Molecular genetics of familial hypercholesterolemia in Israel – revisited

Ronen Durst[1,2], Uche Ken Ibe[3], Shoshi Shpitzen [2], Daniel Schurr [2], Osnat Eliav [2], Marta Futema[3], Ros Whittall[3], Auryan Szalat [4], Vardiella Meiner [5],Hilla Knobler[6], Dov Gavish[7], Yaakov Henkin [8], Avishay Ellis[9], Ardon Rubinstein[10], Dror Harats[11], Rafael Bitzur[11], Bruno Hershkovitz[12], Steve E Humphries[3 *], Eran Leitersdorf [2*].

1. Department of Cardiology, Hadassah Hebrew University Medical Center, Jerusalem, Israel Center for Research Prevention and Treatment of atherosclerosis, Department of Medicine, Hadassah Hebrew University Medical Center, Jerusalem, Israel
2. Center for Research Prevention and Treatment of atherosclerosis, Department of Medicine, Hadassah Hebrew University Medical Center, Jerusalem, Israel
3. Centre for Cardiovascular Genetics, British Heart Foundation Laboratories, Institute of Cardiovascular Sciences, University College London, 5 University Street, London, WC1E 6JF, UK
4. Internal Medicine Department, Mount Scopus, Hadassah Hebrew University Medical Center.
5. Department of Genetics and Metabolic Diseases, Hadassah Hebrew University Medical Center, Jerusalem, Israel
6. Diabetes and Metabolic Disease Unit, Kaplan Medical Center, Rehovot, Israel.
7. Department of Medicine, Wolfson Medical Center, Holon, Israel.
8. Department of Cardiology, Soroka University Medical Center, Beer-Sheva, Israel.
9. Internal Medicine, Rabin Medical Center, Petah Tikva, Israel.
10. Department of Metabolic Clinic, Tel Aviv Medical Center, Tel Aviv, Israel
11. Bert W. Strassburger Lipid Center, the Chaim Sheba Medical Center, Tel Hashomer, Israel.
12. Maccabi health services, Israel.

*Equal contribution as senior authors.

Corresponding author:
Ronen Durst, MD
Cardiology Department
Hadassah Hebrew University Medical Center
Ein Kerem Campus
Jerusalem Isreal, POB 92110
Email: durst@hadassah.org.il

Abstract

Objective: Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by mutations in the genes for LDL receptor (*LDLR*), apolipoprotein B (*APOB*) and Proprotein convertase subtilisin/kexin type9 (*PCSK9).* The purpose of the current investigation was to define the current spectrum of mutations causing FH in Israel.

Methods: New families were collected through the MEDPED (Make Early Diagnosis Prevent Early Death) FH program. Molecular analysis of the *LDLR, PCSK9* and *APOB* genes were done using High Resolution Melt and direct sequencing in 67 index cases. A 6-SNP LDL-C gene score calculation for polygenic hypercholesterolaemia was done using TaqMan genotyping.

Results: Mean serum cholesterol was 7.48±1.89mmol/L and the mean serum LDL-C was 5.99±1.89mmol/L. Mutations in the *LDLR* and *APOB* gene were found in 24 cases (35.8%), with 16 in *LDLR,* none in *PCSK9* and one, p.(R3527Q) in the *APOB* gene, which is the first *APOB* mutation carrier identified in the Israeli population. Of the *LDLR* mutations, two were novel; p.(E140A) and a promoter variant, c.-191C>A. The c.2479G>A p.(V827I) in exon 17 of the *LDLR* gene was found in 8 patients (33.3% of the mutations) with modestly elevated LDL-C but also in a compound heterozygous patient with a clinical homozygous FH phenotype, consistent with this being a "mild" FH-causing variant. A significantly higher 6-SNP LDL-C score was found in mutation-negative cases compared with a normal Caucasian cohort (p=0.03), confirming that polygenic inheritance of common LDL-C raising SNPs can produce an FH phenocopy.

Conclusions: The results indicate a different spectrum of genetic causes of FH from that found previously, in concordance with the heterogeneous and changing origins of the Israeli population, and confirm that a polygenic cause is also contributing to the FH phenotype in Israel.

Key words: familial hypercholesterolemia, LDL receptor, *LDLR* mutation, hyperlipidemia

Background

Familial Hypercholesterolaemia (FH, [OMIM #143890]) is a genetic disorder, inherited in an autosomal dominant fashion, characterized by the defective plasma clearance of low-density lipoprotein cholesterol (LDL-C), and may be caused by mutations in three genes: *LDLR*, *APOB*, and *PCSK9* (1). A recessive form of FH due to mutations in *LDLRAP1* is also known (2, 3) . FH is estimated to affect 1 in 230-250 individuals (4-6), and if untreated leads to premature coronary heart disease (CHD). Previous studies revealed that in several relatively isolated populations there is an increased frequency of FH (7-9). In some subpopulations, specific *LDLR* mutations were found to be common (10-17).

Israel is comprised of many populations in which a founder effect has been well described, such as the Ashkenazi Jewish population, where the founder effect has been dated back to the 14th century (15, 18, 19). Two decades ago we reported all identified mutations in the Israeli population causing FH (20). The *LDLR* gene was systematically screened for mutations using methods available at the time such as single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DDGE). Families of patients with clinical characteristics of FH based on the Simone Broome criteria were recruited through the MED-PED (Make Early Diagnosis Prevent Early Death) FH program (21). Of the 192 families available for screening 15 different mutations were found in 81 individuals (42%). The FH patients harboring these 15 mutations originated from 10 countries (Israel, Hungary, Iraq, Russia, Lebanon, Lithuania, Morocco, Poland, Rumania and Syria). Since this publication, no systematic effort was made to expand our knowledge of FH-causing mutations in the Israeli population.

We tested for the mutation in the index cases of 67 newly recruited Israeli FH families as part of the MED-PED project (21) using high-throughput detection methods and Multiplex Ligation-dependent Probe Amplification (MLPA) for exon deletions or insertions. However, it is well known that about 60% of clinically diagnosed FH patients are mutation-negative (4), and it was hypothesized that FH can also be caused by an accumulation of LDL-C raising alleles each having small contributive effect (22). Using common variants affecting LDL-C concentration identified in the meta-analysis of genome-wide association studies by the Global Lipid Genetic Consortium (GLGC) (23), a gene score method based on twelve common LDL-C raising SNPs has been developed (23) and subsequently refined to include only 6 SNPs (30). We therefore also genotyped our cohort for the effect of this 6-SNP score in our subjects.

Methods:

Recruitment of FH patients and families

Families of patients with clinical characteristics of FH are continuously recruited through the MED-PED (Make Early Diagnosis Prevent Early Death) FH program (21). Inclusion criteria for the index cases are mostly based on hypercholesterolemia with a plasma LDL-C level above the 95th percentile for age and gender. Tendon xanthomas and history of premature (age under 60) ischemic heart disease in the patient or in a first-degree relative are recorded but are not required for recruitment. Biochemical determinations plasma triglyceride, total cholesterol and high density lipoprotein (HDL) cholesterol levels were determined using commercially available diagnostic kits (Boehringer Mannheim, Germany). Plasma LDL-C levels were calculated according to the Friedewald- Levy formula (24). For patients where untreated lipid levels were unavailable, the treated lipid levels have been adjusted for the recorded dose and type of statin being used (see table 2 in Supplementary data).

Molecular Genetic Analysis

High Resolution Melt (HRM): Oligonucleotide primers for PCR-HRM were designed (25) to cover the promoter and coding regions of *LDLR* (intron-exon junctions and up to 40 bp of the intron) together with a fragment of exon 26 of *APOB* to cover the area for common mutation p.(R3527Q), and exon 7 of *PCSK9* to cover p.(D374Y). Exon 10 of *LDLR* was screened by two overlapping PCR fragments and exon 4 by four overlapping fragments. PCR and subsequent HRM were carried out (25) using the Rotor-Gene 6000 (Qiagen) using AccuMelt HRM SuperMix (from Quanta BioScience, DNA-saturating dye; SYTO 9) with 25 ng of gDNA and 4 pmol per µl of each primer in a final volume of 10 µl.

Restriction Fragment Length Polymorphism (RFLP): The samples were genotyped for common polymorphisms in *LDLR* by RFLP and their genotypes compared with the HRM result as described in Whittall *et al.* 2010 (25). Those with shifts due to polymorphism on the HRM were not examined further. However, samples without the polymorphism but with a shift were sequenced to find the cause of the melt temperature shift. The HRM-PCR products were used for digestion with the appropriate restriction enzyme (NEB (UK) Ltd. Hitchin, Herts) using 5 µl of HRM-PCR product and 3U of appropriate enzyme (supplementary Table S2) in a total volume of 15 µl and run on a 1.5% agarose gel in 1xTBE buffer.

Sequence Analysis: HRM-PCR products PCR products that showed a shift in the melt profile and melt temperature were Sanger sequenced. The DNA purification was performed using Illustra GFX PCR DNA and Gel Band Purification Kit (from GE Healthcare). The same primers used for HRM-PCR were used for sequencing, which was performed by Source BioSciences, LifeSciences.

Multiplex Ligation Dependent Probe Amplification (MLPA): To detect rearrangements within the coding sequence of *LDLR*, MLPA was performed according to the manufacturer's protocol (MRC-Holland, Amsterdam, the Netherlands cat number P062-LDLR).

In silico variant analysis: To predict the pathogenicity of novel *LDLR* variants, *in silico* mutation prediction tools, including Polymorphism Phenotyping version2 (PolyPhen-2) (26), Sorting Intolerant From Tolerant(SIFT) (27), Berkeley Drosophila Genome Project - Splice Site Prediction (BDGP) (28)and Mutation Taster (29) were used. The data from Exome Variant Server (http://evs.gs.washington.edu/EVS/) and UCL website https://grenada.lumc.nl/LOVD2/UCL-Heart/home.php?select_db=LDLR were used to check if mutations were novel. Mutation nucleotide numbers were designated using the *LDLR* reference sequence reported (www.ucl.ac.uk/fh). Mutations were designated according to recommendations from Human Genome Variation Society (www.hgvs.org)

LDL-C gene score calculations for polygenic hypercholesterolaemia: To assess the polygenic cause of hypercholesterolaemia, patients were genotyped for 6 LDL-C raising SNPs based on recent findings (22, 23, 30). KASPar PCR technique (Kbiosciences, UK Hoddesdon, Herts, UK) or TaqMan assays (Life Technologies, Carlsbad, California, US) and genotype calling for all assays was carried out using an automated system, the results of which were checked manually by study personnel using SNPviewer software. One SNP (rs4299376) could not be genotyped and a proxy was used instead (rs6544731). The LDL-C gene score was calculated using weighted. A group of 3,020 healthy volunteers (participants of the UK Whitehall II (WHII) study (31) was used for comparison.

Results:

The cohort of 67 unrelated index patients (36 men and 31 women) with an average age of 45±15 (youngest 10yrs, eldest 72yrs) were studied. A summary of the clinical characteristics and combined lipid data of the cohort is presented in Table 1. For patients where untreated lipid levels were unavailable, the treated lipid levels have been adjusted for the recorded dose and type of statin being used. Overall, the mean serum total cholesterol was 7.48±1.89mmol/L (3.6 - 15.0 mmol/L), and the mean serum LDL-cholesterol was 5.52±1.91 mmol/L (1.0 - 13.1 mmol/L). 15 of the recruited patients have levels of untreated (or statin dose adjusted) LDL-C below the Simon Broome cut-off of 4.9mmol/l (supplementary Table 1) and are therefore strictly not subjects with a formal diagnosis of FH.

Mutations of the *LDLR* and *APOB* gene were found in 24 patients which comprises 36% of the cohort (table 2, and Figure 1). If the 15 subjects who do not confirm to the SB diagnostic criteria are excluded, (ie with LDL-C <4.9mmol/l) the overall mutation detection rate increases to 23/52= 46%. As expected, the mean total cholesterol and LDL-cholesterol (adjusted for statin use) was significantly higher in the group of patients with a mutation than in those without a mutation, (40% higher p= 0.02 for LDL-C and 32% higher p = 0.01 for TC). Among the twenty-four, 16 different *LDLR* mutations were found of which two were novel, and the p.(R3527Q) *APOB* mutation was also identified. This is the first reported *APOB* mutation carrier identified in the Israeli population. No *PCSK9* mutations were identified. Two mutations, p.(G219del) and p.(D167H), which are founder mutations in Ashkenazi and Sephardic Jewish ethnic group respectively, were observed in two probands (Is681 and Is687) (20). Three probands (IS 537, IS544, and IS 595) were found to carry a 10 base pair deletion (c.1570_1579del) in exon 10 of *LDLR* which leads to a frameshift change ending in a stop codon after twenty-one residues. This deletion, which was first seen in a Jewish Iraqi FH patient (20) (and was incorrectly described as a 9bp deletion), occurs in the EGF precursor homology domain of the LDLR and is predicted to be pathogenic.

Two novel *LDLR* mutations were identified: c.-191C>A in the promoter region, and p.(E140A), in exon 4. The relatives of these index cases were recruited to provide evidence for segregation of the mutations with elevated LDL-C (figure 2). In the pedigree shown in Figure 2A, the proband is clinically homozygous with LDL-C of 11.1mmol/l and is a compound heterozygote for both *LDLR* c.1570-1579del and c.-191C>A. The two offspring both carry the c.-191C>A mutation and have LDL-C levels in the range of heterozygous FH. In Figure 2B, for the p.(E140A)

mutation, the co-segregation of the mutation with elevated LDL-C levels is shown in a three generation family. Two additional families were studied. Pedigree 2C is of a Druze family with the exons 7-14del mutation showing segregation of the mutation with the trait with 18 family members carrying the deletion and also having elevated TC/LDL-C. In the pedigree shown in Figure D the proband has passed the p.(C121S) allele to his daughter who has elevated LDL-C levels.

p.(V827I) was found in 8 individuals in our cohort. This variant has been reported before as causing FH (32, 33). LDL-C levels in these subjects were 4.1, 4.8, 4.91, 5.38, 5.72 5.9, 6.2 and 6.5mmol/l (supplementary Table 1), with a mean of 5.4mmol/l suggesting a mild mutation. However this mutation was found as a compound heterozygous mutation with p.(E140A) (above) with a clinical homozygous FH phenotype. To explore this further, we genotyped all the Israeli cohort for six common LDL-C raising SNPs previously reported, to see if polygenes could explain the increased LDL-C concentration in the no mutation group as well as the p.(V827I) group. Figure 3 presents the mean gene scores in the Israeli mutation-positive and mutation-negative groups, compared with those of healthy UK WHII cohort. The highest mean 6 SNP score was observed in the FH mutation negative group compared to the UK healthy control group (0.682 (0.14) vs 0.632 (0.22), p=0.03). We have estimated that FH mutation-negative patients who have the SNP score above the top quartile of the score distribution have a more than 95% probability of having their hypercholesterolaemia due to a polygenic cause (30). Based on the WHII population 6 SNPs score top quartile (=0.51), in our study 36 out of the 41 mutation negative samples with sufficient genotyping data (87.8%) have a score above the cutoff, compatible with their hypercholesterolaemia having a polygenic and not a monogenic etiology.

By contrast, both the FH mutation positive group and the individuals who carry the *LDLR* p.(V827I) variant had a similar intermediate mean score (0.656 (0.15) and 0.648 (0.14) respectively, p = 0.9), not significantly higher than the control group (p=0.55). However, six out of the eight p.(V827I) carriers (75%) had a 6 SNP score above the top quartile cutoff. This finding suggests that the effect of the p.(V827I) variant on its own is insufficient to cause the full phenotype of FH, without an additional "polygenic" contribution.

Discussion.

The molecular detection and identification of mutant LDL receptor alleles is important from several aspects. First, it provides the only definitive diagnosis of FH. Second, the correct estimation of the prevalence of FH in a specific population is only possible through screening for the actual molecular defects. Third, it enables a high rate (50%) of screening success of first degree relatives of index cases, enabling early diagnosis and prevention of cardiovascular disease (34). The Israeli population is very heterogeneous, consisting of a multitude of origin groups. In 1996 we published the first paper on the systematic mutation screening for FH patients (20). In the current paper we report the results of mutation systematic screening of 67 new FH index cases recruited through the MED-PED project.

Our results show that the FH mutation spectrum in Israel has changed considerably over the last 20 years. We identified 16 different *LDLR* mutations and the first reported *APOB* p.R35227Q carrier in the Israeli population, with an overall mutation detection rate of 36%, rising to 46% if we apply the strict LDL-C cut-off suggested by the Simon Broome criteria. Previously (20), we reported on mutation detection in 95 index cases, and detected 15 different mutations in 49 cases (detection rate 52%). Three mutations explained 34% of index cases, (Gdel219 (FH-Lithuania), D1687H (FH-Sephardic), and C681X (Lebanese)), and these were regarded as founder mutations in the three respective groups. Here we found only one individual with Gdel219 and one D168H and none with C681X, with the three most common mutations seen earlier now only explaining 8% of mutation carriers (2/24). We found two novel *LDLR* mutations. One variant in exon 4 p.(E140A), and a promoter variant, c.-191C>A, have not been seen in published literature, but both showed co-segregation compatible with being FH-causing. We also noted a probably novel major deletion of exons 7-14, found in a pedigree of Druze ancestry, although it is not possible to be absolutely certain that the deletion seen here is different from similar deletions reported previously (35) since break points have not been established. We found that a mutation p.(V827I), previously reported in subjects of Russian origin Russia (33), to be the most common variant in our sample explaining 8/24 or 33% of the detected mutations. This variant was not seen previously in Israel and is likely to result from recent changes in the population demographics. In the group where no mutation was identified there is highly likely to be a polygenic cause of their elevated LDL-C and FH diagnosis, based on a 6-SNP gene score.

Pathogenicity of novel mutations

Individual Is556-03 is carrying the novel mutation, p.(E140A). It is predicted to be damaging by both PolyPhen™, SIFT™ and Mutation Taster™ and the mutation segregates with elevated LDL-C levels. Glutamic acid140 is present in the ligand-binding domain of the LDL-receptor. A different variant substitution at position c.419, an A to G base change leading to a glutamic acid to glycine substitution, was reported as novel in 2001 by Fouchier and colleagues in Netherland FH patients (36). Comparative proteogenomics analysis across several species show a high level of conservation of Glutamic acid at position p.140. Thus, p.(E140A) is very likely to be a mutation.

Individual Is 537 is carrying c.-191C>A, in the promoter region of the *LDLR*. The variant is located within the repeat 1 (RP1) sequence which has been shown to be a binding site for Sterol Regulatory Element Binding Protein (SREBP) and is therefore very likely to be FH causing (37). Is537 is carrying another, well described mutation, (c.1570_1579del). To better define if c.-191C>A causes FH, we genotyped several other relatives in the Is 537 pedigree. The index case is a compound heterozygote and has LDL-C levels in the range of homozygous FH. Her two children inherited only the c.-191C>A mutation, and have LDL-C levels in the range of heterozygote FH. Thus, it is likely that both mutations contribute to the compound heterozygote of the index case.

Individual Is697 carries a deletion of exons 7-14 as shown by MLPA analysis. We expanded the pedigree and demonstrated perfect segregation of the deletion with hypercholesterolemia. Thus, this major deletion seems to be a new *LDLR* mutation within the Druze population.

We also tested the available relatives of individual Is660 who is carrying the mutation p.(C121S) first reported in a UK patient in 2013 (57) but where little clinical data is reported. PolyPhen™, ™ and MutationTaster™ all predict the variant to be damaging and disease causing (Table 3) (26, 27, 29). This mutation occurs in the ligand binding domain of the LDL-receptor, a cysteine repeat rich region that is important for disulphide bridge formation. The family tree could only be extended to one more individual carrying the mutation with a phenotype of FH.

p.(V827I) Variant:

The c.2479G>A p.(V827I) in exon 17 of the *LDLR* gene was found in 8 patients and constituted 33.3% of the mutations identified in the Israeli FH cohort. If the eight carriers of the p.(V827I) variant were excluded from the mutation positive patient group, the overall mutation

detection rate would fall from 35.8% to 23.8%. It was first found in an American homozygous FH patient by Hobbs and colleagues (5) as FH-New York 5. This homozygous FH patient was later discovered to be a compound heterozygote, as the patient was seen to have another *LDLR* mutation. In 2005, Zakharova and colleagues identified the same variant in one family in St. Petersburg, Russia (33). This change has also been identified twice as potentially deleterious among 81 healthy individuals randomly assigned for exome sequencing (32). This variant was not identified in the Israeli FH patient samples examined in 1996, and the high frequency of this variant in the current samples studied is likely to reflect the recent immigration to Israel of individuals from Russia.

This substitution occurs in the tetrameric consensus sequence NPxY (where x is any amino acid) that plays a major role in the coated pit-mediated internalization of the LDL-receptor via interaction with the protein autosomal recessive hypercholesterolaemia (ARH) (38, 39). The NPVY sequence in the cytoplasmic domain of the LDL-receptor is a major site of recognition for incorporation into clathrin-coated pits. The stringency at the third position (position p.827 "V") in this tetrameric sequence, is not as marked as that seen at the other three positions. Reduction in LDL-C cellular internalization index to approximately 65% of control values was seen when the valine of the NPVY sequence was replaced with an alanine or a cysteine (38). However, data from the UCSC Genome browser conservation tab and PolyPhen's sequence alignment show the valine of the tetrameric sequence to be highly conserved across species.

One of the carriers of p.(V827I) is a compound heterozygote, and carries both p.(V827I) and p.(E140A) and has the phenotype of an FH homozygote. The LDL-C SNP score analysis showed that individuals who carry the variant have a similar SNP higher score to those who inherited other *LDLR* FH-causing mutations. However, one fact which suggests that the p.(V827I) variant is not FH-causing is that it is seen at a frequency of 0.00076 (92 carriers/121,374 alleles sequenced) in the ExAC database http://exac.broadinstitute.org/variant/19-11240278-G-A. While it is thus one of the more common mis-sense variants in the *LDLR* gene, several other FH-causing variants are found at similar frequency (eg p.(G324S) present in 140/120384 for a frequency of 0.0012), with the *APOB* variant p.(R3527W) is present in 28/121182 alleles for a frequency of 0.00023. While a recently-recruited sample of normolipidaemic Israeli subjects would be useful to determine the frequency of V827I in healthy subjects, this is beyond the scope of the current study. Overall the published data and this study suggests that the p.(V827I) variant is a mild FH-causing variant.

Polygenic Familial Hypercholesterolaemia:

We genotyped the Israeli cohort for the 6 common LDL-C raising SNPs, based on the score suggested by Talmud *et al* and refined by Futema *et al* (22, 30). Effects were similar to those published previously, with a found a significantly higher score in mutation-negative cases compared with the normal Caucasian cohort, with the score suggesting that a polygenic etiology is the likely explanation for the hypercholesterolaemia in ~90% of the mutation negative subjects. . This score was validated before in several European mutation negative FH patient cohorts and found to be consistently higher than in the UK general Caucasian population reference group, and with no significant difference in score between the various FH cohorts (30). Since the patients studied here are of the same ethnic background as the UK sample used previously, the comparison is unlikely to be confounded by ethnicity. In such "polygenic hypercholesterolaemia" patients, first degree relatives are less likely to have high LDL-C than in mutation-positive patients. Thus, for successful screening of first degree relatives of FH patients, it is likely to be cost effective to ascertain that the index individual carries a known FH-causing mutation. By contrast, whole genome or exome sequencing in the low SNP score no-mutation patients may reveal novel genetic causes of FH.

Our study has some limitations. While we have previously demonstrated that HRM is a sensitive and specific technique (25) for mutation detection in FH patients, we acknowledge that Next Generation Sequencing (NGS) is now the preferred method of choice for molecular diagnosis, but this work was commenced in 2013 when such methods were not widely available and NGS is still an expensive technique and beyond the funds available for this research project. The overall mutation detection rate in our sample is relatively low compared with other similar published cohorts. (40-42), although similar mutation detection rates were recently reported in Korea (43). This may be because our inclusion criteria were less stringent than in other studies resulting in fewer "monogenic" FH patients and a lower mutation detection rate, and exclusion of those with LDL-C below the diagnostic threshold of 4.5mmol/l increased the mutation detection rate to 46%. This may, however, also be because of population differences in FH mutation prevalence. Also, while we screened the regions of the *PCSK9* and *APOB* genes known to contain pathogenic mutations (25), it is possible that a small proportion of patients may have mutations in the exons not covered here. While pathogenic variants

in other regions of *APOB* have been reported (44) these are very rare. Similarly, gain-of-function FH causing mutations in *PCSK9* are also rare, for example explaining less than 2% of FH patients in the UK, and none have been found in Israel. We therefore estimate that complete coverage of these two genes may at best have increased the overall detection rate by 2-3%. Finally, a limitation of the conclusion that the mean LDL-SNP score in the no-mutation subjects is significantly higher than in healthy controls is that we do not have a comparison group of healthy Israeli subjects and have used a sample of UK healthy controls. Since both groups are of European origin and both are white Caucasians we believe it is unlikely that the result is a false positive, and the mean score in the mutation positive subjects is similar to that in the UK healthy group as has been reported for other mutation positive patients (22, 30).

Conclusion

The FH mutation spectrum in Israel has changed very considerably over the last 20 years. While a sample of 67 patients attending a single lipid clinic in Jerusalem will not be fully representative of all Israeli FH subjects, we clearly show that the current mutation distribution is very different compared to the 1996 study. It is likely that a much larger sample would find a wider range of mutations (both reported and novel), and also possible that patients from lipid clinics in other metropolitan centres may have a different mixture of Ashkenaz, Sephardic and Palestinian-Arab subjects. (RONEN IS THIS OK??) The most common mutation in the FH patients was p.(V827I) which appears to be of mild effect. In the vast majority of subjects were no mutation could be identified, there is likely to be a polygenic cause of their elevated LDL-C and FH diagnosis. These data can help design a future strategy for early screening for FH in our population.

Acknowledgements: This work was supported by a Marie Curie reintegration grant (MVP-GEN) provided to Dr. Ronen Durst, and by an Israeli Science foundation grant (1053/12) provided to Dr. Ronen Durst. SEH is a British Heart Foundation Professor and he and RW and MF are supported by the British Heart Foundation (RG008/08) and by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. We thank Prof Nick

Lench and Dr Emma Ashton for their assistance in using of MLPA equipment in the Regional Genetics Laboratory Great Ormond Street Hospital for Children.

Table 1. Showing the combined clinical characteristics (Mean ±SD) of the Israeli FH cohort.

		All FH Patients (n=67)	**Patients with mutation(n=24)**	**Patients without mutation(n=43)**	**Difference b/w mutation +ves & -ves *P*-value**
Age	years	45±15	41±15	47±14	0.15
Sex	*Female* n (%)	36(53.7)	13(54.2)	23(53.5)	0.96
	Male n (%)	31(46.3)	11(45.8)	20(46.5)	
HDL-C	*mmol/L*	1.20 ± 0.28	1.14 ± 0.31	1.23 ± 0.26	0.18
TG		1.62 ± 0.60	1.67 ± 0.66	1.59 ± 0.57	0.59
LDL-C		5.52 ± 1.91	6.00 ± 1.89	5.26 ± 1.89	0.12
TC		7.48 ± 1.89	7.90 ± 1.88	7.25 ± 1.87	0.18
On Statin	n (%)	18(26.9)	7(29.2)	11(25.6)	
LDL-C before treat		5.99 ± 1.89	7.27 ± 1.60	5.18 ± 1.72	0.02
		(n=18)	(n=7)	(n=11)	
LDL-C Paired t-test before/after treatment			0.001	0.000	significant
TC before treat		8.64 ± 2.20	10.16 ± 1.76	7.68 ± 1.93	0.01
		(n=18)	(n=7)	(n=11)	
TC Paired t-test before/after treatment			0.001	0.000	significant

S.D, standard deviation; TG, triglycerides; TC, total cholesterol; +ves/-ves, positives/negatives.

Table 2. Nucleotide changes identified by HRM, RFLP and sequencing in the LDLR and APOB gene among 67 Israeli FH patients

			TC	HDL	LDL		Identified variant			
Patient ID	***Sex***	***Age***	*(mmol/l)*	*(mmol/l)*	*(mmol/l)*	***Statin***	AA change (variant 1)	Nucleotide change	AA change (variant 2)	Nucleotide change
Is694	F	38	9.2	1.2	6.5	N	p.(V827I)	c.2479G>A		
Is537	F	33	12.67	0.91	11.35	N	n/a	c.-191C>A	p.(V524Ffs*21)	c.1570_1579del
Is522	F	50	5.97	1.97	3.41	Y	p.(V827I)	c.2479G>A		
Is556-03	F	25	13	0.74	11.3	N	p.(E140A)	c.419A>C	p.(V827I)	c.2479G>A
Is544	F	37	6.7	1.24	5.12	Y	p.(V524Ffs*21)*	c.1570_1579del		
Is579	M	46	7.5	0.78	5.90	N	p.(V827I)	c.2479G>A		
Is564	F	45	5.82	1.68	3.52	Y	p.(G314R)	c.940G>A		
Is589	F	35	8.56	0.88	6.72	N	n/a	c.-156C>T		
Is634	M	39	7.27	1.24	4.91	N	p.(V827I)	c.2479G>A		
Is628	M	62	6.13	1.03	4.40	Y	p.(V827I)	c.2479G>A		
Is678	M	60	6.8	0.72	4.8	N	p.(V827I)	c.2479G>A		
Is636	F	29	7.32	1.47	5.38	N	p.(V827I)	c.2479G>A		
Is595	F	43	9.62	1.09	7.78	N	p.(V524Ffs*21)*	c.1570_1579del		
Is588	M	10	9.26	1.24	7.29	N	p.(P685L)	c.2054C>T		
Is662	F	32	5.2	1.16	3.65	Y	*APOB p.(*R3527Q)	c.10580G>A		
Is671	M	25	7.81	1.03	6.10	N	p.(G207=)	c.621C>T		
Is660	F	72	6.9	1.06	5.20	Y	p.(C121S)	c.362G>C		
Is637	M	55	5.07	0.75	3.41	Y	p.(V797M)	c.2389G>A		
Is658	F	54	7.96	1.06	6.21	Y	p.(A431T)	c.1291G>A		
Is681	M	23	6.88	1.01	5.30	Y	p.(G219del)*	c.654_656del		
Is685	F	31	6.9	1.14	5.12	N	p.(R416W)	c.1246C>T		
Is699	M	71	4.94	0.91	3.34	Y	p.(E277K)	c.829G>A		
Is697	M	49	4.76	1.09	2.95	Y	exons 7-14 del	c.941-?_2140+?del		
Is687	M	30	5.43	1.11	3.96	Y	p.(D168H)*	c.502G>C		

TC, total serum cholesterol; HDL, high-density lipoprotein; LDL, Low-density lipoprotein; AA, amino acid

*Mutation previously reported in Israeli FH patients (20).

Table 3. Nucleotide changes identified in the Israeli FH cohort and in silico prediction of their pathogenicity.

†Percentage of all detected *LDLR* and *APOB* mutation ‡Reference shown is for population of first discovery * abbreviations are according to the ISO 3166 standard for country codes and their subdivisions available at

					Protein Prediction		Splice Prediction					
No.	**AA Change**	**Nucleotide change**	**Location**	**Occurrence in other populations***	**PolyPhen™ Prediction**	**SIFT™ Prediction**	**Mutation T@ster Prediction**	**BDGP**	**Human Splice Finder (%)**	**No. of patients with the mutation (%)†**	**Overall/*in silico* Pathogenic Prediction**	**Reference‡**
	***LDLR* Promoter variant**											
1	c.-191C>A		Prom	Novel					23.2	1(4.2)	Probably	New
2	c.-156C>T		Prom	DE					10.4	1(4.2)	Probably	(53)
	***LDLR* missense variant**											
3	p.(C121S)	c.362G>C	Exon 4	GB	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(57)
4	p.(D168H)	c.502G>C	Exon 4	IL, NL	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(55)
5	p.(E140A)	c.419A>C	Exon 4	Novel	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	New
6	p.(E277K)	c.829G>A	Exon 6	CU,SE,ES IN,TR	Benign	Tolerated	Disease causing			1(4.2)	Yes	(45)
7	p.(R416W)	c.1246C>T	Exon 9	GB,NO,AT DE, NL	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(46)
8	p.(A431T)	c.1291G>A	Exon 9	DZ,JP,NL CN, GR	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(47)
9	p.(P685L)	c.2054C>T	Exon 14	ZM, GB, JP NL, CN	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(48)
10	p.(V827I)	c.2479G>A	Exon 17	US, NL RU, DK	Probably damaging	Tolerated	Disease causing			8(33.3)	Uncertain	(5)
	Variants predicted to affect splicing											
11	p.(G207=)	c.621C>T	Exon 4	NL	n/a	Tolerated	Disease causing	C = 0 T = 0.99	C = 65.5 T = 92.3	1(4.2)	Yes	(54)
12	p.(G314R)	c.940G>A	Exon 6	AT	Benign	Tolerated	Disease causing	G = 0.98 A = 0.70	G = 45.4 A = 74.3	1(4.2)	Yes	(58)
13	p.(V797M)	c.2389G>A	Exon 16	CU,CN,NL FR, MX	Benign	Tolerated	Disease causing	G = 1.00 A = 0.89	G = 57.5 A = 86.4	1(4.2)	Yes	(45)
	***LDLR* In-frame deletion**											
14	G219del	c.654_656del3	Exon 4	US,GB,IL DE,PL	n/a	n/a	Disease causing			1(4.2)	Yes	(47)
	***LDLR* Large rearrangement**											
15	Exons 7-14 del	c.941-?_2140+?del	Exons 4-14	Novel	n/a	n/a	n/a			1(4.2)	n/a	New
	***LDLR* frame-shift mutation**											
16	p.(V524Ffs*21)	c.1570_1579del	Exon 10	IL	n/a	n/a	Disease causing			3(12.5)	Yes	(20)
	***APOB* variant**											
17	p.(R3527Q)	c.10580G>A	Exon 26	Caucasian	Probably damaging	Damaging	Disease causing			1(4.2)	Yes	(56)

http://www.iso.org/iso/home/standards/country_codes.htm and details of full references for each variant is available on the UCL *LDLR* database https://grenada.lumc.nl/LOVD2/UCL-Heart/home.php?select_db=LDLR.

Figure 1 Diagrammatic representation of the *LDLR* annotated with identified sequence changes in the Israeli FH cohort. The stars indicate novel variants

Figure 2: Four pedigrees with FH. Pedigree A shows that the proband is clinically homozygous for both *LDLR* del 1570-1579 and the novel *LDLR* Cc.-191C>A. Pedigree B showing segregation of the novel p.(E140A) mutation. Pedigree C is of a Druze family with Ex. 7-14 del mutation showing segregation of the mutation with the trait. Pedigree D of a small family segregating the *LDLR* p.(C121S) mutation

Figure 3: Boxplot of the 6 SNP LDL-C score in FH patients by a presence of an FH mutation in comparison to the WHII control cohort. Only samples where all 6 SNP genotypes were obtained are included. The highest mean (SD) 6 SNP score was observed in the FH mutation negative group (0.682 (0.14)), which was significantly lower (p=0.03) than the control group (0.632 (0.22). Individuals who carry the *LDLR* p.(V827I) variant have intermediate mean score (0.648 (0.14)), not different from other mutation carriers (p=0.9).

References

1. Marks D, Thorogood M, Neil HA, Humphries SE. A review on the diagnosis, natural history, and treatment of familial hypercholesterolaemia. Atherosclerosis. 2003;168(1):1-14.
2. Ciccarese M, Pacifico A, Tonolo G, Pintus P, Nikoshkov A, Zuliani G, et al. A new locus for autosomal recessive hypercholesterolemia maps to human chromosome 15q25-q26. Am J Hum Genet. 2000;66(2):453-60.
3. Garcia CK, Wilund K, Arca M, Zuliani G, Fellin R, Maioli M, et al. Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. Science. 2001;292(5520):1394-8.
4. Taylor A, Patel K, Tsedeke J, Humphries SE, Norbury G. Mutation screening in patients for familial hypercholesterolaemia (ADH). Clin Genet. 2010;77(1):97-9.
5. Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat. 1992;1(6):445-66.
6. Knowles JW, O'Brien EC, Greendale K, Wilemon K, Genest J, Sperling LS, et al. Reducing the burden of disease and death from familial hypercholesterolemia: a call to action. Am Heart J. 2014;168(6):807-11.
7. Khachadurian AK, Uthman SM. Experiences with the homozygous cases of familial hypercholesterolemia. A report of 52 patients. Nutr Metab. 1973;15(1):132-40.
8. Moorjani S, Roy M, Gagne C, Davignon J, Brun D, Toussaint M, et al. Homozygous familial hypercholesterolemia among French Canadians in Quebec Province. Arteriosclerosis. 1989;9(2):211-6.
9. Seftel HC, Baker SG, Sandler MP, Forman MB, Joffe BI, Mendelsohn D, et al. A host of hypercholesterolaemic homozygotes in South Africa. Br Med J. 1980;281(6241):633-6.
10. Lehrman MA, Schneider WJ, Brown MS, Davis CG, Elhammer A, Russell DW, et al. The Lebanese allele at the low density lipoprotein receptor locus. Nonsense mutation produces truncated receptor that is retained in endoplasmic reticulum. J Biol Chem. 1987;262(1):401-10.
11. Hobbs HH, Brown MS, Russell DW, Davignon J, Goldstein JL. Deletion in the gene for the low-density-lipoprotein receptor in a majority of French Canadians with familial hypercholesterolemia. N Engl J Med. 1987;317(12):734-7.
12. Leitersdorf E, Van der Westhuyzen DR, Coetzee GA, Hobbs HH. Two common low density lipoprotein receptor gene mutations cause familial hypercholesterolemia in Afrikaners. J Clin Invest. 1989;84(3):954-61.
13. Aalto-Setala K, Helve E, Kovanen PT, Kontula K. Finnish type of low density lipoprotein receptor gene mutation (FH-Helsinki) deletes exons encoding the carboxy-terminal part of the receptor and creates an internalization-defective phenotype. J Clin Invest. 1989;84(2):499-505.
14. Oppenheim A, Friedlander Y, Dann EJ, Berkman N, Schwartz SP, Leitersdorf E. Hypercholesterolemia in five Israeli Christian-Arab kindreds is caused by the "Lebanese" allele at the low density lipoprotein receptor gene locus and by an additional independent major factor. Hum Genet. 1991;88(1):75-84.
15. Durst R, Colombo R, Shpitzen S, Avi LB, Friedlander Y, Wexler R, et al. Recent origin and spread of a common Lithuanian mutation, G197del LDLR, causing familial hypercholesterolemia: positive selection is not always necessary to account for disease incidence among Ashkenazi Jews. Am J Hum Genet. 2001;68(5):1172-88.
16. Landsberger D, Meiner V, Reshef A, Levy Y, van der Westhuyzen DR, Coetzee GA, et al. A nonsense mutation in the LDL receptor gene leads to familial hypercholesterolemia in the Druze sect. Am J Hum Genet. 1992;50(2):427-33.

17. Maruyama T, Miyake Y, Tajima S, Harada-Shiba M, Yamamura T, Tsushima M, et al. Common Mutations in the Low-Density-Lipoprotein–Receptor Gene Causing Familial Hypercholesterolemia in the Japanese Population. Arteriosclerosis, Thrombosis, and Vascular Biology. 1995;15(10):1713-8.
18. Risch N, Tang H, Katzenstein H, Ekstein J. Geographic distribution of disease mutations in the Ashkenazi Jewish population supports genetic drift over selection. Am J Hum Genet. 2003;72(4):812-22.
19. Karpati M, Gazit E, Goldman B, Frisch A, Colombo R, Peleg L. Specific mutations in the HEXA gene among Iraqi Jewish Tay-Sachs disease carriers: dating of founder ancestor. Neurogenetics. 2004;5(1):35-40.
20. Reshef A, Nissen H, Triger L, Hensen TS, Eliav O, Schurr D, et al. Molecular genetics of familial hypercholesterolemia in Israel. Hum Genet. 1996;98(5):581-6.
21. Williams RR, Hamilton-Craig I, Kostner GM, Hegele RA, Hayden MR, Pimstone SN, et al. MED-PED: An Integrated Genetic Strategy for Preventing Early Deaths. In: Berg K, Boulyjenkov V, Christen Y, editors. Genetic Approaches to Noncommunicable Diseases: Springer Berlin Heidelberg; 1996. p. 35-45.
22. Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet. 2013;381(9874):1293-301.
23. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466(7307):707-13.
24. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972;18(6):499-502.
25. Whittall RA, Scartezini M, Li K, Hubbart C, Reiner Z, Abraha A, et al. Development of a high-resolution melting method for mutation detection in familial hypercholesterolaemia patients. Ann Clin Biochem. 2010;47(Pt 1):44-55.
26. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-9.
27. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-81.
28. Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comput Biol. 1997;4(3):311-23.
29. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods. 2010;7(8):575-6.
30. Futema M, Shah S, Cooper JA, Li K, Whittall RA, Sharifi M, et al. Refinement of Variant Selection for the LDL Cholesterol Genetic Risk Score in the Diagnosis of the Polygenic Form of Clinical Familial Hypercholesterolemia and Replication in Samples from 6 Countries. Clin Chem. 2015;61(1):231-8.
31. Marmot MG, Smith GD, Stansfeld S, Patel C, North F, Head J, et al. Health inequalities among British civil servants: the Whitehall II study. Lancet. 1991;337(8754):1387-93.
32. Gonzalez-Garay ML, McGuire AL, Pereira S, Caskey CT. Personalized genomic disease risk of volunteers. Proc Natl Acad Sci U S A. 2013;110(42):16957-62.
33. Zakharova FM, Damgaard D, Mandelshtam MY, Golubkov VI, Nissen PH, Nilsen GG, et al. Familial hypercholesterolemia in St-Petersburg: the known and novel mutations found in the low density lipoprotein receptor gene in Russia. BMC Med Genet. 2005;6:6.

34. Marks D, Thorogood M, Neil HAW, Wonderling D, Humphries SE. Comparing costs and benefits over a 10 year period of strategies for familial hypercholesterolaemia screening. Journal of Public Health. 2003;25(1):47-52.
35. Usifo E, Leigh SE, Whittall RA, Lench N, Taylor A, Yeats C, et al. Low-density lipoprotein receptor gene familial hypercholesterolemia variant database: update and pathological assessment. Ann Hum Genet. 2012;76(5):387-401.
36. Fouchier SW, Defesche JC, Umans-Eckenhausen MW, Kastelein JP. The molecular basis of familial hypercholesterolemia in The Netherlands. Hum Genet. 2001;109(6):602-15.
37. De Castro-Oros I, Pampin S, Bolado-Carrancio A, De Cubas A, Palacios L, Plana N, et al. Functional analysis of LDLR promoter and 5' UTR mutations in subjects with clinical diagnosis of familial hypercholesterolemia. Hum Mutat. 2011;32(8):868-72.
38. Chen WJ, Goldstein JL, Brown MS. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J Biol Chem. 1990;265(6):3116-23.
39. Dvir H, Shah M, Girardi E, Guo L, Farquhar MG, Zajonc DM. Atomic structure of the autosomal recessive hypercholesterolemia phosphotyrosine-binding domain in complex with the LDL-receptor tail. Proc Natl Acad Sci U S A. 2012;109(18):6916-21.
40. Humphries SE, Whittall RA, Hubbart CS, Maplebeck S, Cooper JA, Soutar AK, et al. Genetic causes of familial hypercholesterolaemia in patients in the UK: relation to plasma lipid levels and coronary heart disease risk. Journal of Medical Genetics. 2006;43(12):943-9.
41. Taylor A, Wang D, Patel K, Whittall R, Wood G, Farrer M, et al. Mutation detection rate and spectrum in familial hypercholesterolaemia patients in the UK pilot cascade project. Clinical Genetics. 2010;77(6):572-80.
42. Miyake Y, Yamamura T, Sakai N, Miyata T, Kokubo Y, Yamamoto A. Update of Japanese common LDLR gene mutations and their phenotypes: Mild type mutation L547V might predominate in the Japanese population. Atherosclerosis.203(1):153-60.
43. Han SM, Hwang B, Park TG, Kim DI, Rhee MY, Lee BK, et al. Genetic testing of korean familial hypercholesterolemia using whole-exome sequencing. PLoS One. 2015;10(5):e0126706.
44. Thomas ERA, Atanur SS, Norsworthy PJ, Encheva V, Snijders AP, Game L, et al. Identification and biochemical analysis of a novel APOB mutation that causes autosomal dominant hypercholesterolemia. Molecular Genetics & Genomic Medicine. 2013;1(3):155-61.
45. Pereira E, Ferreira R, Hermelin B, Thomas G, Bernard C, Bertrand V, et al. Recurrent and novel LDL receptor gene mutations causing heterozygous familial hypercholesterolemia in La Habana. Hum Genet. 1995;96(3):319-22.
46. Day INM, Whittall RA, O'Dell SD, Haddad L, Bolla MK, Gudnason V, et al. Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolemia. Human Mutation. 1997;10(2):116-27.
47. Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL Receptor Locus in Familial Hypercholesterolemia: Mutational Analysis of a Membrane Protein. Annual Review of Genetics. 1990;24(1):133-70.
48. Soutar AK, Knight BL, Patel DD. Identification of a point mutation in growth factor repeat C of the low density lipoprotein-receptor gene in a patient with homozygous familial hypercholesterolemia that affects ligand binding and intracellular movement of receptors. Proceedings of the National Academy of Sciences. 1989;86(11):4166-70.