used, for example every referred index case in the Czech Republic was tested for p.(Arg3527Gln) but only those with a clinical diagnosis of FH were tested for *LDLR* mutations. In children with an *APOB* gene mutation, 97% of the reported variants were p.(Arg3527Gln), with seven other reported variants identified in only one or two individuals. Although detailed molecular studies of some of these variants have not been carried out, the pathogenicity of some of these variants (p.(Thr3826Met), p.(Arg1164Thr), p.(Gln4494del)) has been determined (35-37). Data from all reported *APOB* variants were combined, but excluding the data of the seven variants did not significantly alter the sample mean characteristics or the statistical significance of the *LDLR* vs. *APOB* contrasts. As previously reported in adults with FH (3), the prevalence of GoF mutations in *PCSK9* was low in the children from all countries, which may be in part because of only partial coverage of the gene in some diagnostic laboratories. In Greece, where no screening of the *PCSK9* gene was performed, no individuals carrying such variants have been reported (8) although these data are based on a small sample size and it is possible that such variants may be found in rare cases. However, because of reported higher CHD risk in adults carrying such variants (3, 38), sequencing of the *PCSK9* gene is also recommended (1, 17, 19).

The genotype-phenotype analysis showed that children who carry an *LDLR* mutation had higher mean untreated total and LDL-C (15.1% and 18.5% higher respectively) and a 2.3 fold higher prevalence of a family history of CHD than whose carrying an *APOB* mutation. This confirms reports in adults with FH (3) and from population-based studies (39). While we do not have data to address this specifically, it is likely that the roughly 1mmol/l higher mean LDL-C concentrations seen in *LDLR*-FH vs *APOB*-FH children is also seen in their adult relatives, and the resulting additional “LDL-C Burden” is the major contributor to the higher prevalence of a family history of CHD in the adult relatives of the LDLR-FH children. The low overall prevalence of a family history of CHD in this cohort has been noted previously (21) and is in part explained by the young age of the children, which means that their parents have not yet reached an age where CHD might be more common. It may also reflect the greater availability and benefit of lipid lowering therapy for these parents. Surprisingly, in contrast to adult FH patients, the seven children carrying a GoF *PCSK9* mutation had the lowest mean LDL-C, but the sample size is too small for this conclusion to be robust, and further work is required to confirm or refute this. The median age of diagnosis was also significantly different between groups, with the lowest age in the *LDLR* children (5 vs. 7 years in the *APOB* group), but this finding was strongly influenced by the Greek sample, where all children had an *LDLR* mutation and because of the identification strategy through pediatric clinics, were found at a considerably younger age than in other countries.

When looking at the association of baseline LDL-C levels in children with different functional classes of *LDLR* mutations, our analysis showed that carriers of the class 1 mutation have the highest median LDL-C, followed by those with class 2A mutations. To our knowledge this is the first report of the effect of the functional mutation classes on the LDL-C in children. There was a considerable range of untreated mean LDL-C concentrations in groups of children with

different *LDLR* mutations, with those with the common Greek mutation p.(Val429Met) having 60% higher concentrations than those with a common mutation in Austria p.(Tyr828Cys), and 41% higher than those with a common Czech mutation p.(Gly592Glu). Since LDL-C is a causal factor for development of CHD, it would be expected that, if untreated, these differences in LDL-C would translate into a similar difference in the accumulating LDL-C burden (40) and in the subsequent risk of CHD in the child as they grow up, and in their relatives. Such differences in prevalence of CHD have been reported in carriers of different *LDLR* mutations in studies from the Netherlands (41).

One issue that this analysis has identified is that of the 2252 reported *LDLR* mutations, based on the designation given to the registering centres by their local diagnostic laboratories, 16 (0.7%) were classified as "probably benign" (score 2) and 44 (2%) as a VUS (score 3). Similarly for *APOB*, a small proportion of children (7/2531 (0.3%) mutation positive) were reported as carrying an FH-causing mutation, but with ClinVar reporting these as "benign", or with conflicting evidence for pathogenicity. This highlights the need for better standardisation of variant prediction and classification across diagnostic laboratories, as is being attempted by the ClinGen programme (42), and for development of laboratory assays to characterise the functional impact of those variants on LDL-C metabolism. Although some of the *LDLR/APOB* variants called as VUS may actually be pathogenic, caution needs to be exercised in counselling their families, since the information of an affected/not affected diagnosis as a result of cascade testing using the VUS may not be accurate. A similar concern relates to the families carrying a variant designated as probably benign. In support of the classification of these variants as "benign" and VUS, their median LDL-C was significantly lower than the group carrying a definitely pathogenic variant (34% and 21%, respectively).

In the total cohort of DNA-tested children 335 (12%) had no mutation identified, and these children are therefore most likely to have a polygenic aetiology for their elevated LDL-C concentrations, as has been shown in both adults and children with clinical FH (11, 12). In some countries the molecular testing of the child had been carried out some years ago, and therefore not all regions of all three genes had been comprehensively screened for mutations. It is therefore possible that use of state-of-the-art next generation sequencing methods may identify a causative mutation in *LDLR/APOB/PCSK9* in a small proportion of these mutation-negative children, and thus identify an underlying monogenic cause for their clinical phenotype. However, compared to the group with an identified *LDLR* mutation, the no-mutation group have 11.9% lower mean LDL-C concentrations and 23.7% higher triglyceride concentrations, which is a similar finding as the characteristics of other "no-mutation" adult FH cohorts (3, 43) suggesting that many of these children may have a polygenic and not a monogenic cause of their hypercholesterolaemia.

We also attempted to examine if the response to lipid-lowering therapy might be different between *LDLR* mutation classes and those with an *APOB* mutation. A direct comparison of the fall in LDL-C concentrations from baseline to "on-treatment" concentrations would be confounded by the fact that clinical guidance is for treatment to an LDL-C target of below 3.5mmol/l, and this may be achieved by increasing the dose of a non-potent statin, by

switching to a more potent statin and or by adding another agent such as ezetimibe. The choice of which of these approaches to adopt is made based on clinician as well as patient and parent preferences. In addition, we acknowledge that the children from Greece appear to show a particularly severe phenotype, and for example have the highest mean untreated LDL-C concentration and were identified at a younger age than in other countries (21) As expected, the mean untreated LDL-C concentration seen in carriers of two of the most common Greek mutations (p.(Val429Met) and p.(Gly549Asp)) were the highest of the common mutations in the whole cohort, but the third (p.(Ser286Arg)) was ranked only 13/21. Since inclusion of the Greek data may have incorrectly influenced the inferences made regarding genotype-phenotype comparisons, we have presented analysis with and without these data.

Although this observational data need to be interpreted with caution, taken together, the overall inference from these analyses are that the response to statin therapy is equally good in those with *LDLR* or *APOB* causative mutations, and in those with different functional classes of mutations, but that in children with mutations where little or no LDL-receptors reach the surface (Class 1 and 2) response may be poor and fewer children achieve a treated LDL-C concentration below 3.5mmol/l. This is also the case for children with mutations that result in high untreated LDL-C (such as seen in those with the common Greek mutations), where a good percentage reduction in LDL-C may still be inadequate to lower LDL-C below 3.5mmol/l. Thus, while all children showed a clinically useful LDL-C lowering when on lipid-lowering therapy, these data suggest that those with a class 1 *LDLR* mutation (38% of the non-Greek *LDLR* mutation positive subjects) may represent a group where particular care may be needed to achieve target lipid lowering.

Strengths and Limitations

The main strength of this study is the large size of the cohort, which has enabled the comparison of the mutation spectrum across eight European countries and genotype-phenotype comparisons in a statistically robust manner. The main limitation is because of the different recruitment approaches used in the different countries, and by the different selection processes used for example in Greece, where only children with a known pathological mutation were registered and in others by inclusion of all tested children. Thus some of the differences in lipid concentrations seen in those with different mutations could be explained in part by these issues. A second limitation in the analysis is that different mutation testing strategies have been used in the different countries, with only some using next generation sequencing approaches and many only testing for specific mutations (eg in the UK for p.(Asp374Trp) in *PCSK9* and only for certain regions of the *APOB* gene. Except for Portugal, no country had systematically included the *APOE* gene to test for the p.(Leu167del) so no accurate estimates of the prevalence of *PCSK9* or *APO*E mutations can be made from this data. A third limitation is the large genetic heterogeneity in the mutation spectrum, so even in this cohort some specific mutations and some mutation classes are still relatively small. This is particularly so for the *PCSK9* gene, where further studies to determine the natural history of carriers of GoF mutation carriers through childhood and adolescent are

lacking. There is also a limitation to the analysis of the relationship between mutation class and response to treatment, since treatment regimens differ considerably across the eight countries, and choice of lipid lowering therapies is dependent on both clinician and patient/parent choice. Finally, none of the analyses were adjusted for the relatedness of the recruited children, although this is a possible issue since in some countries a large proportion of the children are carriers of identical mutations. This is because in some countries there are "founder" effects, and not due to selection of multiple children from extended pedigrees. Therefore we do not believe that the analyses presented are confounded by the presence of "hidden" relatedness in the dataset, and also do not believe that any such relatedness would materially influence any of the inferences made from the data.

Conclusions

The most common cause of FH in children from eight European countries was an *LDLR* mutation, but the single most common cause of FH in this cohort was the *APOB* p.(Arg3527Gln) mutation, although the prevalence of this mutation varied significantly across countries. In children, *LDLR*-FH is associated with higher concentrations of LDL-C and of a family history of CHD compared to those with *APOB*-FH. In all countries a comprehensive DNA diagnostic strategy should include sequencing of the entire *LDLR* gene so that missing *LDLR* mutation carriers is avoided. Although only 2-3% of reported *LDLR/APOB* mutations in this cohort are unlikely to be pathogenic, this highlights the need for better standardisation of variant calling across diagnostic laboratories, as is being attempted by the Clinical Genome Resource (https://clinicalgenome.org/working-groups/gene-curation/). (42).

Author Contributions and Acknowledgements

Marta Futema carried out the statistical analysis and co-wrote the manuscript with Steve Humphries and with the help of a writing group comprised of Uma Ramaswami, Martin Bogsrud, Mafalda Bourbon and Tomas Freiberger. Lukas Tichy characterised all the mutations according to functional class. All co-authors commented on drafts and approved the final version of the manuscript. We thank the additional steering committee members for their support for the Register; Joep Defesche, Jules Payne (HEART UK), Phil Rowlands (Wales).

Conflict of interest

Authors of this study do not have any conflicts of interest related to the presented work.

Funding sources

The European Register is supported by a grant from the International Atherosclerosis Society (Pfizer number 24052829). The UK register is supported by funds from the British Heart

Foundation (BHF); HEART UK, Cardiac Network Co-ordinating Group Wales and the Royal College of Physicians. SEH is a BHF Professor and is funded by RG08/008, and by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. MF is funded by the Fondation Leducq Transatlantic Networks of Excellence Program grant (no. 14 CVD03). MV is supported by the Ministry of Health, Czechia, project No. 64165, General University Hospital in Prague. TF and LT are supported by the Ministry of Health, Czechia, grant number NU20-02-00261. The Austrian FH register has been supported by funds from the Austrian Heart Foundation and the Tyrolean Regional Government. The Portuguese FH Study has been supported by grants from the Portuguese Science and Technology Foundation and grants from the Portuguese Cardiology Society. AMM was supported by the Portuguese Science and Technology Foundation, grant number SFRH/BD/113017/2015. The study sponsors had no role in study design, the collection, analysis, and interpretation of data, the writing of the report or the decision to submit the manuscript for publication. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

Figure legends

Figure 1. Proportions of mutations in *LDLR, APOB* and *PCSK9* by country.

Figure 2. Box-Whisker plot of baseline LDL-C in children with:

2A. A detected mutation in *LDLR/APOB/PCSK9* and in those with no mutation reported. The box represents the interquartile range and the line the median. Individual outliers are shown as open circles. Numbers are *LDLR* = 2260, *APOB* = 264, *PCSK9* = 7, Mutation –ve = 335.

2B. Most common *LDLR* mutations, sorted by median baseline LDL-C (data in Supplementary Table S5),

2C. Different types of mutations, according to their effect on the LDLR protein sequence (data in Supplementary Table S6).

2D. A detected mutation in *LDLR* by ACGS class, designated as probably benign (score 2, n = 16), variant of unknown significance (VUS) score 3, n = 44), likely pathogenic (score 4, n=1838) and definitely pathogenic (score 5=362). Overall difference $p < 2.2 \times 10^{-16}$ (Supplementary Table S7A).

2E. A detected mutation in *LDLR* by "functional" class, designated as classes 1-5, as described in Methods: Mutation Classification. Data shown excluding the Greek children. Overall difference $p < 2.2 \times 10^{-16}$ (Supplementary Table S8B).

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Figure1

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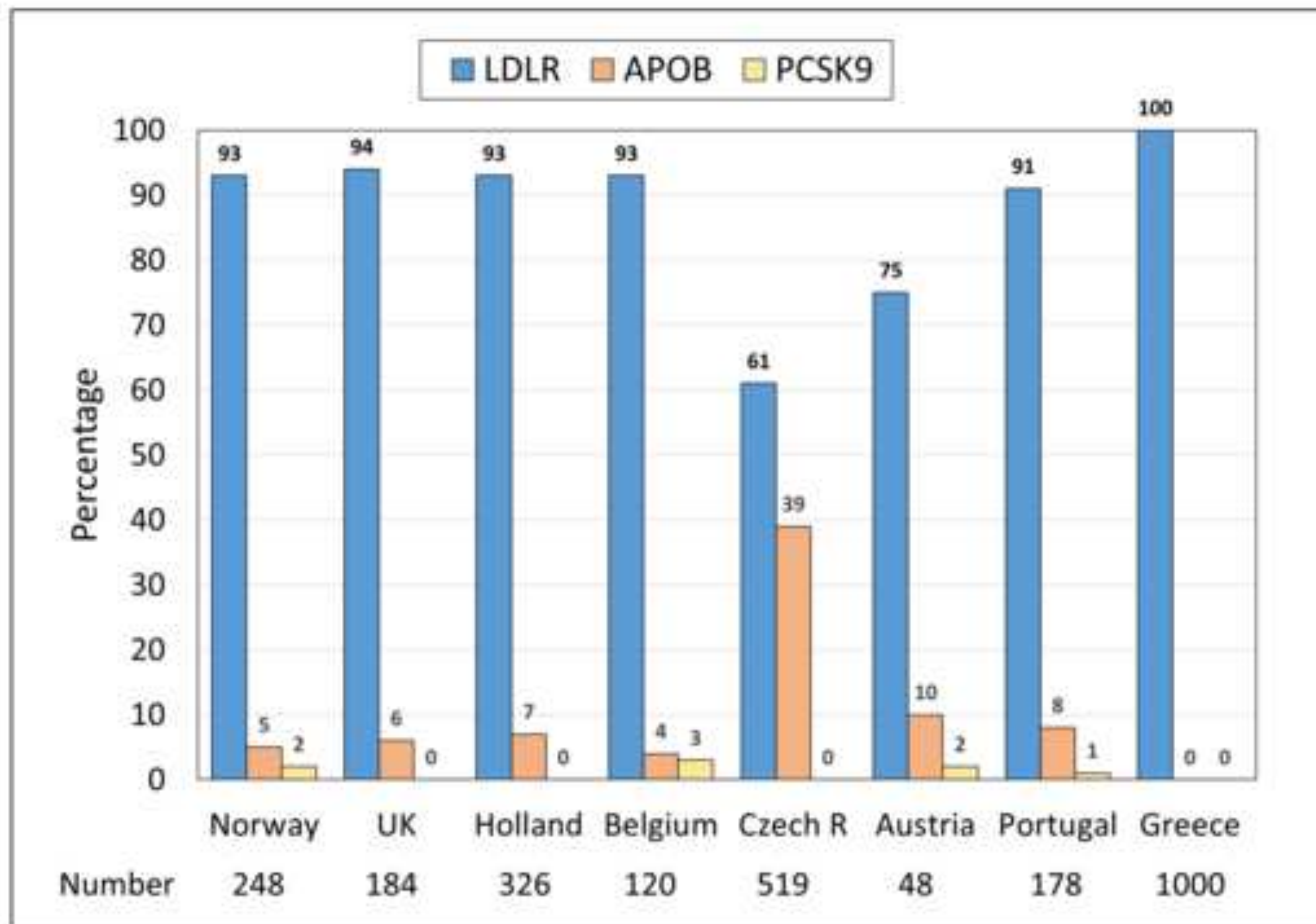

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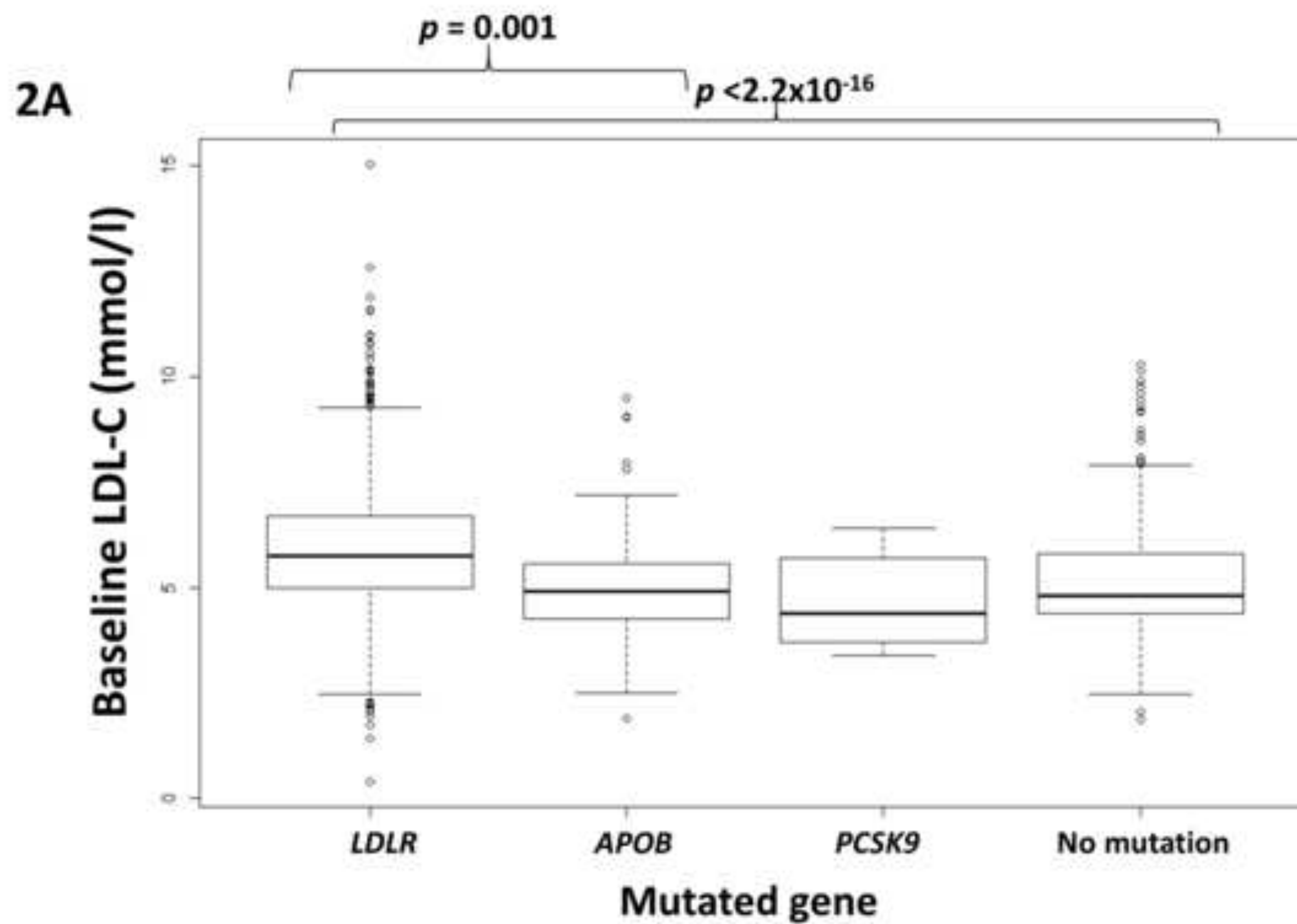

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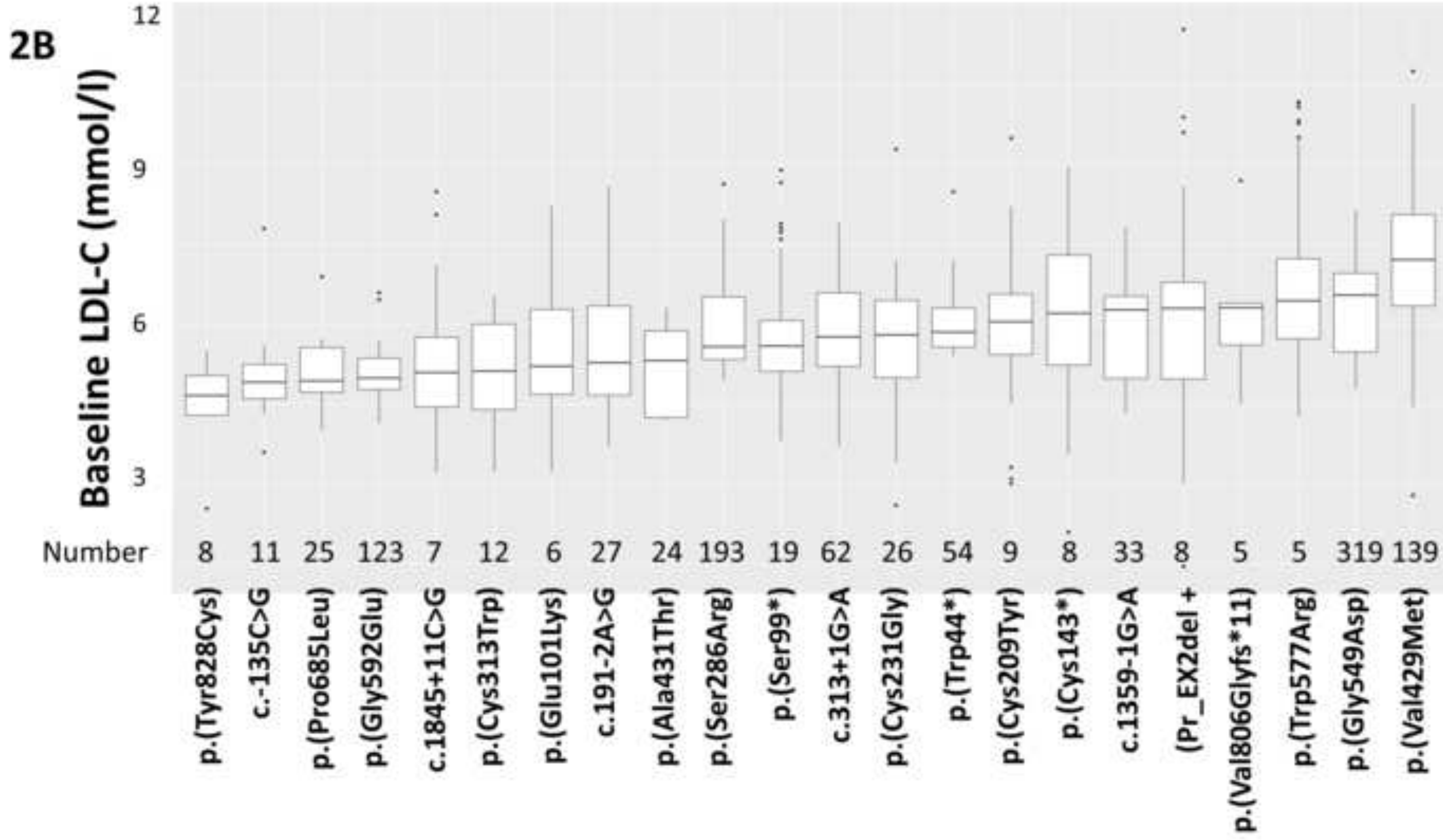

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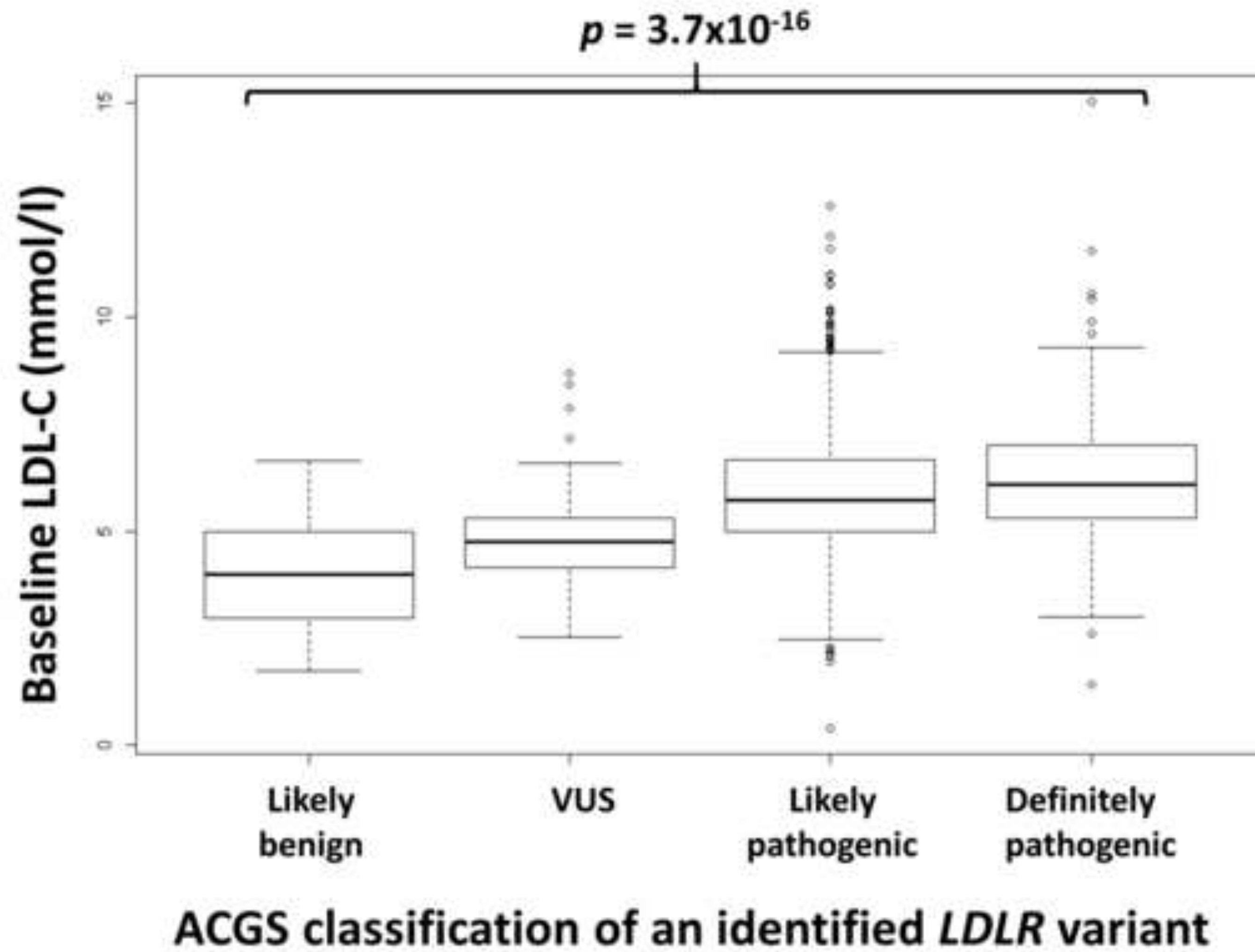

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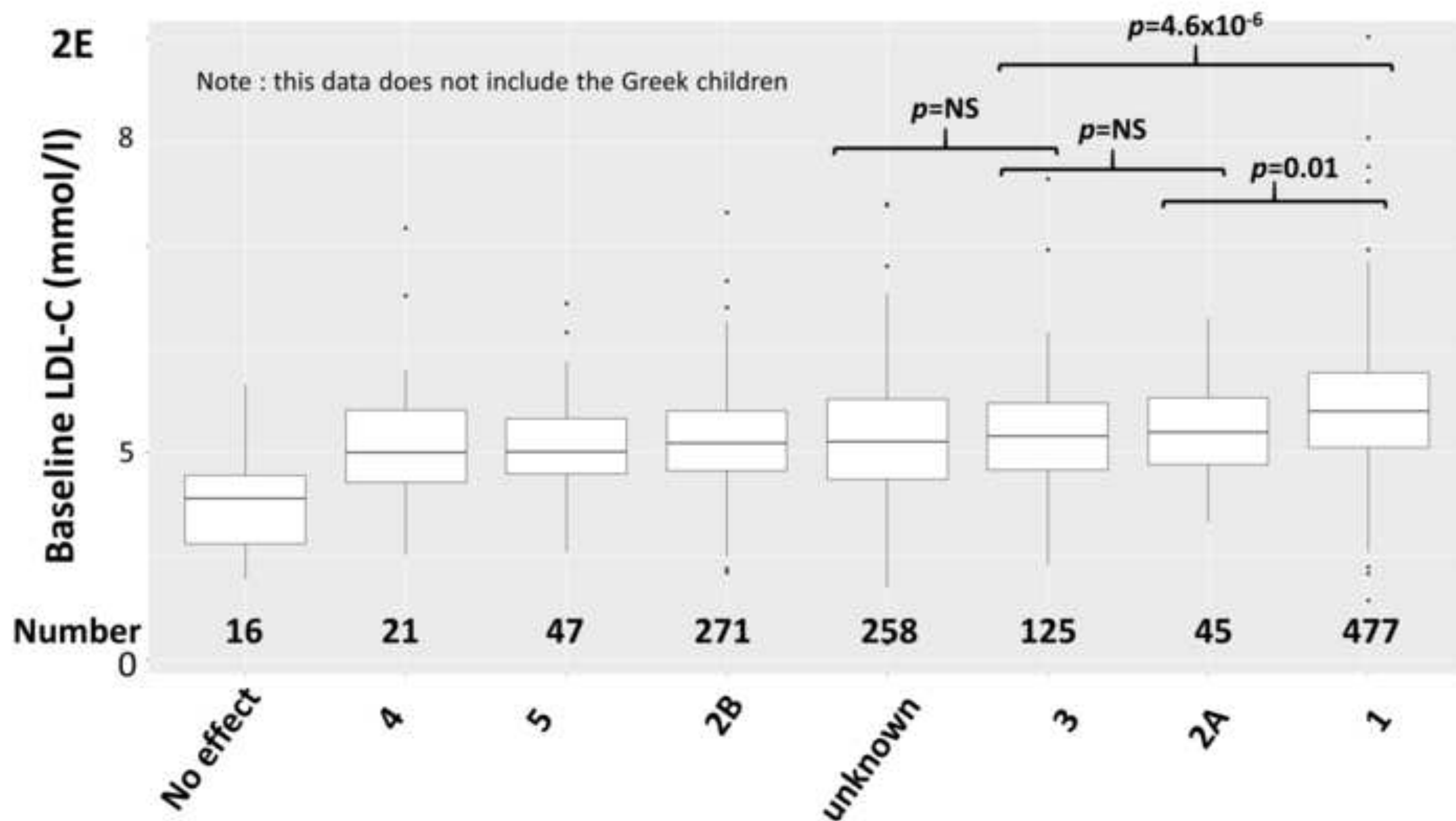

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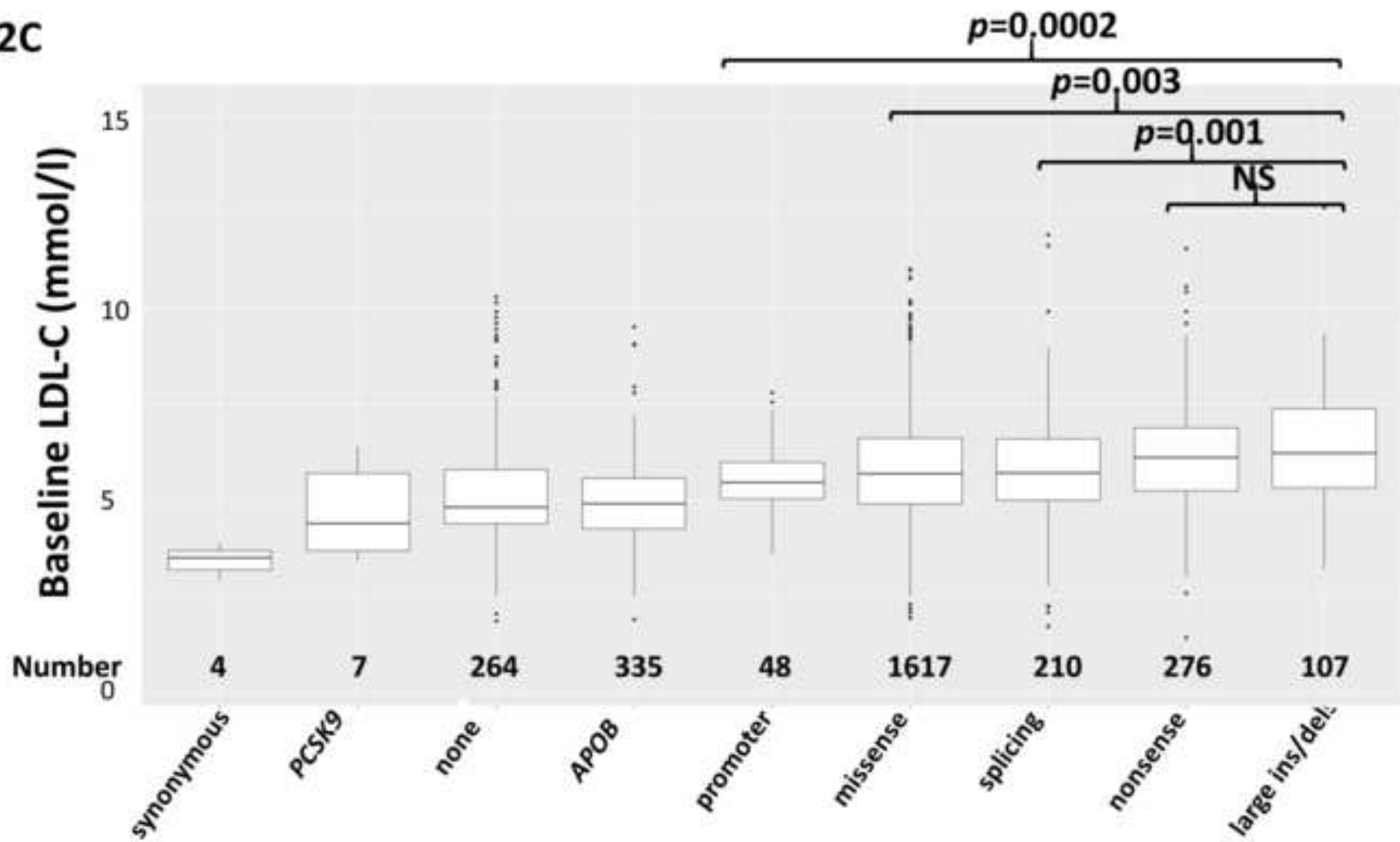

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Table 1. The total number of different *LDLR* mutations and the three commonest *LDLR* mutations (as a percentage of the total number of *LDLR* mutations found) by country.

Country	Total different (Number)	Most common Mutation (%age of total)	2nd most common Mutation (%age of total)	3rd most common mutation (%age of total)	Sum of all three
Norway	47 (224)	c.313+1G>A, splice site (25.5%)	c.691T>G; p.(Cys231Gly) (11.6%)	c.296C>G; p.(Ser99*) (8.4%)	45.5%
UK	67 (154)	c.2054C>T; p.(Pro685Leu) (5.2%)	c.1845+11C>G; splice site (4.5%)	c.301G>A; p.(Glu101Lys) (3.9%)	13.6%
The Netherlands	55 (280)	c.131G>A; p.(Trp44*) (11.8%)	c.191-2A>G; splice site (9.6%)	c.313+1G>A; splice site (9.3%)	30.7%
Belgium	44 (102)	c.1359-1G>A; splice site (8.8%)	c.939C>G; p.(Cys313Trp) (8.8%)	c.429C>A; p.(Cys143*) (7.8%)	25.4%
Czech Republic	81 (310)	c.1775G>A; p.(Gly592Glu) (17.7%)	c.798T>A; p.(Asp266Glu) (14.5%)	c.131G>A; p.(Trp44*) (4.2%)	36.4%
Austria	17 (32)	c.2483A>G ; p.(Tyr828Cys) (25.0%)	c.1516_1562del; p.(Val506Hisfs*14) (12.5%)	c.1729T>C; p.(Trp577Arg) (9.4%)	46.9%
Portugal	56 (159)	c.1291G>A; p.(Ala431Thr) (11.3%)	c.-135C>G; promoter (7.5%)	c.670G>A; p.(Asp224Asn) (6.3%)	25.1%
Greece	16 (1000)	c.1646G>A; p.(Gly549Asp) (31.6%)	c.858C>A; p.(Ser286Arg) (19.2%)	c.1285G>A; p.(Val429Met) (13.1%)	63.9%

Table 2. Baseline characteristics and pre- and post-treatment lipid concentrations by gene mutation.

Number (%)	No Mut 335 (12)	*LDLR* 2260 (79)	*APOB* 264 (9)	*PCSK9* 7 (0.2)	*p* (overall difference)	*p* (*LDLR* vs. *APOB*)
Median Age (IQR) at diagnosis (years)	10 (6)	5 (7)	11 (6.7)	8 (3)	$<2.2x10^{-16}$	$<2.2x10^{-16}$
Number of boys (%)	145 (43)	1120 (50)	115 (44)	2 (29)	0.04	NS
Number with family history of CHD (%)[a]	45 (14)	167 (16)	18 (7)	NA	0.01	0.0005
Baseline lipids ((+SD (mmol/l):						
Total Cholesterol (mmol/l)	7.15 (1.38)	7.76 (1.44)	6.74 (1.19)	6.33 (1.15)	$<2.2x10^{-16}$	$<2.2x10^{-16}$
LDL-Cholesterol (mmol/l)	5.18 (1.30)	5.88 (1.41)	4.96 (1.08)	4.71 (1.22)	$<2.2x10^{-16}$*	$<2.2x10^{-16}$*
HDL-Cholesterol (mmol/l)	1.51 (0.43)	1.44 (0.34)	1.41 (0.36)	1.34 (0.16)	0.003	NS
Triglycerides (mmol/l)	1.15 (0.65)	0.93 (0.48)	0.84 (0.38)	0.90 (0.37)	$6.2x10^{-15}$	0.0009
Number (%) with LDL-C≥4.0mmol/l	289 (86)	2085 (92)	219 (83)	4 (57)	$4.8x10^{-10}$	$1.2x10^{-8}$
On treatment Lipid profile (+SD)						
Number receiving statins (%)[b]	89 (27)	1469 (66)	53 (21)	4 (57)	$<2.2x10^{-16}$	$<2.2x10^{-16}$
Number receiving +ezetimibe (%)[2]	6 (7)	747 (51)	10 (18)	0 (0)	$<2.2x10^{-16}$	$<2.2x10^{-16}$
On treatment LDL-C (mmol/l)	3.66 (1.26)	3.09 (1.08)	3.65 (0.93)	2.44 (0.52)	$3.2x10^{-6}$*	$7.8x10^{-6}$*
LDL-C reduction (mmol/l)	2.07 (1.39)	3.00 (1.59)	1.52 (1.18)	3.11 (0.62)	0.0003	$6.3x10^{-10}$
LDL-C reduction (%)	34.9 (20.7)	47.4 (20)	27.8 (20)	56.2 (6.5)	$9.8x10^{-5}$	$7.4x10^{-8}$
Number (%) with LDL-C<3.5mmol/l[c]	43 (49)	898 (74)	22 (42)	4 (100)	$1.4x10^{-11}$	$6.4x10^{-7}$

**p* value adjusted for age (since age was statistically different between mutation classes and might therefore be a potential confounder). IQR = Inter Quartile Range, SD=standard deviation [a]data available for 1618 children. [b]data available for 2820 children. [c]statin treated children >10yrs only.