

Technical advance

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## Detection of large deletions in the LDL receptor gene with quantitative PCR methods

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### Abstract

**Background:** Familial Hypercholesterolemia (FH) is a common genetic disease and at the molecular level most often due to mutations in the LDL receptor gene. In genetically heterogeneous populations, major structural rearrangements account for about 5% of patients with LDL receptor gene mutations.

**Methods:** In this study we tested the ability of two different quantitative PCR methods, i.e. Real-Time PCR and Multiplex Ligation-Dependent Probe Amplification (MLPA), to detect deletions in the LDL receptor gene. We also reassessed the contribution of major structural rearrangements to the mutational spectrum of the LDL receptor gene in Denmark.

**Results:** With both methods it was possible to discriminate between one and two copies of the LDL receptor gene exon 5, but the MLPA method was cheaper, and it was far more accurate and precise than Real-Time PCR. In five of 318 patients with an FH phenotype, MLPA analysis revealed five different deletions in the LDL receptor gene.

**Conclusion:** The MLPA method was accurate, precise and at the same time effective in screening a large number of FH patients for large deletions in the LDL receptor gene.

### Background

Familial Hypercholesterolemia (FH) is clinically characterised by elevated concentrations of LDL-cholesterol in plasma, tendon xanthomas and an increased risk of developing coronary artery disease inherited in an autosomal dominant manner [1]. At the molecular level FH is most commonly due to mutations in the LDL receptor gene, in which more than 900 different mutations in the LDL

receptor gene have been reported worldwide [2,3]. Approximately 90 of them are various major structural rearrangements such as deletions and insertions of sizes ranging from 37 bp to 25 kb [2,3]. Major structural rearrangements account for approximately 5% of identified mutations in the LDL receptor gene in genetically heterogeneous populations [1,4,5]. In Denmark four different deletions in the LDL receptor gene have been described

[6-8]. They were found in four of 97 patients tested [8]. Southern blotting, followed by hybridisation with LDLR probes, has been used to screen for major structural rearrangements [6], but this method is labour-intensive and requires large amounts of DNA. Alternatively long-range PCR analysis has been used to amplify segments of the LDL receptor gene up to approximately 20 kb in lengths [9,10]. The major drawback of long-range PCR is that the deletion or duplication has to be within the borders of the primers of the amplified segment, because the allele containing the deletion or duplication will otherwise not be amplified, and the analysis result will falsely appear to be homozygous wild-type (normal). With quantitative PCR methods, it is possible to relate the number of allele copies to that of appropriate controls independently of the extent of the deletion /insertion. Only small amounts of DNA are required, and the methods are faster than Southern blotting analysis.

This study aimed at testing and comparing the abilities of two quantitative PCR methods, i.e. Real-Time PCR and Multiplex Ligation-dependent Probe Amplification (MLPA) for detection of deletions in the LDL receptor gene in a group of patients in whom deletions in the LDL receptor gene had been demonstrated by Southern blotting or long-range PCR analyses. If one of the methods proved valid and effective, the study also aimed at re-estimating the relative contribution of major structural rearrangements to the spectrum of LDL receptor gene mutations in FH patients in Denmark.

## Results

For testing and comparing Real-Time PCR and MLPA, seventeen patients with a deletion of LDL receptor gene exon 5 were used as positive controls. The deletion of exon 5 was due either to a 9 kb deletion of exons 3-6 (14 patients from one family) or to a 1 kb deletion only of exon 5 (three patients from two families) described by Rüdiger et al and Jensen et al [6,8]. These mutations had been demonstrated earlier by either Southern blotting or long-range PCR analysis. Twelve individuals from the family with the 9 kb deletion that have been tested negative for the deletion were used as negative controls.

### Real-Time PCR

Real-Time PCR is a quantitative PCR method, which quantifies the molecular concentration of DNA that can serve as template for amplification [11]. The amplification progress was measured by binding of SYBR Green to double stranded DNA. SYBR Green is an intercalating dye, the fluorescence of which is higher in the bound than in the free-state [12]. The fluorescence signal is measured real-time in the extension phase of the PCR reaction cycle, and the measurement, proportional to the amount of double stranded DNA, is plotted as an amplification curve against

cycle number. A threshold value of fluorescence in the exponential part of the amplification curve is selected, and for each sample the number of cycles needed for the signal to reach the threshold is measured (threshold cycle ( $C_T$ )).

Quantification of the molecular concentration of template DNA was performed with the standard curve method [13]. Data analysis was performed with the iCycler™ iQ Optical System Software Version 3.0a (BIO-RAD), and the results were exported to Excel sheets for storing and further processing. The copy number of exon 5 in the LDL receptor gene was divided by the copy number of the reference gene (albumin). The ratio is 2:2 (i.e. 1) if both alleles of both genes are present, and the ratio is 1:2 (i.e. 0.5) if one of the alleles of the target gene is absent. In contrast, the ratio is 3:2 (i.e. 1.5) if one of the alleles of the target gene has been duplicated.

### Multiplex Ligation-dependent Probe Amplification (MLPA)

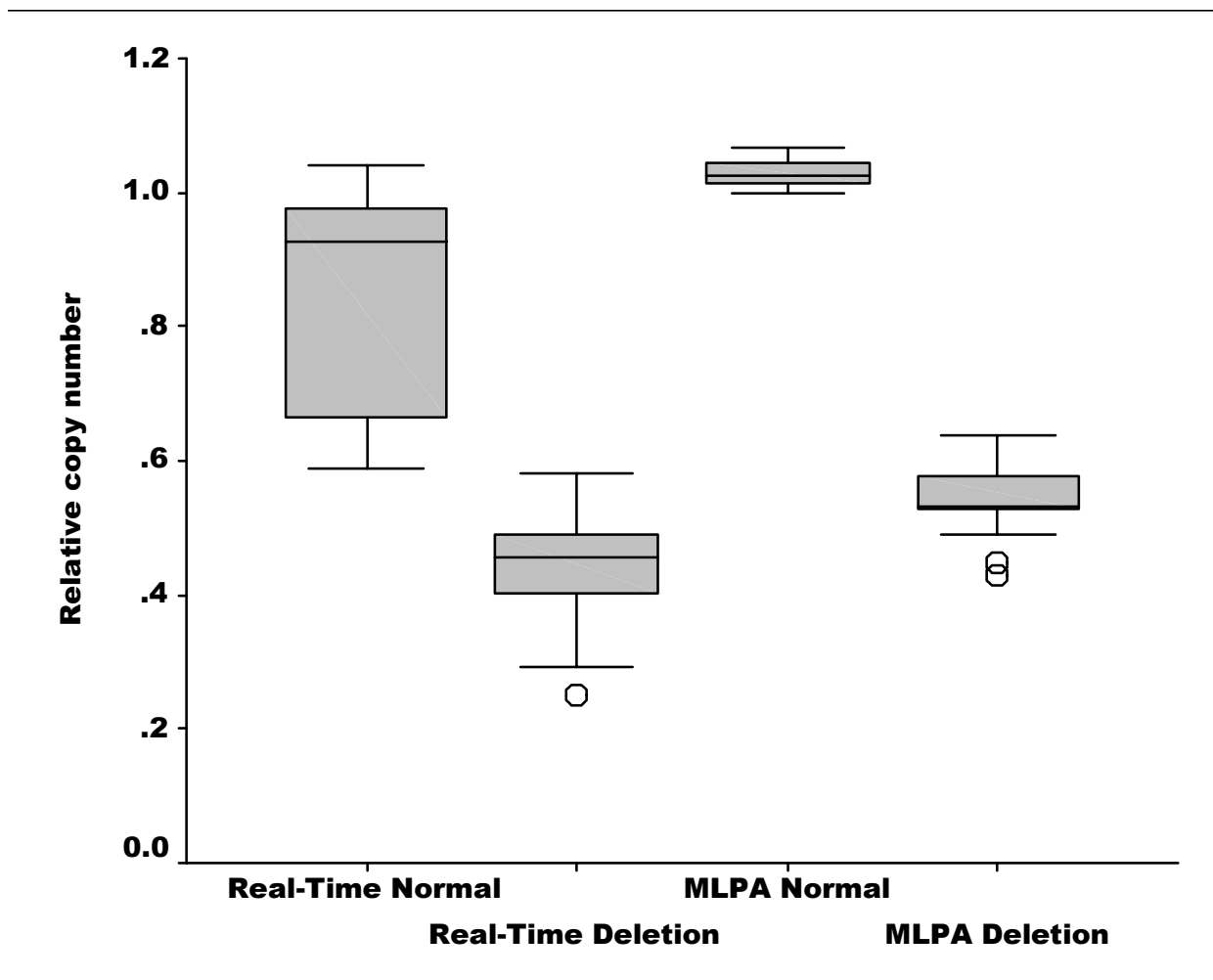
With the MLPA method developed by MRC-Holland [14], relative quantification of up to 40 different DNA sequences in one reaction is possible. Each MLPA probe consists of two fluorescently labelled oligonucleotides that can hybridise, adjacent to each other, to a target gene sequence. When hybridised, the two oligonucleotides are joined by a ligase, and the probe can then be amplified by PCR. To each probe is attached a set of universal primers, and one of the oligonucleotides contains a stuffer sequence of variable length that enables us to separate the single fragments according to their length by gel electrophoresis [14,15]. The area under peak for each fragment was measured with the GeneScan Analysis Software Version 3.1.2 and Genotyper Software Version 2.5 (Applied Biosystems) and exported to Excel sheets for storing and for further processing. The peak area was normalised by dividing it by the combined area of all peaks in that lane. This normalised peak area was then divided by average normalised peak area from five normal control subjects. With this method the results are given as allele copy numbers as compared to normal controls, and a ratio of 1 is obtained if both alleles are present, a ratio of 0.5 if one allele is absent, and a ratio of 1.5 if one allele is duplicated.

A comparison of Real-Time PCR to MLPA for detection of known deletions of LDL receptor gene exon 5 is given in Table 1 and illustrated in Figure 1. With both Real-Time PCR and MLPA analysis, it was possible to distinguish between one and two copies of the LDL receptor gene exon 5, but the coefficients of variation were larger for the Real-Time PCR method than for the MLPA analysis. The results of the MLPA analysis of 16 out of 18 exons in 14 patients with a 9 kb deletion of exons 3-6 are shown in Figure 2 as wells as the results for 12 negative controls.

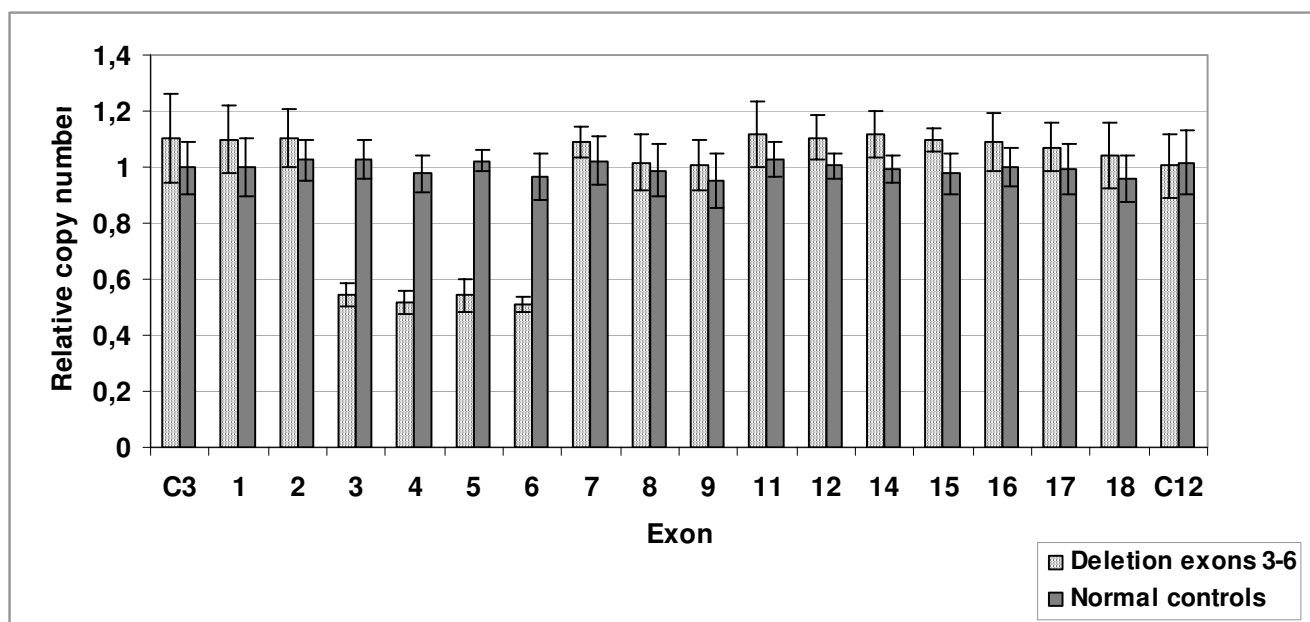
**Table 1: Relative copy number of LDL receptor gene exon 5 measured with Real-Time PCR and MLPA**

Method	Sample population	N	Mean	SD	CV (%)
Real-Time PCR	Normal	12	0.84	0.17	20
	Deletion	17	0.44	0.097	22
MLPA	Normal	12	1.03	0.02	2
	Deletion	17	0.54	0.05	10

Mean relative allele copy number in 12 normal individuals and in 17 individuals with deletion of exon 5. SD: standard deviation. CV (%): coefficient of variation in percent.



**Figure 1**  
**Relative copy number of LDL receptor gene exon 5.** Boxplots of relative copy number of LDL receptor gene exon 5 measured with Real-Time PCR Analysis and MLPA analysis showing median; box: 25<sup>th</sup>-75<sup>th</sup> percentile; bars: largest and smallest values within 1.5 box lengths; circles: outliers.



**Figure 2**  
**Mean relative copy numbers of exons in the LDL receptor gene.** Mean relative allele copy number  $\pm$  SD of LDL receptor gene exons 1–9, exons 11–12, exons 14–18, 5' (C3) and 3' (C12) flanking control fragments in 14 patients with a 9 kb deletion of exons 3–6 measured with MLPA analysis compared to mean relative copy number in 12 normal controls.

**Table 2: Cost-analysis for detection of major structural rearrangements in the LDL receptor gene with quantitative PCR methods**

	Real-Time PCR	MLPA
Price reagents (in euros)	1120	110
Time laboratory technician (hours)	56	14

Data are given for a batch of 10 samples tested in 16 exons in the LDL receptor gene with the Real-Time PCR method and with the MPLA method. Costs of data analysis and interpretation of results were the same for the two methods.

A cost-analysis and comparison of Real-Time PCR analysis and MLPA analysis are given in Table 2. The cost analysis included costs of reagents and laboratory technician time. When 10 samples were analysed together, the MLPA method was considerably cheaper as well as faster than the Real-Time PCR method.

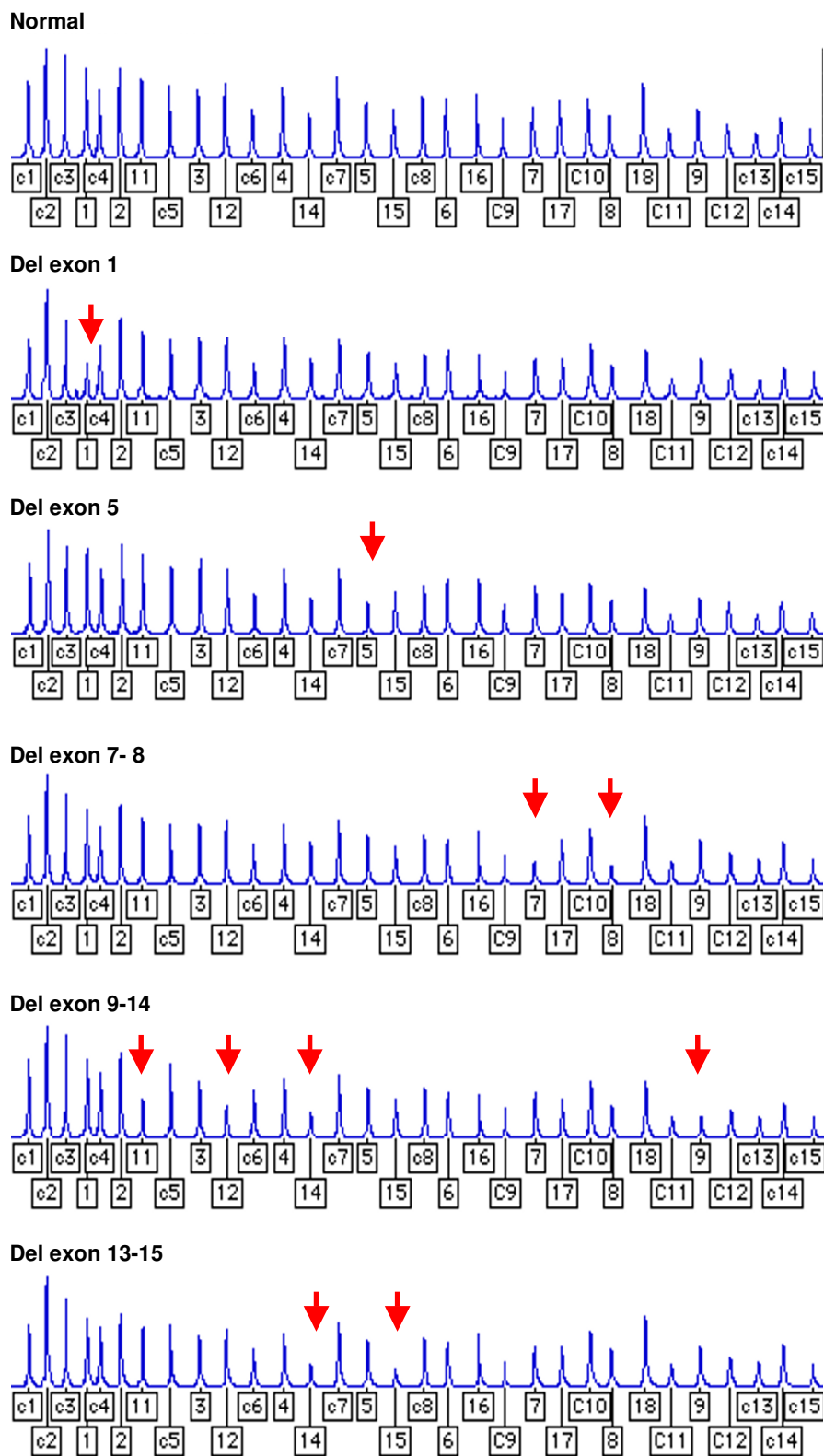
To expand our assessment of the contribution of major structural rearrangements in the LDL receptor gene to the spectrum of mutations causing FH [8], we further studied, by MLPA analysis, 318 patients with an FH phenotype referred for molecular genetic analysis to Aarhus Sygehus,

Aarhus University Hospital in the period January 1995 to June 2004, in whom no mutations in the LDL receptor gene had been detected by SSCP analysis and in whom the apoB R3500Q mutation also had been excluded. Two-hundred and seventy-six of these 318 patients have been described previously [16].

A deletion of the LDL receptor gene was found in five patients tested with the MLPA method. The five deletions were a 9.3 kb deletion of the promoter and exon 1 [16], a 1 kb deletion of exon 5 [6], a 3 kb deletion of exons 7–8 [16,17], a 9.5 kb deletion of exons 9–14 [16] and 5 kb deletion of exons 13–15 [17]. The graphical results of the MLPA analysis are given in Figure 3. The detection of the deletions was confirmed by long-range PCR analysis with amplification of segments that MLPA analysis had suggested might contain the deletion (data not shown).

**Discussion**

This study showed that the MLPA method unambiguously discriminates between one and two copies of LDL receptor gene exon 5. MPLA also enabled us to distinguish clearly between deleted and non-deleted exons in patients with a known 9 kb deletion of exons 3–6. Finally the MLPA method proved efficient in screening of large numbers of patients for major structural rearrangements of



**Figure 3**  
**Graphical results of MLPA analysis.** Peak profiles of the MLPA analysis in one normal individual and in the five patients with the deletions described in the results section.

almost the whole LDL receptor gene in one PCR reaction. The protocol described by MRC Holland was followed, and no further optimisation was necessary.

It was also possible to distinguish between carriers and non-carriers of the exon 5 deletions with the Real-Time PCR method. The coefficients of variations (CV) for measurement of relative copy numbers with the Real-Time PCR method were larger than with the MLPA method, however. The CV's found in this study are comparable to other studies of measurements of relative allele copy numbers with Real-Time PCR and the use of SYBR green fluorescence in other genes [18,19], but higher than coefficients of variation reported with the use of TaqMan probes in Real-Time PCR analysis [20,21]. This difference could be due to multiplexing the PCR reactions when TaqMan probes are used such that both test gene and reference gene are amplified in the same reaction, which is not possible when SYBR Green fluorescence is used. It might have been possible to reduce the coefficients of variation of the Real-Time PCR analysis, with further optimisation or test of more primer sets. However, with the present data the intervals of mean  $\pm$  2SD's of relative allele copy numbers in carriers and non-carriers are overlapping, and in a considerable number of cases it was not possible to distinguish between one and two copies of exon 5 with the same degree of certainty as with the MLPA method. If screening of the entire LDL receptor gene was to be performed, a segment representative of each exon had to be amplified, and the need of optimisation would have been extensive. That would also have been the case if TaqMan probes were used and the PCR reactions were multiplexed.

Thus, in our hands, the MLPA method was cheaper, and it was more accurate and more precise than Real-Time PCR.

Previous work has not identified major structural rearrangements in the LDL receptor gene as a common cause of FH in Denmark [8]. It has therefore been necessary to employ a method for screening of the whole LDL receptor gene for pathogenic variations. In the period January 1995 to June 2004, a mutation in the LDL receptor gene was identified in 162 patients (data not shown) and large deletions accounted for 3.1% (5 of 162) of these LDL receptor gene mutation carriers, a finding similar to that obtained in other genetically heterogeneous populations [1,4,5]. In a study of Wang et al [22] eight different abnormal patterns of MLPA analysis were found in 12 of 21 patients with a clinical diagnosis of FH in whom sequencing of the LDL receptor gene had not revealed any mutations. In five patients with the same abnormal pattern, this was confirmed as a deletion with another method.

## Conclusion

The MLPA method was accurate, precise and at the same time effective in screening a large number of patients for large deletions in the LDL receptor gene. Large deletions accounted for the 3.1 % of LDL receptor mutation carriers in the population studied.

## Methods

### DNA

Genomic DNA was extracted from EDTA stabilised blood with the PUREGENE Genomic DNA Purification Kit (Gentra Systems).

### Real-Time PCR

A fragment of 116 bp located in exon 5 of the LDL receptor gene was amplified with the following primer set: forward exon 5 primer: 5'CCCTGCTTGTITTTTCTCTGG 3' and reverse exon 5 primer: 5'TGCAGTTTCCATCAGAGCAC 3'. For relative quantification of number of alleles in exon 5 of the LDL receptor gene, we used albumin as an internal reference gene with the primers described by Laurendeau et al [20]. PCR was carried out in reaction volumes of 25  $\mu$ L with 12.5  $\mu$ L of iQ™ SYBR Green Supermix (BIO-RAD), 300 nM of each primer and 4 ng of DNA. All samples were analysed in triplicate, and each run included separate standard curves for both primer pairs resulting from the amplification of serially diluted (50 ng, 10 ng, 2 ng, 0.4 ng and 0.08 ng) control DNA. Thermal cycling was performed on the iCycler™ iQ system (BIO-RAD) with a first denaturation step of 90 s at 95 °C, followed by 40 cycles at 94 °C for 10 s, 61 °C for 20 s and 72 °C for 20 seconds. PCR efficiencies in both reactions (exon 5 and albumin) were approximately 90%. Melting curve analysis was performed to exclude amplification of non-specific products.

### Multiplex Ligation-dependent Probe Amplification (MLPA)

A kit for screening the LDL receptor gene for deletions or duplications was obtained from MRC-Holland (SALSA P062 LDLR exon deletion test kit). It contains probes for 16 out of 18 exons in the LDL receptor gene as well as two probes for genes located just upstream and downstream of the LDL receptor gene. It also contains 13 probes for other human genes located on different chromosomes as controls. MLPA was performed as described by Schouten et al [14] and the manufacturer. Samples consisted of approximately 100 ng of genomic DNA. The amplified fragments were run on an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

### Long-range PCR analysis

When the results of MLPA analysis suggested that a major structural rearrangement was present in the LDL receptor gene, the results were confirmed with long-range PCR

analysis [10] using the Expand 20 kb<sup>PLUS</sup>PCR System (Roche). The sizes of deletions were estimated from the difference in size of the amplified allele with the deletion and the normal allele with long range PCR.

### Competing interests

The author(s) declare that they have no competing interests.

### Authors' contributions

DD designed the study, interpreted the data and drafted the manuscript. PHN conceived of the study and participated in the design of the study, the interpretation of data and helped to draft the manuscript. LGJ participated in the design of the study and helped to draft the manuscript. GGN and AS carried out the molecular genetic studies. MLL and OF evaluated the patients clinically, and OF helped to draft the manuscript. All authors read and approved the final manuscript.

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