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The study of familial hypercholesterolemia in Italy: A narrative review

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Abstract

In this review we outline our experience in the clinical and molecular diagnosis of familial hypercholesterolemia (FH), built up over more than three decades. We started our work by selecting FH patients on the basis of stringent clinical criteria, including extensive family studies. In most patients we confirmed the clinical diagnosis by showing a reduced LDLR activity in skin fibroblasts. After the isolation of *LDLR* cDNA and the characterization of the corresponding gene by the Dallas group, we started a systematic molecular investigation of our patients first using Southern blotting, and, subsequently Sanger sequencing. Up to now we have been able to identify 260 mutations of *LDLR* gene in more than 1000 genotyped FH patients, including 68 homozygotes. During this survey we identified 13 mutation clusters located in different geographical districts, which gave us the chance to compare the phenotype of patients carrying the most common mutations. We also found that mutations in *APOB* and *PCSK9* genes were a rare cause of FH in our cohort. Despite our efforts, we failed to identify mutations in candidate genes in ∼20% of cases of definite FH. An exome-wide study, conducted within the context of an international collaboration, excluded the presence of other major genes in our unexplained FH cases. Recently, we have adopted sequencing technology of the next generation (NGS) with the parallel sequencing of a panel of FH targeted genes as a way of obtaining a more comprehensive picture of the gene variants potentially involved in the disease.

Keywords: Familial hypercholesterolemia; *LDLR*; *APOB*; *PCSK9* gene mutations; Genotype-phenotype correlation; Mutation clusters

1. Study of FH in pre-genomic era

The elegant studies of Goldstein and Brown in the early 70s, which led to the discovery of the LDL receptor (LDLR) and demonstrated that a defect of this receptor was the cause of homozygous Familial Hypercholesterolemia (FH) [1], aroused our interest in the study of FH from both a clinical and biochemical stand point. In the late 70s, one of us (S. B., head of the Lipid Clinic at the University Hospital in Genova) started the systematic recruitment of individuals suspected to have FH on the basis of an LDL-cholesterol (LDL-C) level >95th percentile of the distribution in Italian population (>4.90 mmol/L in adults and >3.50−4.00 mmol/L in children/adolescents) and the belonging to a family where hypercholesterolemia was transmitted as dominant trait (vertical transmission of hypercholesterolemia and bimodal distribution of cholesterol levels in the family). Additional validation criteria for the diagnosis were the occurrence of tendon xanthomas and

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premature coronary heart disease (CHD) in the index case or in some family members or the presence of high cholesterol level in family members <18 years of age. The basic assumption was that by applying these multiple criteria one could select individuals likely to have a genetically homogenous dominant disorder. Retrospectively, these criteria for FH diagnosis were fairly similar to those proposed by the Simon Broome registry developed in the UK in 1980 [2]. From these early clinical studies, it soon became clear that the extension of plasma lipid analysis to index patient's family members was not only instrumental for diagnosis of FH but appeared to be an efficient way to discover other individuals with hypercholesterolemia. All hypercholesterolemic subjects were able to take advantage of dietary/life style interventions and treatment with the hypolipidemic drugs available at the time (cholestyramine, probucol, fenofibrate, and niacin; children and adolescents intolerant to cholestyramine were treated with pantethine). This survey also led to the identification of some patients whose clinical features were consistent with the diagnosis of homozygous FH (HoFH) who were treated with plasma exchange and starting from 1985 with selective LDL-apheresis.

The clear demonstration by the Dallas group that the LDLR defect was manifest in cultured skin fibroblasts, prompted one of us (S.C, head of the laboratory of Clinical Pathology at the University of Modena) to set up a cell culture system as a tool to define whether an LDLR defect was indeed the cause of FH in our patients. By applying the experimental assay developed in Dallas [3], we confirmed that most of our FH heterozygous patients (about 30 carefully selected patients) had a reduced LDLR activity (measured as ^{125}I-LDL degradation), ranging from 30 to 60% of that observed in control fibroblasts. These results were corroborated by the observation that in fibroblasts of a couple of patients with homozygous FH the LDLR activity was very low (<5% of control value) and fully confirmed that in our patients the cause of the disorder was to be ascribed to an LDLR defect. Over the years we have been able to set up a bank of fibroblasts isolated from more than 100 Italian FH patients, including 45 FH homozygotes.

2. Study of FH in the early genomic era

2.1. Segregation analysis of hypercholesterolemia in families using restriction fragment length polymorphisms (RFLPs) of LDLR gene

The cloning of *LDLR* cDNA and the isolation of the corresponding gene in the early 80s by the Dallas group paved the way to a multitude of molecular studies in many laboratories throughout the world. Thanks to the gift of *LDLR* cDNA from the Dallas group and taking advantage of the data on the restriction pattern of *LDLR* gene, the first approach for the genetic diagnosis of FH was to use some RFLPs of *LDLR* gene to construct the haplotype co-segregating with hypercholesterolemia and to follow the inheritance of the defective allele(s) in families [4,5]. In one of these studies we were able to identify the haplotype of the mutant allele in 57 FH families and to follow its inheritance in 67% of the cases [5]; in some families with this methodology it was possible to detect fragments of abnormal size co-segregating with the disease, increasing the probability of unequivocal diagnosis (see below) [5].

The RFLPs approach was used also for the pre-natal diagnosis of homozygous FH in a family where a young couple had had two children, one normocholesterolemic and one with homozygous FH. At the beginning of the third pregnancy we were asked to perform pre-natal diagnosis on chorionic villi DNA even though at that time we had not yet identified the causative mutation in the homozygous FH child. We investigated the *LDLR* gene restriction pattern in DNA isolated from chronic villi at the 10th week of gestation, using an approach similar to that adopted by Reshef A. et al. [6]. We were able to identify the informative haplotype co-segregating with the defect, which indicated that the HoFH child was a true homozygote and the fetus of the third pregnancy was unaffected by the disease. The newborn's plasma LDL cholesterol was 30 mg/dl (0.77 mmol/L) and the LDLR activity in umbilical cord fibroblasts was within the control range [7].

2.2. Identification of major rearrangements of LDLR gene

To take full advantage of the availability of the cDNA probe, in the late 80s our two research groups joined their efforts with the purpose of conducting a systematic analysis of genomic DNA in our cohort of FH patients, starting from those found to have a reduced LDLR activity in cultured fibroblasts. We started this investigation by looking for major rearrangements of the *LDLR* gene. We performed countless Southern blotting experiments, confident that we would discover the mutations causing FH in most of our patients. The meticulous inspection of the restriction patterns obtained with multiple cDNA probes (specific for a single exon or group of exons) led to the characterization of several major rearrangements of *LDLR* in heterozygous as well as in homozygous patients (examples of these studies are given in Refs. [8−10]). In some cases we complemented the genomic study with the analysis of mutant *LDLR* mRNA and the characterization of the encoded mutant receptors. In this respect we specifically investigated at cellular level two large duplications (designated FH-Salerno and FH-Caltanissetta) and one minute duplication (FH-Chieti-2), which did not disrupt the reading frame and were predicted to produce elongated receptors. In collaboration with Dr D. Patel of the Hammersmith Group in London (UK), we found that the two receptors containing large duplications reached the cell surface but were degraded much more rapidly than the

normal receptor, whereas the mutant receptor containing a short amino acid duplication was not processed to the mature form and did not reach the cell surface [11,12]. Thus, these examples demonstrated that duplications of amino acid sequences in the LDLR may disrupt in different ways the intracellular transport and catabolism of the receptor.

During the systematic search for gene rearrangements, we realized that some of them were found in apparently unrelated patients living in or coming from specific geographical districts of Italy, giving us the first clue about the presence of mutation clusters in our country (see below). Following the idea that patients sharing the same rearrangement might be related, we conducted a systematic study of three families living in northern Italy, whose members had been found to carry a 25 kb deletion spanning from exon 2 to exon 12 of *LDLR* gene. Tracing these three families back to the 17th century, we found their common ancestor and the possible geographic origin of this mutation in a region close to the ancient city of Pavia [13]. Subsequently, nine other apparently unrelated families carrying this mutation were identified in the same area of Italy. A married couple of heterozygous subjects belonging to two of these families (whose common ancestor had lived in the early 1800s) have had two homozygous children with extremely severe hypercholesterolemia (26.89 and 22.50 mmol/L respectively at 1 year of age).

However, to our great disappointment we soon realized that less than 10% of our patients showed an abnormal restriction pattern of *LDLR* gene, an indication that gross rearrangements were a minor cause of FH in our cohort and we had to adopt other gene analysis techniques to discover minute/point mutations.

3. Study of FH in the middle genomic era

3.1. The re-sequencing of LDLR gene

From the very beginning we adopted the strategy of exon by exon sequence analysis of the whole *LDLR* gene (Sanger sequencing), which, although time consuming and expensive, appeared to be the most reliable method for point/minute mutation detection. Over the years we have been able to identify a large number of *LDLR* gene mutations in more than 1000 FH heterozygotes as well as in 68 homozygotes who have been recruited in several Lipid Clinics throughout the country. One of the major problems we had to face in the early days was the attribution of pathogenicity to the numerous genomic variants found in our survey. While nonsense variants, small deletions/insertions producing truncated proteins and splice site variants could reasonably be considered pathogenic, this was not the case for the minute in frame insertions/deletions, missense or intronic variants. The assessment of pathogenicity was based upon the screening of a patient's family to ascertain if a novel variant co-segregated with hypercholesterolemia or, in the absence of family studies, had been found in other unrelated FH patients of our cohort or in patients of other countries. In recent years the advent of numerous algorithms designed for *in silico* "functional" analysis has greatly improved our capacity of predicting the possible deleterious effect of novel missense and intronic variants, specifically when three or more of these algorithms were consistent in predicting a damaging effect. Table 1 shows an update of the types of pathogenic variants we have found so far in Italian FH patients.

3.2. LDLR mutations in FH homozygotes

In 1998–99 we conducted the first genotype survey in 39 FH homozygotes identified at the time in Italian Lipid Clinics on the basis of LDL-C $\geq$ 12 mmol/L, tendon and cutaneous xanthomas in infancy, and the presence of both parents with hypercholesterolemia [9]. In this survey we were able to correlate the type of *LDLR* gene mutation with the residual LDL receptor activity in cultured fibroblasts. The LDLR assay revealed that in some patients the LDLR activity was virtually undetectable (^{125}I-LDL degradation <5% of the control value), while in others we could detect a measurable residual activity (up to 30%). This observation supported the concept, originally proposed by the Dallas group, that homozygous FH patients could be separated into two categories defined as receptor-negative and receptor-defective mutation carriers. It turned out that receptor-negative patients had higher LDL-C levels and a higher frequency of premature CHD than receptor-defective patients [9]. Receptor-negative mutations included large gene rearrangements, minute insertions/deletions resulting in frameshift, nonsense and splice site mutations as well as some missense mutations, while receptor-defective mutations included mostly missense variants. In the first survey we identified both mutant *LDLR* alleles in 36 patients; in three patients we were unable to identify the second mutant allele [9].

Table 1
Pathogenic mutations of *LDLR* gene found in Italy.

Type of mutation	Number	Percent
Major rearrangements		
Exon deletions	20	9.6%
Exon duplications	5	
Minute mutations		
Deletions	18	11.5%
Insertions/Duplications	9	
Deletions/Insertions	3	
Point mutations in the coding sequence		
Missense	132	50.7%
Nonsense	31	11.9%
Single nucleotide deletions	9	6.1%
Single nucleotide insertions	7	
Splicing mutations	26	10.0%
TOTAL	260	

3.3. APOB gene mutations in FH patients (familial defective ApoB, FDB)

In FH heterozygotes negative for *LDLR* mutations we sequenced exon 26 of *APOB* gene searching for the presence of p.(Arg3527Gln) mutation in apolipoprotein B, as well as other less frequent mutations of this peptide known to be a rare cause of a defective binding of LDL-apoB to the LDLR (Familial Defective ApoB or FDB). Against our expectations, only 2.2% of the patients of our cohort were found to carry the p.(Arg3527Gln) mutation or other apoB mutations known to cause FDB; thus, we concluded that these mutations were a rare cause of FH in our selected patients referred to the lipid clinics [14].

3.4. Discovery of some exceptions to the rules

Mutation screening in families was performed whenever possible, taking advantage of the fact that the majority of index patients were willing to collaborate with us (especially after the introduction of the use of statins) in the identification of other family members who might have FH and may have timely and effective treatment. The systematic family studies led us to the discovery of some apparent exceptions to the rules governing the transmission of a monogenic dominant disorder, as specified below.

3.4.1. de novo mutations

In two FH index patients, heterozygous carriers of pathogenic mutations, we could not trace the transmission of the mutations in the families. After extensive genetic investigations and exclusion of non paternity, we concluded that these patients were carriers of "de novo" mutations of *LDLR* gene [15,16]. The haplotype analysis of the *LDLR* locus revealed that in one of these patients the "de novo" mutation had occurred in the paternal germ line [16]. To the best of our knowledge, only five FH patients carrying de novo *LDLR* mutations have been reported so far [17–19].

3.4.2. Inconsistency in parent to offspring transmission

One of our patients with the clinical diagnosis of homozygous FH was found to be homozygous for a mutation in exon 12 [c.1775G>A, p.(Gly592Glu)] known to be pathogenic and heterozygous for a rare variant in intron 14 (c.2140 +5G>A). Surprisingly, the patient's daughter with definite heterozygous FH carried only the intron 14 variant. To explain this inconsistency we suspected that the proband was a carrier of a partial deletion of *LDLR* gene. This intuition was found to be correct as both the proband and her daughter were heterozygous for a deletion of exons 11 and 12, which accounted for the apparent homozygosity of the c.1775G>A mutation in exon 12 found in the proband. The Ex11_12 deletion was linked to the c.2140 +5G>A variant in intron 14 [20]. Other FH patients, heterozygotes for c.2140 +5G>A variant, were found to carry the Ex11_12 deletion or other pathogenic mutations. Thus, the study of this pedigree indicated that: i) the apparent homozygosity for a missense mutation may derive from the presence of a partial or complete gene deletion on the other allele; ii) the intronic variant c.2140 +5G>A was probably not pathogenic "per se" [20].

3.4.3. Homozygous FH with a recessive transmission

During the survey of homozygous FH we came across some young patients with the clinical phenotype of homozygous FH who were born from normocholesterolemic parents and had normal LDLR activity in cultured skin fibroblasts. We soon realized that similar patients had been identified by other Italian investigators among homozygous FH patients living in the island of Sardinia. An extensive study of these patients performed by our colleagues led to the demonstration that this disorder was inherited as a recessive trait (autosomal recessive hypercholesterolemia, ARH) and did not involve the *LDLR* gene [21,22]. A collaborative molecular study of these families coordinated in Dallas by Dr H. Hobbs led to the discovery of the gene responsible for this disorder (*LDLRAP1*) which turned out to encode an adaptor protein (LDLR Adaptor Protein 1) required for the function of LDLR in hepatocytes [23]. A complete history of the discovery of this disorder has recently been published [24].

These three examples emphasize the paramount importance of the family study to dissect the molecular bases of a monogenic disorder.

3.5. Contribution of modifying genes to LDL-C variability in FH

The great variability of LDL cholesterol levels in genotyped patients suggested a possible contribution of common sequence variants in other genes affecting LDL metabolism. We tested this hypothesis by looking at the distribution of common SNPs of *APOE*, *MTTP* and *APOB* in a large group of molecularly characterized heterozygous FH patients. We found that *APOE* gene polymorphism affected the LDL-C level in our FH patients, as it does in the general population. However, in FH patients this polymorphism explained only 2.8% of the total variability of LDL-C, whereas in a sample of the Italian population this contribution was higher (12.8%). The FH subjects with the −493TT genotype of *MTTP* gene had LDL-C levels 12.5% and 8% lower than the carriers of GG and GT genotype, respectively. With regard to the −516C/T polymorphism of *APOB* gene, we found that the rare genotype −516TT was associated with 25.5% and 20.5% increase in LDL-C levels compared to CC and CT genotypes, respectively. The combined results of *APOE*, *MTTP* and *APOB* polymorphisms indicated, therefore, that in FH heterozygotes, despite the large effect on LDL-C produced by the *LDLR* gene mutations, the influence of these modifying genes was similar to that observed in the general population. However, the percentage of the total

variance of LDL-C level attributable to the investigated SNPs was less than 10% [25].

3.6. The LDL lowering effect of the beta-thalassemia trait

One of the most relevant aspects encountered during the search for modifying genes was the discovery that the carriage of beta-thalassemia trait had a strong LDL lowering effect in FH patients. Our curiosity was aroused by a previous observation that Sardinian beta(0)-thalassemia carriers [i.e carriers of a unique *HBB* gene mutation of the beta-globin: c.118C>T, p.(Q40*)] had lower plasma cholesterol than non-carriers. We investigated 63 FH heterozygous patients carrying 7 different mutations of the *LDLR* gene, two of which were found in several individuals/families (mutation clusters). In one these clusters, [(c.1778delG, p.(Gly593Alafs*72)], a receptor-negative mutation, the plasma LDL-C level was 5.76 ± 1.08 mmol/L in subjects with beta(0)-thalassemia trait and 8.25 ± 1.66 mmol/L in subjects without this trait ($P < 0.001$). The LDL lowering effect of beta(0)-thalassemia trait emerged also when we pooled the data from all FH subjects with and without beta(0)-thalassemia trait, regardless of the type of mutation in the *LDLR* gene (5.64 ± 1.16 vs 7.78 ± 1.53; $P < 0.001$). We suggested that LDL-lowering effect of beta(0)-thalassemia might be related to: i) the increased plasma LDL removal by the bone marrow due to the mild erythroid hyperplasia usually present in beta(0)-thalassemia carriers and ii) the hemolysis-induced chronic activation of the monocyte-macrophage system, which causes an increased secretion of some cytokines (interleukin-1, interleukin-6 and tumor necrosis factor-alpha), known to affect the hepatic secretion and the receptor-mediated removal of apolipoprotein B-containing lipoproteins [26]. We proposed that the lifelong LDL-lowering effect of beta(0)-thalassemia trait might delay the development and progression of coronary atherosclerosis in FH.

4. The discovery of mutation clusters

4.1. LDLR mutation clusters: geographical distribution and historical perspectives

During the genotype survey of FH patients we discovered that, despite a great allelic heterogeneity (largely expected due to the heterogeneous genetic background of the Italian population), several mutations were recurrent in apparently unrelated patients whose families had been living in or had come from specific geographical districts of the country. By extending the clinical and genetic investigation to all available family members of a recurrent mutation carrier, we tried to establish the geographical origin of the family and, consequently, of the mutation – going some generations back in an attempt to discover the oldest carrier of the genetic defect. In this search we took advantage of the fact that internal migration of families (from the rural regions of southern Italy to the industrialized areas of northern Italy) has been a relatively recent event, occurring mostly over the past century [27]. So far we had identified 13 *LDLR* mutation clusters (defined as groups of patients consisting of at least 10 unrelated families sharing the same mutation) [14]. The identification of these clusters, in addition to providing relevant information on the different clinical impact of the most common mutations, allows some speculation about historical aspects of mutation origin.

Among the clusters identified in northern Italy, two deserve consideration. The first, c.662A>G, p.(D221G) (designated FH Padua-1), includes at present 90 apparently unrelated families, 181 heterozygotes and 4 homozygotes. These families are mostly distributed in the north-east areas of Italy, which in the XVI century were part of the territory of the Republic of Venice. By using intragenic markers and multiallelic microsatellites close to the *LDLR* gene (D19S394 and D19S221) [28], our genetic studies indicated quite a historic origin of this mutation, which, considering the chance of recombination events, probably dated back more than one thousand years [27,29].

The second cluster (c.1415_1418dupACAT, p.(Q474Hfs*63) was designated FH Savona-1, as the first identified families came from small villages in the mountains (Ligurian Apennines) overhanging the coastal city of Savona (Fig. 1). Over the years many other carrier families were detected and the cluster now includes 50 families and 191 heterozygotes. Interestingly, we found that most of these families came from villages located along the "High Road", a mountain pathway (located at an altitude of about 1000 m above sea level) which encompasses the whole territory of the Liguria region and is connected with a network of other ancient roads leading eastward to the interior of the country (Fig. 1). Other families, that now live in some villages on the sea side (the famous "Cinque Terre" area) had moved over the last century from the mountain villages located along the "High Road". Interestingly, up to the XVIII century the "High Road" was preferentially used by Ligurian merchants to transport goods by mule to the northern countries since it was considered safer and better protected than the coast roads, a prey to pirate raids along the coasts. Using the above mentioned genetic markers, we confirmed the previous observation [27] that in all 50 families the Savona-1 mutation co-segregated with the same haplotype and was in complete linkage disequilibrium with the 251 bp allele of the D19S394 microsatellite. This finding suggests the presence of a common ancestor who, approximately one thousand years ago, lived in the areas close to the "High Road". Interestingly, the FH-Savona-1 mutation appears to be confined to the Liguria region, since it has never been found in other areas of Italy.

Among the clusters identified in southern Italy the largest in terms of number of families and subjects

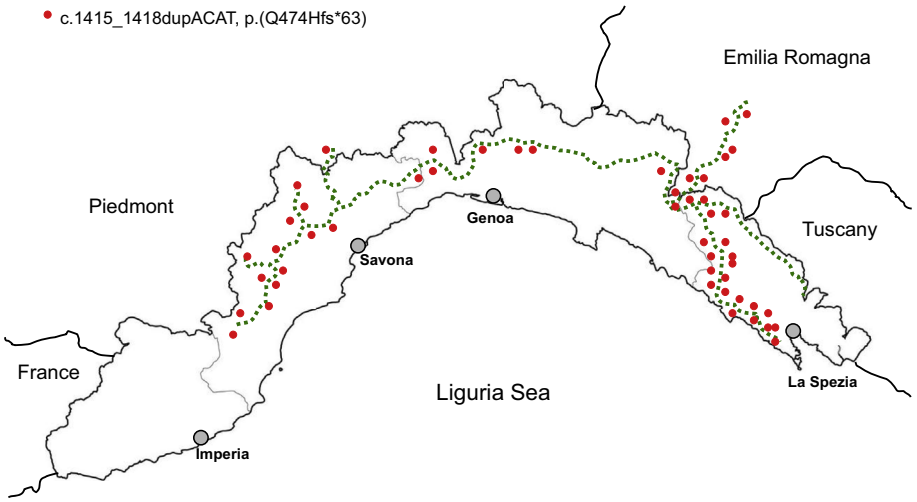

Fig. 1. **Geographical location of the LDLR mutation cluster FH-Savona-1.** The figure shows the map of the Liguria Region. The Ligurian Sea is a streak of the Mediterranean Sea between the Italian Riviera (Liguria and Tuscany) and the island of Corsica. The dotted green line traces the "High Road" (situated at an altitude of 1000 m above sea level), which was the major line of communication and transport of goods up to the XVIII century. The red dots along the "High Road" indicate the villages where the original families carrying the FH Savona-1 mutation were living. The migration of some of these families from the mountains to the major cities of the Ligurian coastline has occurred over the past century.

analyzed was the c.1646G>A, p.(G549D), which was named FH Palermo-1 from the origin of the first families we identified. Up to now this cluster includes 78 families, three homozygotes, four compound heterozygotes and 163 heterozygotes. These FH families were mainly distributed in the southern regions of mainland Italy and in Sicily. This distribution largely corresponds to the areas of Italy colonized by the Greeks from the VIII to the IV century B.C. (and subsequently designated "Magna Graecia" by the Romans). The hypothesis that the mutation was "imported" from Greece over that time period is supported by the observation that applying in our families the above mentioned genetic markers [27] the mutation could be dated back to more than two thousand years ago. This concept is also supported by a series of studies showing that FH-Palermo-1 mutation is the most prevalent among FH Greek patients (with frequency ranging from 23% to 30%) [30,31].

Mutation cluster identification gave us the opportunity to investigate the phenotypic variability among patients with the same LDLR mutation and to better define phenotypic differences among carriers of different mutations, in terms of LDL cholesterol levels (Fig. 2) and prevalence of clinical manifestations such as tendon xanthomas and premature CHD [14].

5. The study of FH in late genomic era

5.1. Implementation of the cascade screening

Since the early days, we have systematically extended our investigation to index cases' family members as a way to define the transmission of hypercholesterolemia. In the last decade we have extended the molecular diagnosis to family members (cascade screening) as an efficient way to identify mutation carriers and offer appropriate treatment and genetic counseling. Obviously, the success of the cascade screening implies a collaborative index case, good family relations, a favorable logistic context and the cooperation of general practitioners and primary care providers [32]. Fig. 3 shows a typical example of a successful cascade screening in a large pedigree. Recently, we have implemented reverse genetic testing starting from index FH children, identified by pediatricians, to detect other mutations carriers (both children and adults) within family [14].

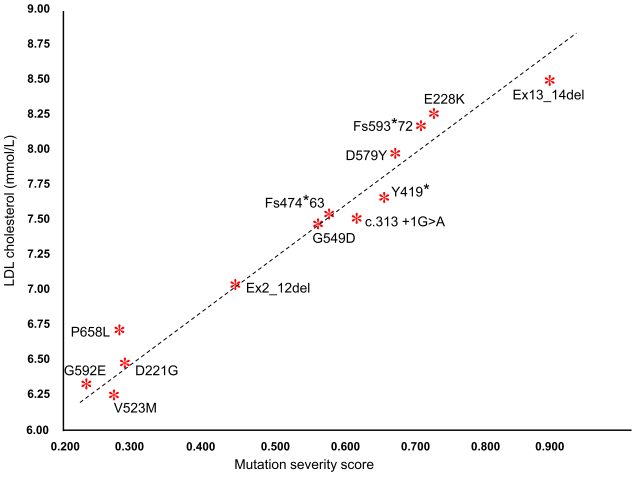

Fig. 2. **Plasma LDL in *LDLR* mutation clusters.** The figure shows the relationship between the mean LDL-C level of each mutation cluster and the corresponding LDL severity score. The LDL severity score is given by the ratio between the number of subjects with adjusted LDL-C values above the median value of the whole patients sample (n. 1600 genotyped FH patients belonging to 13 clusters) and the total number of subjects carrying the same mutation (see Ref. [14] for details).

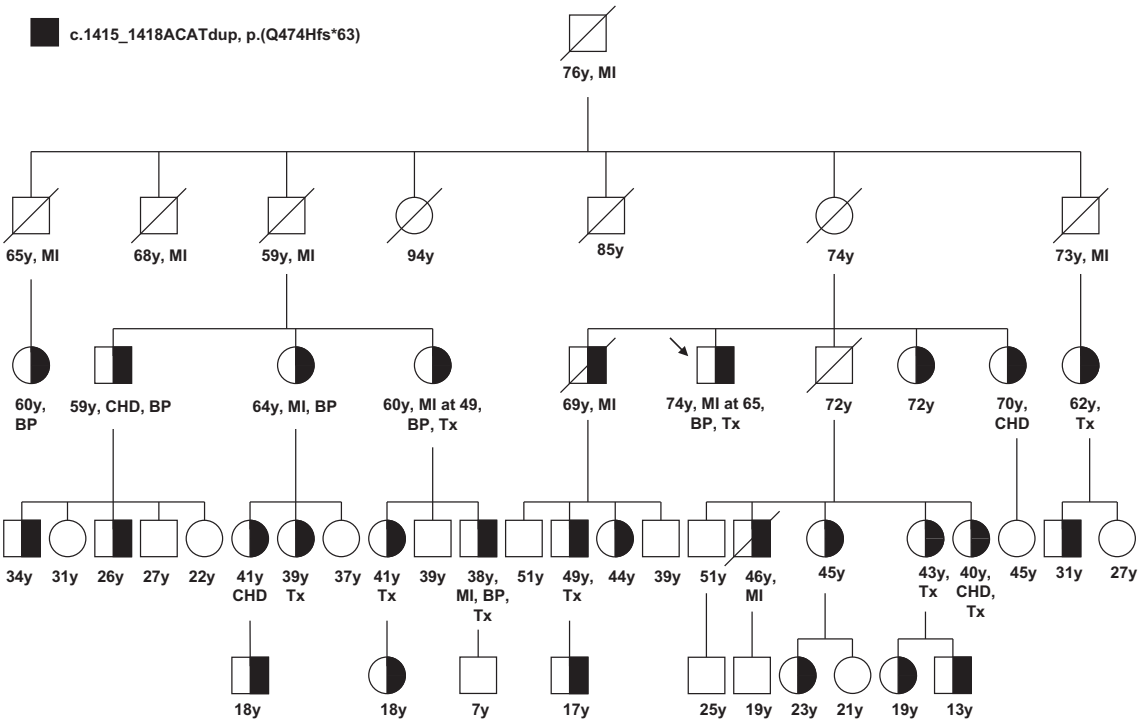

Fig. 3. **Genetic cascade screening of FH.** The figure shows one of the largest FH families we investigated whose ancestors had lived in a small village in the mountains between Liguria and Emilia-Romagna. Thanks to the collaboration of the index patient (indicated by the arrow), we were able to extend the genetic screening to all living relatives. The black symbol indicates the carriers of the *LDLR* mutation. The age of the subjects at the time of the screening or the age of death is indicated below each symbol. MI: myocardial infarction; BP: coronary artery bypass graft; Tx: tendon xanthomas.

5.2. Looking for PCSK9 mutations

Needless to say that the discovery of *PCSK9* as third candidate gene in FH [33] prompted us to sequence this gene in FH patients who did not carry *LDLR* or *APOB* mutations. At the beginning of this adventure we were confident that we would discover several patients with mutations in this gene, an expectation shared by many laboratories in Europe. However, this was not the case and, after an intensive search, we now can say that gain of function mutations of PCSK9 are an exceedingly rare cause of FH in our cohort. However, during this survey we identified three patients (two of whom siblings) with severe hypercholesterolemia (LDL-C ranging from 10.0 to 13.3 mmol/L), who turned out to be double heterozygotes for an LDLR mutation [p.(E228K) and p.(Y419*), respectively] inherited from one hypercholesterolemic parent and a novel PCSK9 variant [p.(R496W) and p.(N425S), respectively] inherited from the other hypercholesterolemic parent. The LDL levels in the double heterozygotes were 56% and 44% higher than those found in simple heterozygous carriers of the two LDLR mutations, respectively [34]. In view of these findings and the FH-like phenotype found in simple heterozygous carriers of the *PCSK9* variants, we assumed that these novel variants were pathogenic (i.e. they are gain of function mutations). In our series of FH patients we found only two other patients heterozygous for a gain of function mutation of PCSK9 p.(S127R) [14].

5.3. Definite FH without mutations in candidate genes

As found in other laboratories, the systematic analysis of patients with the clinical diagnosis of definite FH showed that in approximately 20% of the cases we fail to detect mutations in the candidate genes for dominant as well as recessive hypercholesterolemias, raising the question of the existence of other unknown major genes. The successful application of whole exome sequencing (WES), which had led to the discovery of loss of function mutations of ANGPTL3 as the cause of Familial Combined Hypolipidemia [35], promoted an active search of new genes in unexplained FH patients. An international consortium coordinated by Prof. S. Humphries in London (UK) focused on this search in 125 FH heterozygous patients negative for *LDLR/APOB/PCSK9* mutations. Despite a great effort, this study did not lead to the identification of novel major genes responsible for FH, even though it showed an excess of novel variants in 18 genes mostly not directly related to cholesterol/lipoprotein metabolism. The conclusion was that the unexplained cases of FH were likely to be very heterogeneous and the lack of suitable large families for linkage analysis makes it difficult to ascertain whether a specific gene variant co-segregates with FH [36].

5.4. Targeted Next Generation Sequencing (NGS)

Until recently DNA samples of FH patients were assayed using automated Sanger sequencing of the coding regions of

one candidate gene at a time, an operation which requires several weeks to report results. The scenario has changed with the introduction of massive parallel high-throughput DNA sequencing (Next Generation Sequencing) [37]. Specific gene targeted protocols have been developed to detect mutations in a set of cholesterol-related genes, including all known candidate genes for dominant and recessive forms of hypercholesterolemia, as well as other genes related to cholesterol metabolism. In this context the time needed to sequence a set of candidate genes has decreased from several weeks to a few days.

At present our comprehensive screening of familial hypercholesterolemia includes the targeted NGS resequencing of several genes (*LDLR, APOB, PCSK9, STAP1, APOE, ABCG5, ABCG8, LDLRAP1, CH25H, SREBF1, LIPA, SCAP, FGFR4* and *MYLIP*), complemented with strategies (e.g. MLPA) for the identification of copy number variations (CNVs) in *LDLR* gene.

The use of targeted NGS for FH has revealed a new scenario with regard to the interpretation of the data generated by the system. The simplest situation is given by the presence of a known pathogenic or a likely pathogenic variant in heterozygous state in one of the FH candidate genes (*LDLR, APOB*, *PCSK9*). A more complex situation occurs when a patient turns out to be heterozygous for a pathogenic variant in other genes (e.g. *LIPA*, *ABCG5* or *ABCG8*), a condition which theoretically should not be regarded per se as sufficient to cause an FH-like phenotype. Another puzzling situation is the discovery of rare missense variants in *APOB*, which are found to be deleterious or not tolerated *in silico* but may either be the cause of hypercholesterolemia (defective binding of LDL to the LDLR) or hypobetalipoproteinemia (defective secretion of apoB containing lipoproteins). So the large number of data generated by NGS technology is a challenge to our capacity to give clinically meaningful information.

5.5. Novel strategies for the identification of FH patients: role for Clinical Chemistry Laboratory

Technological advances have considerably improved the productivity of Clinical Chemistry Laboratories of major hospitals, in such a way that nowadays these laboratories provide routine examinations for large population samples. This activity translates into the production of huge laboratory databases which represent powerful resources which can be exploited for clinical research purposes, particularly if they are linked to clinical and demographic data.

ESC/EAS guidelines for the management of dyslipidemias recommend baseline lipid assessment (total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol) in adult men ≥40 years of age and in women ≥50 years of age or postmenopausal, especially in the presence of other risk factors [38]. On the other hand, in 2011 the US National Heart Lung and Blood Institute (NHLBI) developed guidelines for cardiovascular health in children that strongly recommend universal screening for children comprised between 9 and 11 years of age [39]. Following those recommendations, an important role is given to the clinical chemistry laboratories in alerting physicians, primary care providers and patients about the possibility of FH diagnosis on the basis of a high plasma LDL-C (>4.9 mmol/L) or high Total Cholesterol (≥7 mmol/L). The introduction of interpretative commenting to laboratory lipid profile for LDL-C higher than 6.5 mmol/L (Dutch Score ≥5) has shown to increase specialist referrals and molecular FH diagnosis and to be associated, over time, with a significant reduction in LDL-C concentration in patients [40].

Following these concepts we have recently activated collaboration with the Clinical Chemistry Laboratory of a major hospital associated with the University of Modena and Reggio Emilia. In 2014 this laboratory performed almost 110,000 LDL-C determinations in approximately to 88,000 subjects (about 1 out of 6 subjects of a population of 534,845 individuals has had at least one LDL-C determination). On the basis of the laboratory data, 3.5% of these subjects (3122) could be considered as "possible FH" having an LDL-C level higher than 4.9 mmol/L (Dutch Score ≥3) and 0.17% of them (150 subjects) could be considered as "probable FH" having an LDL-C level higher than 6.5 mmol/L (Dutch Score ≥5). The scrutiny of the laboratory data revealed that a large proportion (88%) of subjects classified as "possible FH" was composed of outpatients, i.e. patients whose LDL-C determination was not requested by physicians working within the hospital setting. The majority of "possible FH" patients were female (57.5%), especially among subjects older than 60 years of age (Fig. 4). Only few LDL-C determinations were requested in children aged between 9 and 11.

Although these data cannot be directly extended to the entire Italian population, it is reasonable to assume that thousands of Italian subjects receive every year a laboratory

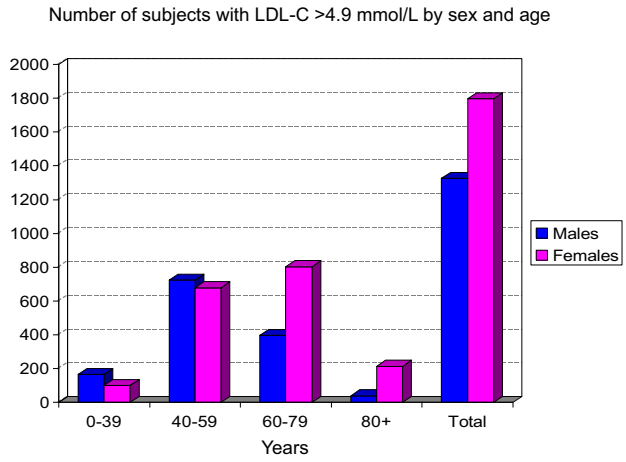

Fig. 4. The figure shows the number of subjects with LDL-C levels (determined by the Clinical Chemistry Laboratory) above 4.9 mmol/L (Dutch Score 3) in relation to age and gender.

report showing an LDL-C level higher than 4.9 mmol/L. In these subjects FH diagnosis should be taken into consideration with the support of laboratory interpretative commenting, alerting both patients and physicians. We are confident that the implementation of this collaboration with a large Clinical Chemistry Laboratory will extend the capacity of general practitioners and primary care providers to identify putative FH patients/families, who can be directed to genetic diagnosis and appropriate clinical counseling.

6. Conclusions

We have summarized our experience in the study of the clinical and molecular aspects of FH in our country, built up over a period of more than three decades. Although we now have a deep knowledge of the genetic and pathophysiology of FH, many questions remain unanswered such as the molecular basis of the phenotypic variability among patients sharing the same *LDLR* mutation or the set of genes involved in the unexplained definite FH cases. We expect that new data will emerge from the implementation of new technologies of gene sequencing and exome/genome analysis. From the clinical standpoint we recommend that new strategies must be devised to identify FH patients who may benefit of appropriate treatment. Finally, the results of our studies emphasize the key value of long standing collaboration between clinicians and basic science researchers who share the same curiosity about the basic mechanisms of a monogenic disease. We are also pleased that our studies have aroused the interest of young investigators in our country who are now actively involved in answering some open questions in the FH field.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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