Molecular diagnosis of familial hypercholesterolemia: an important tool for cardiovascular risk stratification [59]

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ABSTRACT

Familial hypercholesterolemia (FH) is associated with an increased risk of premature coronary heart disease. Molecular identification of these patients can reduce the burden of mortality from cardiovascular disorders simply by the correct identification of the disease early in life, followed by counseling and appropriate lifestyle modifications, and therapeutic measures when required. Recent studies show that, in Portugal, this disease is severely under-diagnosed. After more than 10 years of research through the Portuguese FH Study, it is now possible to translate the original research results into clinical application.

Aims: The main aims of the present work were to determine whether clinical characterization is sufficient to identify these individuals at high risk of developing CHD and to evaluate the clinical applicability of molecular diagnosis for FH.

Methods: All patients described in this study were recruited for the Portuguese FH Study. The diagnostic criteria used to select the index patients were adapted from the Simon Broome Heart Research Trust. To analyze the usefulness of the molecular diagnosis, graphs of total and LDL

Diagnóstico molecular de hipercolesterolemia familiar: uma ferramenta importante para a estratificação do risco cardiovascular

RESUMO

Introdução: A Hipercolesterolemia Familiar (FH) associa-se a um aumento do risco de doença coronária prematura. A identificação molecular dos indivíduos afectados pode contribuir para a redução da morbi-mortalidade por doença cardiovascular, já que permite a correcta identificação da patologia em idades mais jovens e o subsequente aconselhamento e a adopção de um estilo de vida mais saudável, bem como a introdução de terapêutica farmacológica mais precocemente se necessário. Estudos recentes demonstram que em Portugal a FH encontra-se sub-diagnosticada. Depois de mais de 10 anos de investigação no âmbito do "Estudo Português de Hipercolesterolemia Familiar" é agora possível fazer a translação dos resultados de investigação original para a aplicação clínica.

Objectivo: O principal objectivo deste trabalho foi determinar se a caracterização clínica é suficiente para identificar os indivíduos com Hipercolesterolemia Familiar e avaliar a aplicabilidade clínica

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cholesterol values by age were constructed for 622 possible FH patients. The lipid profile of patients genetically identified as having FH, before and under medication, were analyzed to assess whether these patients were receiving appropriate treatment. The data are shown separately for children and adults and for female and male propositi (index cases and hypercholesterolemic relatives), both with and without a detectable mutation in the LDLR gene.

Results: The Portuguese FH Study has already genetically identified 404 individuals (171 index patients and 233 relatives) among more than one thousand individuals sent for study. A total of 78 different mutations in the LDLR gene were found in 171 index patients, 2 different mutations were found in the apoB gene of 4 patients and 2 patients had a unique PCSK9 mutation. Statistical analysis revealed that there are significant differences between total cholesterol ($p<0.001$) and apoB ($p=0.026$) values in the group of children (male and female) with and without a mutation in LDLR. For female children LDL values were also significantly different ($p<0.001$) between subgroups but for male children this difference did not reach statistical significance. In adult women there is a statistically significant difference for total cholesterol ($p=0.049$), LDL cholesterol ($p=0.031$) and apoB ($p=0.003$) values in the subgroups with and without a LDLR mutation. In adult males there is a statistical difference for total cholesterol ($p=0.002$). LDL cholesterol ($p=0.003$) and apoB ($p=0.0023$) in subgroups with and without an LDLR mutation. Nevertheless there was considerable dispersion of values and individually it is not possible to distinguish between patients with and without a mutation in the LDLR gene, based only on lipid profile.

Conclusions: By analysis of the clinical data of 696 possible FH patients, the present report shows evidence that clinical characterization is not sufficient to do diagnóstico molecular da FH.

Material e Métodos: Todos os doentes em estudo foram recrutados do "Estudo Portugês de Hipercolesterolemia Familiar". Os critérios de diagnóstico utilizados para a selecção dos casos-índex foram adaptados do "Simon Broome Heart Research Trust". De modo a se analisar a utilidade do diagnóstico molecular efectuaram-se gráficos com o colesterol total e LDL dos 622 indivíduos com diagnóstico clínico de FH. O perfil lipídico dos doentes identificados molecularmente com FH, antes e após tratamento, foi analisado de modo a se perceber se estavam a receber o tratamento adequado. Os resultados são apresentados separadamente para o grupo das crianças e adultos, *propositi* mulheres e homens" (casos-índex e familiares hipercolesterolémicos), ambos com e sem mutação no gene LDLR.

Resultados: O Estudo Português de Hipercolesterolemia Familiar foi estabelecido com sucesso em 1999, tendo já sido identificados geneticamente 404 indivíduos (171 índex e 233 familiares), entre mais de 1000 indivíduos enviados para estudo. Foram encontradas 78 mutações diferentes no gene do receptor das LDL em 171 casos índex, 2 mutações diferentes na apoB em 4 casos-índex e uma única mutação no PCSK9 em 2 casos-índex. Através da análise estatística verificou-se que existem diferenças estatisticamente significativas entre o colesterol total ($p<0,001$) e a apoB ($p=0,026$) no grupo das crianças (mulheres e homens) com e sem mutação no gene LDLR. Nas crianças do sexo feminino para os valores de cLDL existem diferenças estatisticamente significativas ($p<0,001$), no entanto para as crianças do sexo masculino não se verificam. Nos adultos do sexo feminino existem diferenças estatisticamente significativas para os valores de colesterol total ($p= 0,049$), cLDL ($p=0,031$) e apoB ($p=0,003$) nos sub-grupos com e sem mutação no LDLR. Nos adultos homens verificou-se existir também

distinguish between patients with genetic or environmental dyslipidemia, and so molecular diagnosis is useful in clinical practice, allowing correct identification of FH patients and their relatives, and the early implementation of therapeutic measures to reduce the elevated cardiovascular risk of these patients. In general, molecular diagnosis of FH is feasible and could be obtained in 1-2 months if the technology is available. In Portugal the test will be offered to the population by our Institute at a cost of about 500 euros, like many other genetic tests or exams such as nuclear magnetic resonance.

Key words
Familial Hypercholesterolemia; Molecular diagnosis; LDL receptor gene; Premature coronary heart disease; Patient prognosis

diferenças estatisticamente significativas para os valores de colesterol total (p= 0,002). cLDL (p=0,003) e apoB (p=0,023) nos sub-grupos com e sem mutação no LDLR. No entanto quando visto graficamente estes valores do perfil lipidico existe uma grande dispersão e não é possivel distinguir os doentes com ou sem mutação no gene LDLR.
Conclusões: Pela análise dos dados clínicos de 696 indivíduos com diagnóstico clínico de FH o presente estudo demonstrou evidência de que a caracterização clínica não é suficiente para distinguir entre indivíduos com dislipidemia genética e ambiental. O diagnóstico molecular é importante e útil para o clínico, permitindo a identificação correcta dos doentes com FH e seus familiares, bem como a aplicação precoce de medidas terapêuticas para a redução do risco cardiovascular nestes doentes. O diagnóstico molecular da FH em Portugal é viável e efectuado em 1-2 meses, se a tecnologia utilizada estiver disponível. O teste molecular da FH em Portugal é efectuado pelo nosso Instituto e tem um preço aproximado de 500 euros, como muitos outros testes genéticos ou uma ressonância magnética nuclear.

Palavras chave
Hipercolesterolemia familiar; Diagnóstico molecular; Receptor das LDL; Doença coronária prematura; prevenção cardiovascular

INTRODUCTION

Familial hypercholesterolemia (FH) is an inherited disorder of cholesterol metabolism. It is associated with increased risk of premature coronary heart disease (CHD) and early identification of affected individuals can increase their life expectancy and quality, by the early modification of lifestyle and administration of appropriate lipid-lowering treatment. There are about 10,000,000 people with FH worldwide and approximately 85% of males and 50% of females with FH will suffer a coronary event before 65 years of age if appropriate preventive efforts are not implemented [1].

FH is one of the most common monogenic disorders, with a heterozygous frequency of 1/500 in most European populations. It is estimated that Portugal has about 20,000 FH patients, but this disorder is severely underdiagnosed in this country, even though there are clear indications from the World Health Organization for large-scale screening[2]. The early identification of these individuals is essential to decrease their elevated cardiovas-

cular risk. The plasma total cholesterol levels in heterozygous FH patients are increased about 2-3 fold compared to normal subjects and triglycerides are usually within the normal range. Hypercholesterolemia, the earliest manifestation of the disease, is present at birth in virtually all affected subjects and remains throughout life. The finding of severe hypercholesterolemia in children increases the diagnostic probability within the family since environmental factors take longer to show their effect. Tendon xanthomas, CHD and atherosclerosis are usually detected after the ages of 30-40 years [3], but the age of onset of these conditions is dependent on other genetic and/or environmental factors, so patients carrying the same mutation may have completely different plasma total and LDL cholesterol levels.

FH is caused in the majority of cases by mutations in the LDLR gene. Mutations in other genes such as apolipoprotein B and proprotein convertase serine kexin 9 (PCSK9) are rare causes of FH. apoB mutations usually produce a similar, or milder, phenotype to mutations in the LDLR gene but the few patients described with PCSK9 mutations show a very severe phenotype, similar to the phenotype of FH patients homozygous for LDLR mutations [4].

FH patients are at high risk of developing CHD, however recommendations for the general population do not apply to FH patients [5]. For the general population the European Cardiology Society recommends that total cholesterol levels should be below 190 mg/dl and LDL cholesterol below 115 mg/dl. For patients at high risk of developing premature CHD, as is the case of FH patients, the recommendations are to decrease cholesterol levels to below 175 mg/dl and LDL cholesterol to below 100 mg/dl. If these targets cannot be reached, then an extra effort should be made to decrease the overall cardiovascular risk by better management of other risk factors, for example by the implementation of a healthy diet with restricted fat intake and other lifestyle orientations such as physical activity and tobacco cessation. The NICE recommendations for management of familial hypercholesterolemia state that at least a 50% reduction in basal cholesterol levels should be achieved [6] to decrease the elevated cardiovascular risk of these patients.

To overcome the problem of how to reach these targets with FH patients, a panel of international experts has gathered to prepare guidelines for the management of these individuals[5]. Patients with a genetic dyslipidemia are at greater risk of premature cardiovascular disorders since they start to accumulate cholesterol from birth, so the treatment of these patients has to be different and more effective than the treatment of an environmental dyslipidemia. The majority of FH patients can only achieve the desired total and LDL cholesterol when a potent statin is prescribed and usually combination therapy is necessary[6]. Moderate doses of potent statins in combination with ezetimibe, a selective cholesterol intestinal absorption inhibitor, or resins are the first choice for combination when over 50% LDL cholesterol reductions are required or when high doses of statins are not well tolerated [7]. However it is important to note that ezetimibe has not been proved to also reduce cardiovascular events and the progression of arteriosclerotic disease measured by evaluation of intima-media thickness[8]. To decrease mortality from avoidable cardiovascular diseases, the international panel of experts on FH also highlighted the importance of detecting subclinical atherosclerosis for cardiovascular risk stratification, since treatment of silent vascular disease may prevent future clinical events [6]. A healthy lifestyle is also important to achieve a greater reduction in cholesterol levels. FH patients should have a healthy diet, ideal body weight, and moderate but daily physical activity, and should not smoke. Dietary recommendations apply to children after the age of 2-3 years [6].

Their physician should also be able to give lifestyle counseling or should refer patients to a specialized clinic for inherited cardiovascular disease (ICD) with a medical geneticist. This specialist provides a detailed exami-

nation of the family history with the ability to confirm diagnosis in the family, and knowledge and experience in identifying ICD and genetic syndromes that may have cardiac involvement. The medical geneticist also has experience in understanding of the possible underlying molecular pathology of ICD, the likely clinical utility of testing, and interpretation of tests and uncertain results, providing advice to parents about recurrence, and family cascade testing.

Once FH patients are identified they need to start appropriate treatment immediately, and this treatment has to be continued for the rest of their lives, as only in this way can their cardiovascular risk be reduced.

There are no absolutely predictive clinical criteria for the diagnosis of FH, and arbitrary criteria must be used. Some countries have developed their own criteria for the diagnosis of FH, including the USA, the Netherlands and the UK, and other countries use one of these [9]. Some authors argue that fulfillment of the clinical criteria is sufficient to identify a FH patient [10], but lately the need to perform molecular diagnosis has been put forward as the only way to correctly identify these patients [11]. The high cost of the genetic test is always quoted as one reason that molecular diagnosis is not yet offered to large populations, but since the test is only performed once in a lifetime and unlimited relatives can then be identified at low cost, the health benefits are considered by some countries, including the Netherlands and some provinces of Spain, greater than the cost of the test itself [12, 13]. It is important to note that the cost of the genetic test is comparable to the cost of nuclear magnetic resonance (NMR), an exam that is routinely prescribed for different diseases and usually more than once in a lifetime and does not usually improve the patient's prognosis as the genetic test for FH does.

The Portuguese FH Study has been successfully implemented since 1999 and has already genetically identified 404 individuals (171 index patients and 233 relatives) among more than one thousand individuals sent for study. A total of 78 different mutations in the LDL receptor (LDLR) gene were found in 171 index patients, 2 different mutations were found in the apoB gene of 4 patients and 2 patients had a unique PCSK9 mutation [14]. In Portugal the molecular diagnosis of FH has been funded by research grants from the Portuguese Cardiology Society and the Science and Technology Foundation.

The main aim of this project was to determine whether clinical characterization is sufficient to identify these individuals at high risk of developing CHD and to evaluate the clinical applicability of molecular diagnosis for FH.

METHODS

All patients described in this study were recruited for the Portuguese FH Study as previously described [9]. The diagnostic criteria used to select the index patients were adapted from the Simon Broome Heart Research Trust [1, 9].

To analyze the usefulness of molecular diagnosis, graphs of total and LDL cholesterol values by age were constructed for 622 possible FH patients. The lipid profile of patients genetically identified as having FH, before and under medication, were analyzed to assess whether these patients were receiving appropriate treatment. The data are shown separately for children and adults and for female and male propositi (index cases and hypercholesterolemic relatives), both with and without a detectable mutation in the LDLR gene.

Biochemical characterization

The biochemical tests for total cholesterol, LDL cholesterol, HDL cholesterol, lipoprotein (a), apolipoprotein AI and apolipoprotein B were determined as described elsewhere [9].

Analysis of biochemical data

In this study all index and relatives with a clinical diagnosis of possible FH were included, in a total of 696 patients. Graphics were generated using patients' and relatives' biochemical data before treatment, to compare values between possible FH patients (index cases and relatives), with and without a

mutation in the LDLR gene. For diagnostic purposes pre-treatment cholesterol values are the most important, and so for this analysis only cholesterol values of 333 index patients and 289 possible FH relatives were used, since only these patients knew their lipid values before treatment. Since most FH patients have a mutation in the LDLR gene, only patients with a mutation in this gene were analyzed in this study.

All data were analyzed using SPSS software (version 17.0). The t-test was used to test for the difference between means. When this could not be used a non-parametric test was used (Mann-Whitney).

For all tests $p<0.05$ was considered statistically significant.

RESULTS

To date, 482 index patients and 858 relatives (hypercholesterolemic and normolipidemic individuals) have been sent to the Portuguese FH Study. From these, 359 index patients have their study of the LDLR, apoB and PCSK9 gene completed as described elsewhere [14, 15] and the others are under study. The characterization of the 696 possible FH patients presented in this study is represented in Table I.

Clinical versus molecular diagnosis

To analyze the usefulness of molecular diagnosis, graphs of total and LDL cholesterol values by age were constructed for 622 possible FH patients. The data are shown separately for female propositi (index cases and hypercholesterolemic relatives), both with and without a detectable mutation in the LDLR gene, and for male propositi (*Figure 1*). Statistical analysis revealed that there are significant differences between total cholesterol ($p<0.001$) and apoB ($p=0.026$) values in the group of children (male and female) with and without a mutation in LDLR. For female children LDL values were also significantly different ($p<0.001$) between subgroups, but for male children this difference did not reach statistical significance. In adult women there is a statistically significant difference for total cholesterol ($p=0.049$), LDL cholesterol ($p=0.031$), and apoB ($p=0.003$) in the subgroups with and without an LDLR mutation. In adult males there is a statistical difference for total cholesterol ($p=0.002$). LDL cholesterol ($p=0.003$), and apoB ($p=0.023$) in subgroups with and without an LDLR mutation. Nevertheless there was considerable dispersion of values and it is not possible to distinguish individually between patients with and without a mutation in the LDLR gene, based only on lipid profile.

Table 1. Clinical and biochemical characterization of 696 possible FH patients presented in the study. Only pre-treatment values were considered.

	n	Pediatric group	n	Adult group
Age (years)	208	9.84±3.63	488	44.63±13.90
Female (%)	99	47.1	277	56.8
Male (%)	109	52.4	211	43.2
TC (mg/dL)	205	273.99±62.69	417	338.18±69.81
LDL (mg/dL)	174	201.93±60.31	300	250.92±72.19
HDL (mg/dL)	81	54.46±15.40	247	54.57±17.98
TG (mg/dL)	82	131.25±85.39	241	128.63±72.83
apoB (mg/dL)	144	110.29±39.00	124	155.36±49.19
apoAI (mg/dL)	144	144.15±33.33	128	151.84±32.65
Tendon Xanthomas (%)	208	0.0	488	2.5
CHD (%)	208	0.5	488	16.3
Under medication (%)	208	20.2	488	60.5

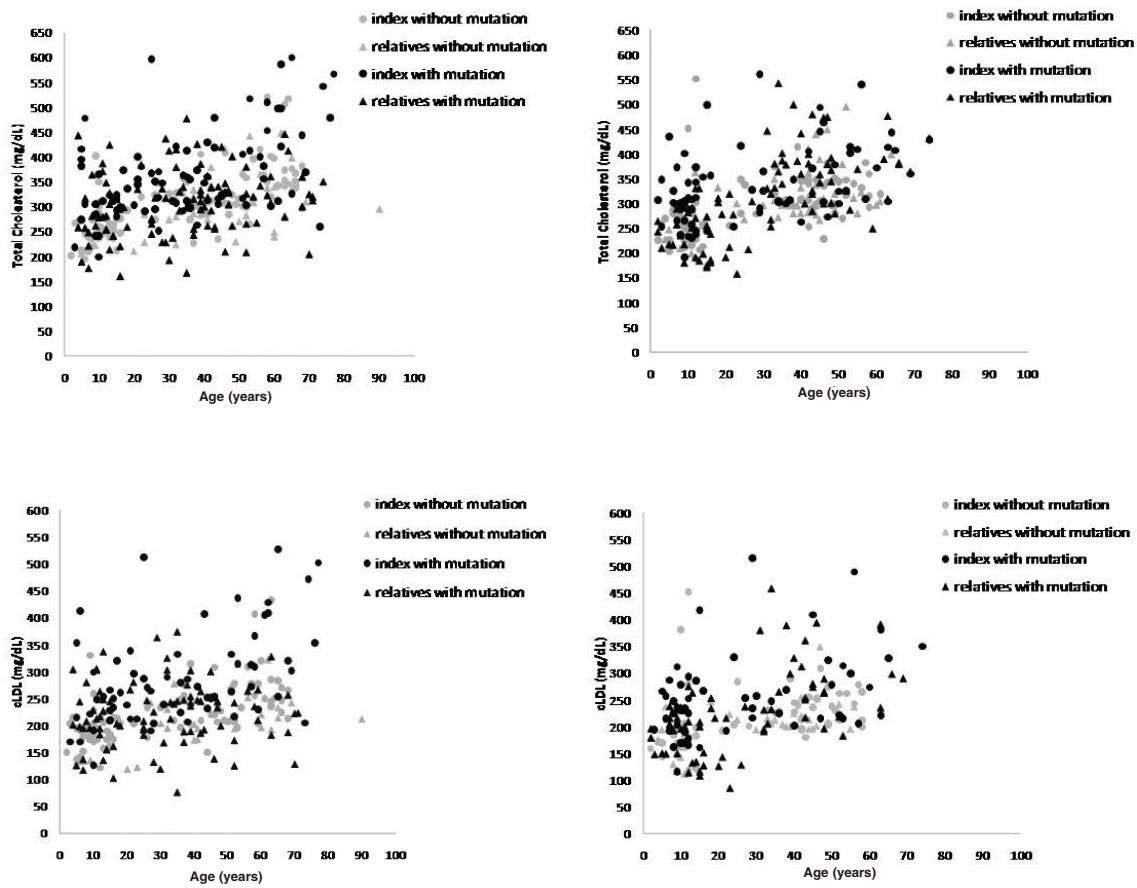

Figure 1. Clinical versus molecular diagnosis. a) Total cholesterol vs. age in females (index and relatives, with and without mutation); b) Total cholesterol vs. age in males (index and relatives, with and without mutation); c) LDL cholesterol (LDL-C) vs. age in females (index and relatives, with and without mutation); d) LDL cholesterol vs. age in males (index and relatives, with and without mutation).

Clinical applicability of molecular diagnosis

From the above results we can assume that FH patients can only be correctly identified when molecular diagnosis is performed. Since the early identification of FH patients is important to determine the therapeutic regime for each patient, and it has been shown that only molecular diagnosis can correctly identify FH patients, the lipid profile of patients genetically identified as having FH, before and under medication (with a statin and with a statin and ezetimibe) were analyzed to assess whether these patients were receiving appropriate treatment (*Figure 2*). As can be seen in Table I, only 60.5% of the adult patients are receiving any kind of treatment, namely 81% index cases and 42.3% of the relatives. It can be seen in Figure 2 that mean total cholesterol and LDL cholesterol values under treatment are above the limits recommended by the European Cardiology Society, especially in patients under treatment with a statin alone. Of these, only 3.3% had total cholesterol levels (2.2% for LDL-C) below the recommendations for the general population, and only 2.9% reached the target for total cholesterol (0.8% for LDL-C) recommended for high-risk patients. These results were not considered strange since from analysis of the clinical questionnaires, most FH patients are being treated with low statin doses. Only a small percentage of patients are receiving combined therapy, and even in this group only 9.8% have reached the target. In the group of patients taking a statin alone,

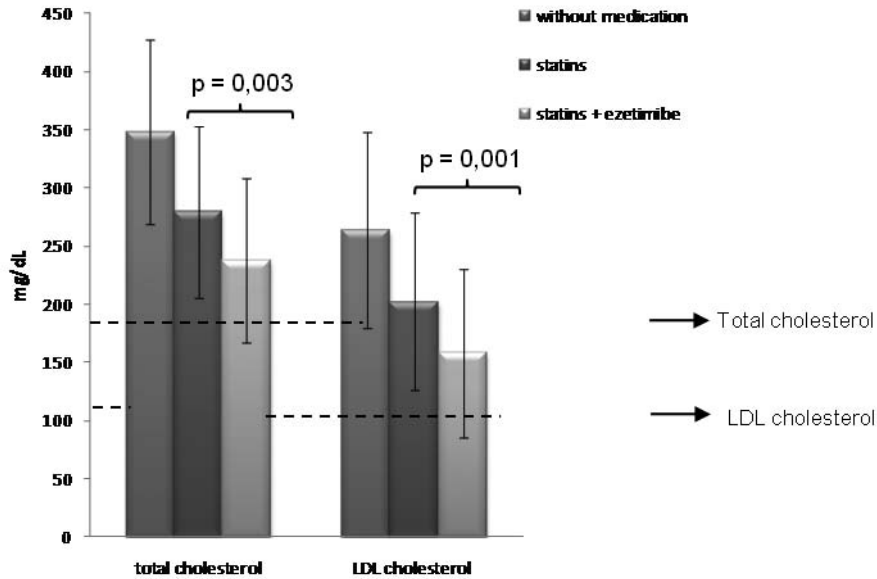

Figure 2. Comparison of patients without medication and taking statins and ezetimibe.

total cholesterol levels decreased by only 20% and LDL cholesterol by only 23%. In patients taking a statin and ezetimibe total cholesterol decreased in 32% and LDL cholesterol in 40%. These results could be improved if a potent statin were prescribed to all these patients, instead of a low dose of a first-generation statin, as is currently recommended by the international experts on FH management [5, 6]. These results show that most FH patients have not complied with the recommendations of the European Cardiology Society or of NICE for FH patients. These numbers also show that these FH patients are not being treated as having a genetic disorder, making molecular diagnosis even more important as a cardiovascular risk stratification tool.

Molecular diagnosis and patient prognosis

In order to illustrate the subject, below we present four clinical cases that well exemplify the impact that molecular diagnosis can have on patient prognosis and attitude towards the severity of the disease.

Clinical case 1

Patient 1 is a 58-year-old man, with multiple risk factors for cardiovascular disease: dyslipidemia, smoking (60 pack/years), physical inactivity, overweight (BMI=28) and family history of premature coronary disease, as well alcohol consumption (60 g/day). He has a history of inferior myocardial infarction at age 38, non-ST-elevation myocardial infarction at age 42 and coronary arterial bypass graft at age 43 years (triple bypass). In spite of his clinical history, the patient did not comply with the lifestyle change measures and therapeutic approach proposed. At age 49 the patient returned to the hospital's cardiology out-patient clinic, with complaints of Canadian Cardiology Society class II angina. The patient repeated cardiac catheterization that highlighted progression of the disease, and an angioplasty of one of the bypasses was required. His lipid profile was the following: total cholesterol, 327 mg/dL; LDL cholesterol, 216 mg/dL; HDL cholesterol, 48 mg/dL; apoAI, 110 mg/dL; apoB, 240 mg/dL; Lp(a) 108 mg/dL; and triglycerides 313 mg/dl. Given his personal and family history of dyslipidemia and premature coronary disease, this patient was enrolled in the Portuguese FH Study. The genetic study showed the existence of a mutation in exon 9 of the LDLR gene (p.Ala431Thr, formerly A410T) [15]. The family study was performed and it was confirmed that the mutation co-segregated in family members who are hypercholesterolemic (*Figure 3A*). After discovering he had a genetic disorder

and understanding his cardiovascular risk, the patient expressed concern about his daughter, who had inherited his mutation. For both, index patient and daughter, the cardiologist prescribed a moderate dose of a potent statin. At present, the index patient and his daughter are complying with the lifestyle measures and prescribed pharmacological therapy, and are clinically asymptomatic.

Clinical case 2

Patient 2 is a 39-year-old woman followed at the lipid clinic since 1997 due to refractory hypercholesterolemia. Since her father was also hypercholesterolemic (*Figure 3B*), she was enrolled in the Portuguese F Study in 2001.

Her lipid profile at the time was the following: total cholesterol 490 mg/dL; LDL cholesterol 435 mg/dL; HDL cholesterol 42 mg/dL; apoAI 117 mg/dL; apoB 245 mg/dL and Lp(a) 100 mg/dL; triglycerides within the normal range. The molecular study revealed that the patient was compound heterozygous, with two distinct mutations (p.Ala431Thr, formerly A410T, and c.313+6C/T), each in a different allele.

After these results were sent to her cardiologist, an extensive range of exams were performed. Clinical studies including ECG, echocardiogram, ECG exercise testing and Achilles tendon ultrasonography were performed and were normal. Carotid and

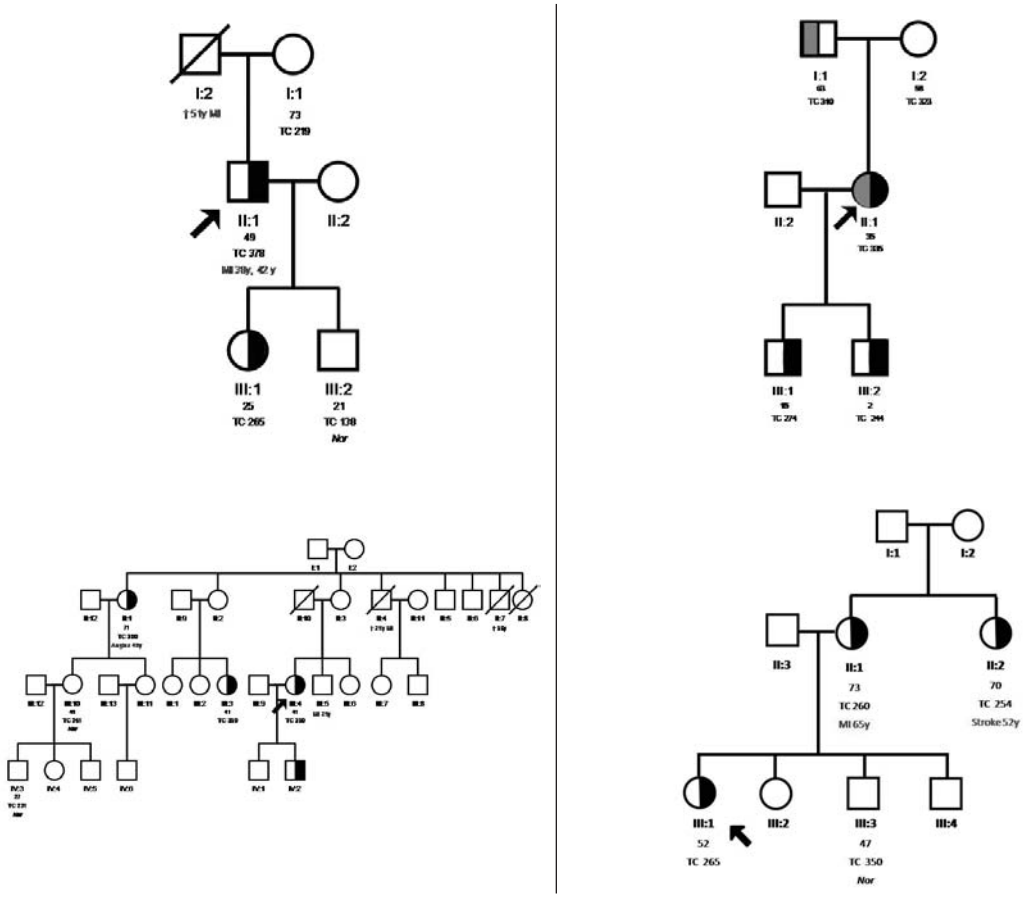

Figure 3. Pedigrees of clinical cases. (A) Clinical case 1. The arrow indicates the index patient; half-filled black symbols represent individuals heterozygous for the mutation p.Ala431Thr; Nor, individuals in whom the family mutation was not found. (B) Clinical case 2. The arrow indicates the index patient; half-filled black symbols represent individuals heterozygous for the mutation p.Ala431Thr; half-filled gray symbols represent individuals heterozygous for the mutation c.313+6 C>T; Nor, individuals where the family mutation was not found. (C) Clinical case 3. The arrow indicates the index patient; half-filled black symbols represent individuals heterozygous for the mutation c.190+4insTG; Nor, individuals in whom the family mutation was not found. (D) Clinical case 4. The arrow indicates index patient; half-filled black symbols represent individuals heterozygous for the mutation c.1359-5C>G; Nor, individuals in whom the family mutation was not found. Below each symbol are generation numbers followed by age and pre-treatment total cholesterol levels in mg/dL. Abbreviations: †, deceased; MI, myocardial infarction; TC, total cholesterol.

femoral arteries were studied with duplex ultrasonography and intimal-medial thickness (IMT) evaluated. IMT was normal in the primitive carotid arteries and increased in the common femoral artery. The clinical phenotype presented by this patient is not that described for homozygous FH patients, nevertheless the molecular diagnosis indicates why this patient did not respond to any treatment available at the time. Due to her molecular diagnosis, LDL apheresis was initiated, but since she had to travel 200 km twice a month for the treatment, was in employment and had to take care of two children, this was not practical. The cardiologist then prescribed a moderate dose of a potent statin and 10 mg of ezetimibe; the patient has begun to respond to treatment, her lipid profile at present being as follows: total cholesterol, 251 mg/dL; LDL cholesterol, 181 mg/dL; HDL cholesterol, 50 mg/dL; apoAI 164 mg/dL; apoB 125 mg/dL and Lp(a) 98 mg/dL; triglycerides within normal range. This molecular finding reveals that many individuals are probably being under-diagnosed; many individuals at risk are not being correctly identified and have an even greater risk of developing premature CHD. Since this patient has been followed for many years by her cardiologist, who is aware of her increased cardiovascular risk, it is probable that, if she continues to take the medication, she will not suffer premature heart disease.

Clinical case 3

Patient 3 is a woman who at 40 years of age sought the advice of a medical geneticist with concerns about her health due to a strong family history of premature CHD, in some cases fatal. Her mother had a myocardial infarction at age 40 and triple coronary bypass/CAGB at age 52, and the family history revealed several premature deaths in maternal uncles (myocardial infarction in individuals between 29-40 years old) (*Figure 3C*).

Even though the patient had been informed that she had high cholesterol levels, she was never advised to take medication until the present consultation.

The lipid profile, at the time of her consultation, was the following: total cholesterol, 354 mg/dL; LDL cholesterol, 211 mg/dL; HDL cholesterol, 37 mg/dL; apoAI 112 mg/dL; apoB 109 mg/dL and Lp(a) 82 mg/dL; triglyceride levels within normal ranges. Echocardiogram and carotid ultrasound with intima-media complex measurement were normal. Physical examination showed no evidence of xanthomas or xanthelasma. The patient was enrolled in the Portuguese FH Study, which revealed the presence of the c.190+4insTG mutation at the 5' end of intron 2 of the LDLR gene. This insertion led to retention of two nucleotides of intron 2. This alteration creates a premature stop codon and consequently a truncated LDLR protein of 206 amino acids that is not functional (14). Other family members were asked to perform the genetic test to confirm their hypercholesterolemia due to an LDLR mutation but not all agreed, even when the benefits were explained of early detection of a genetic disease that is treatable and, when patients receive the correct treatment the progression of CHD can be slowed or even stopped.

After confirmation by molecular analysis that the patient had FH, and the mutation encountered was predicted to produced a severe phenotype, she was placed on a strict diet with reduced fat and cholesterol intake and daily exercise in addition to lipid--lowering therapy of a moderate dose of statin, ezetimibe and acid acetylsalicylic. After 12 months of treatment, total cholesterol levels had decreased to 184 mg/dL; LDL cholesterol, 124 mg/dL; HDL cholesterol, 76 mg/dL; and triglyceride levels were within normal ranges. The patient was encouraged to maintain the lifestyle and treatment prescribed. If she does, her cardiovascular risk can be reduced substantially and she will most probably not develop premature CHD like many members of her family, since at present she shows no evidence of atherosclerotic disease.

Clinical case 4

Patient 4 is a 55-year-old woman with a clinical diagnosis of familial hypercholes-

terolemia, presenting a strong family history of CHD: her mother had a myocardial infarction at 65 years of age and her maternal aunt had a severe stroke at age 52 (*Figure 3D*). The patient was aware of her condition since she was 35 years old and had been treated with different statins but had never reached the target values. On her own initiative, she sought the advice of a medical geneticist due to her family history of premature cardiovascular disease and because she was aware of the difficulty of treating her hypercholesterolemia.

At 50 years of age, under treatment with a first-generation statin, her lipid profile was the following: total cholesterol 265 mg/dL; LDL cholesterol 183 mg/dL; HDL cholesterol 70 mg/dl; and triglyceride levels were within normal ranges. Echocardiogram and carotid ultrasound with intima-media complex measurement were normal. Physical examination showed no evidence of xanthomas or xanthelasma. She was enrolled at the time in the Portuguese FH Study, and a splicing mutation in the LDLR gene was found that co-segregated in the family with hypercholesterolemia, except in a brother with hypertriglyceridemia and hypercholesterolemia, overweight and with "social" alcohol consumption (*Figure 3D*). The mutation found is the c.1359-5C>G substitution, leading to retention of intron 9. RNA studies show that this alteration is predicted to result in a protein with two additional incorrect amino acid residues after those encoded by exon 9 followed by a stop codon and consequently a non-functional truncated protein of 454 amino acids (16). After ascertaining the severity of the mutation that the patient carried, the clinician prescribed a diet with restricted fat intake, daily exercise and walking, a moderate dose of a potent statin, ezetimibe and ezetimibe and acid acetylsalicylic. After 12 months of treatment, total cholesterol decreased to 144 mg/dL, LDL cholesterol to 87 mg/dL, HDL cholesterol to 87 mg/dL and triglyceride levels were within normal ranges. Since total cholesterol values had decreased more than sufficiently, the statin dose was adjusted to decrease possible secondary effects. Again, if the patient maintains the medication and lifestyle advice, her cardiovascular risk will reduce substantially.

DISCUSSION

Clinical versus molecular diagnosis

To identify a patient as having FH can be straightforward when, for example, the patient has severe hypercholesterolemia, presents tendon xanthomas, already has premature heart disease and also has a family history of hypercholesterolemia and premature heart disease in relatives. However, the majority of Portuguese FH patients do not present all these clinical characteristics and in these cases clinical diagnosis can be difficult. Even when an index patient is identified as having inherited hypercholesterolemia based on convincing clinical criteria, it is doubtful whether all relatives at risk can also be identified by clinical criteria alone. Frequently, some of these relatives, especially if they are young, can have borderline cholesterol values and may not show other clinical signs, which will prevent a diagnosis of FH based solely on clinical evidence. On the other hand, some individuals may have increased cholesterol due to environmental factors and can be misdiagnosed as having FH. In these cases molecular diagnosis can be useful. Figure 1 clearly illustrates these situations. The total cholesterol and LDL values in the two groups analyzed are significantly different but the figures show that it is difficult to distinguish between patients with and without a mutation in the LDLR gene based only on clinical evidence. However, penetrance is high, with virtually all mutation carriers having total cholesterol and LDL cholesterol levels well above the recommended levels for the normal population. At adult age several factors can influence cholesterol values, and only genetic testing can give an accurate diagnosis of the origin of the hypercholesterolemia. In children it can be argued that environmental factors will not have had time to play a role in the development of hypercholesterolemia, and so

what is seen is a true phenotype, which in some cases can be used to guess the genotype. Nevertheless, even when a statistical difference is observed, the information this adds may be limited. From the graphic analysis it can be seen that the values overlap in these two groups, making it difficult to distinguish between individuals with and without the mutation. The fact that about 40% of children in the Portuguese FH Study do not have a mutation in the LDLR gene confirms that clinical data alone are not sufficient to determine the cause of the hypercholesterolemia, even in children [14, 15].

In a study in Denmark [16], 76% of mutation carrier relatives had LDL-C levels above the 90th percentile, although 15% of non-mutation carrier relatives also had levels above this cut-off. However, this means that a simple LDL-C measure will not distinguish adequately between mutation carriers and non-carriers. Data from the Dutch data set of over 2000 mutation-positive and negative FH relatives demonstrates that this overlap increases with age, due to the general increase in LDL-C levels seen in normal people as they age, such that by the age of 45-55 years, the false negative rate using the inflection between the LDL-C distribution in mutation carriers and non-carriers is ~45%. The conclusion from these data is that when a relative has inherited an FH-causing mutation, the penetrance is 100%, although other relatives who have not inherited the family mutation may also have elevated LDL-C for polygenic or environmental reasons (e.g. a poor diet) [17].

One of the questions that this work aimed to address was whether molecular diagnosis is indeed necessary to properly assess the CHD risk of a patient already diagnosed with hypercholesterolemia. Evidence has shown that even for a hypercholesterolemic adult who already attends a lipid clinic and is receiving cholesterol-lowering medication, molecular diagnosis can focus more attention on the aggressiveness of the medication, since most patients do not reach the desired targets. Individuals with inherited hypercholesterolemia need more effective treatment than those who only become hypercholesterolemic in middle age, since they have been accumulating cholesterol since birth. Another benefit of molecular diagnosis is the early identification of patients and relatives at high risk of developing CHD, especially children and adolescents whose hypercholesterolemia may otherwise be identified much later in life, sometimes only when the first cardiovascular problems appear. If these children are identified early in life preventive interventions can be made to change lifestyle (and initiate therapy when advisable) to decrease the elevated cardiovascular risk of these children. It is well known that a lifelong change to target cholesterol levels can dramatically reduce the cardiovascular risk of the general population [18] and this also applies to these patients.

Molecular diagnosis for FH - Applicability to clinical practice

FH fulfils the World Health Organization criteria for screening programs (10). Since the introduction of statins (HMG-CoA reductase inhibitors) in the last decade, the prognosis of FH patients has improved substantially and therefore it is considered appropriate to perform systematic screening for FH [20]. Some countries, such as the UK [21] [22] and the Netherlands [23, 24], have established a specialized genetic service for FH and in recent years a cascade screening program for FH has begun. The LDLR gene has been extensively studied and the regions where mutations are most common are now known, and so a strategy can be designed. During recent years the technology has also improved immensely and new methods, such as denaturing high-performance liquid chromatography (DHPLC) and heteroduplex analysis by capillary electrophoresis [25], have proved to be less time-consuming and more cost-effective than other methods and can be used successfully to screen for mutations in the LDL receptor gene. Studies in the cost-effectiveness of performing genetic analysis for FH indicates that screening of 16-year-old

adolescents is the most cost-effective case-finding strategy, followed by family tracing and screening of patients admitted to hospital with premature myocardial infarction [20, 26].

Information on the pathogenicity of each mutation from functional assays is also important to identify patients at an even higher risk of developing CHD and to stratify their cardiovascular risk.

In general, molecular diagnosis of FH is feasible and can be obtained in 1-2 months if the correct technology is available. In Portugal the test will be offered to the population by our Institute at a cost of about 500 euros, like many other genetic tests or exams such as NMR.

Other authors argue that it is cost-effective to identify FH patients through molecular diagnosis, especially those identified by cascade screening once the index case is analyzed. The London IDEAS FH cascade audit project examined the cost of finding and testing an index case. The average cost was £500 (with a range of £330-£2,470 between clinics). Based on their findings and the national FH screening program in the Netherlands, they concluded that cascade testing for FH was highly cost-effective, with an estimated cost of US$8,700 per life year gained (discounted) and so well below the £20,000 per QUALY used by NICE as a benchmark for cost-effectiveness [27].

Ethical issues related to performing a genetic test for a disease are usually more problematic if a treatment for that disease does not exist and so no benefits can be derived from an early diagnosis. FH is a disease for which, fortunately, effective treatment exists and can be offered to patients, thus improving their health and quality of life. The sooner the disorder is diagnosed the better the patient's prognosis. Nowadays, an FH patient correctly diagnosed and treated at an early age may never have CHD [28]. At present, cardiovascular disease is still the leading cause of death in developed countries and the associated morbidity is also substantial. So, in general, it can be said that there are no negative ethical implications of performing genetic testing for FH and, in fact, it can be beneficial for the health of the patient and in reducing the state's treatment burden and hospitalization costs associated with cardiovascular events. In the case of FH, it is thought that 75–90% of at-risk individuals remain unaware of their risk. As the penetrance of FH is very high, intervention may be justified for a substantial proportion of these people since, with potent statins alone or in combination therapy, their risk could be reduced to near the average population risk for coronary heart disease.

CONCLUSIONS

The genetically identified FH patients from the Portuguese FH Study are now receiving counseling and treatment based on their diagnosis, and it is expected that some, especially younger individuals, will never develop premature CHD due to the early detection of their disease through molecular diagnosis. Molecular study has thus helped to improve patient prognosis. Molecular diagnosis also helps to determine the appropriate therapeutic regime for each patient and functional assays identify patients at even higher risk of developing CHD, stratifying the cardiovascular risk for each patient. None of the patients in whom no mutation was found in the LDL receptor gene ceased their medication, as this was not the aim of the study, and the fact that no mutation was found in this gene does not exclude the possibility of hypercholesterolemia due to other gene defects. Nonetheless, several patients saw their medication changed to a more effective therapeutic regime due to a mutation being found in their LDLR gene. Clinicians also stated that patients who were usually lax about taking their medication, after obtaining a molecular diagnosis, understood better that they had a chronic disorder that needed chronic treatment and made an effort not to forget to take the medication.

Unfortunately there are social and economic limitations that lead to patients to givegiving up

their medication at some point in their treatment, and about which scientists and clinicians can do nothing. The state contribution to the expensive medication for FH, such as statins and ezetimibe, is only 40% compared to medication for other chronic or molecular disorders such as diabetes mellitus or cystic fibrosis, for which, in most cases, the costs are almost 100% covered by the state. In Spain, due to the intervention of scientists and clinicians working together with interested politicians, the government decided that FH patients would have all medication they needed, paying only a small fee per month.

They presented calculations that it was more cost-effective to identify and treat these patients at an early age (thus preventing coronary events) than to perform angioplasty or heart surgery later in life[27].

A last consideration is that clinicians, especially pediatric cardiologists, pediatricians, cardiologists, nephrologists, endocrinologists, internists, general physicians and medical geneticists, should all be aware of the benefits of the early identification of these patients and make use of molecular diagnosis to correctly identify the cause of hypercholesterolemia presented by their patients and to assess and treat their cardiovascular risk.

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