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Rapid Diagnosis of Familial Hypercholesterolemia in British Patients

Marianne Stef, PhD

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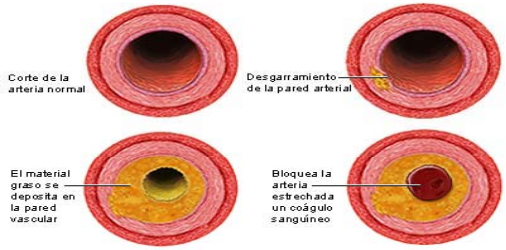
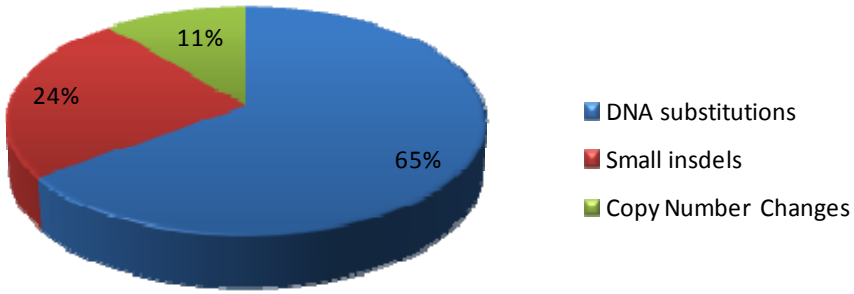
FAMILIAL HYPERCHOLESTEROLEMIA



- **Symptoms / Phenotype:**
  - High level of circulating cholesterol
  - Xanthomas and cholesterol deposits
  - Most frequent genetic cause of premature cardiovascular disease
- **Autosomal dominant disease**
- **Frequency of 1 in 500:**
  - Estimated 10 million affected worldwide (WHO) and 120,000 in the UK
  - Under diagnosed and only 25% effectively treated (statins)
- **Suitable Diagnosis?**
  - Clinical diagnosis: Mild phenotypes at young age go unnoticed and differential diagnosis from other disorders is difficult
  - Genetic diagnosis: provides unequivocal diagnosis

HF MUTATIONS

- Mutations in few genes (LDLR, APOB, PCSK9…)
- > 1000 mutations described: heterogeneity
- Most of the mutations are in the LDLR gene



(Leigh et al., 2008)

GENETIC DIAGNOSIS

- **Point mutations**
  - LDLR gene : 18 exons
  - APOB gene : 2/26 exons (Ligand binding domain)
  - PCSK9 gene : 12 exons (gain of function)

  → **Sequencing or screening + sequencing**

- **Copy Number Changes**

  → **MLPA, RFLP…**

→ **Expensive, long and tedious analysis**

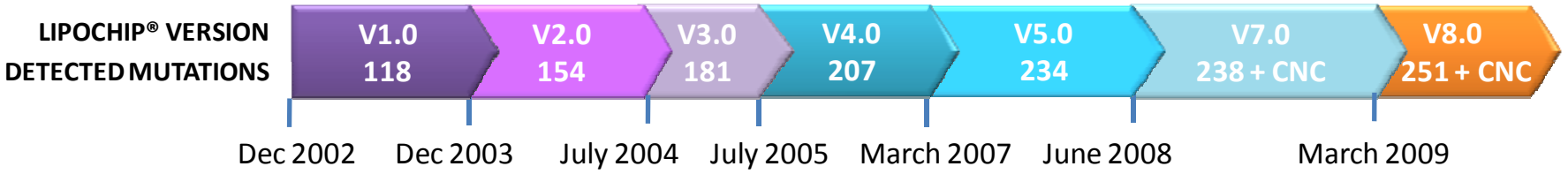
LIPOchip SERVICE PLATFORM WORKFLOW

- Chip: LIPOchip
  - Specific point mutations' detection
  - CNVs detection

- Sequencing in negative samples
  - Prom + 18 exons LDLR gene
  - APOB exons 26

LIPOchip HISTORY

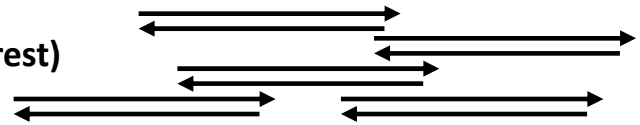
- First designed to detect the most frequent mutations in Spain
- First chip with CE mark for IVD
- Implementation of Copy Number Changes detection in v7.0
- Implementation of detection of European mutations in v8.0



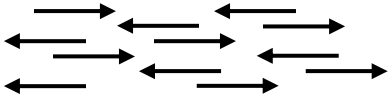
MOLECULAR BASIS OF HYBRIDIZATION

Patient DNA

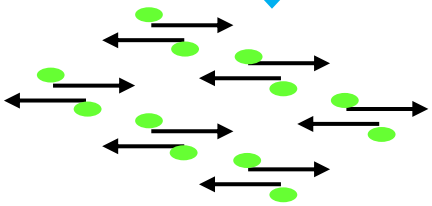
AMPLIFICATION (PCR)
(region of interest)



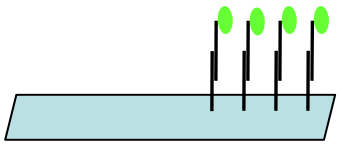
FRAGMENTATION



LABELLING

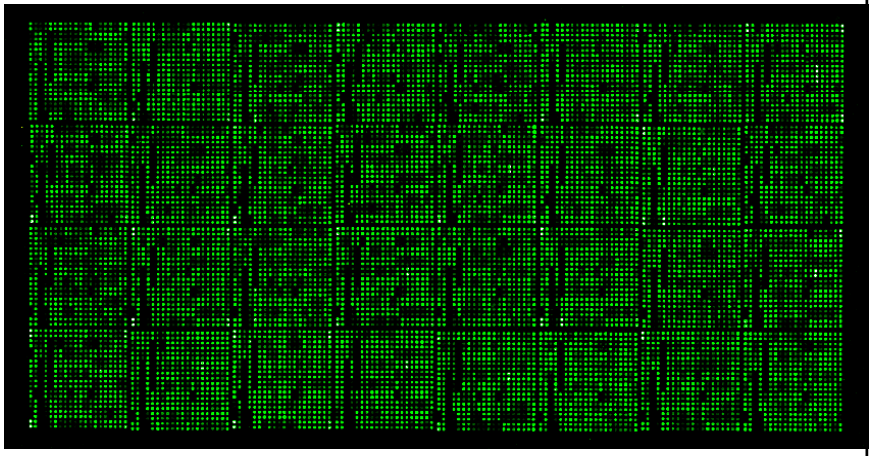


Biotin: Indirect labelling



HYBRIDIZATION

DNAchip
With mutation specific probes



LIPOchip

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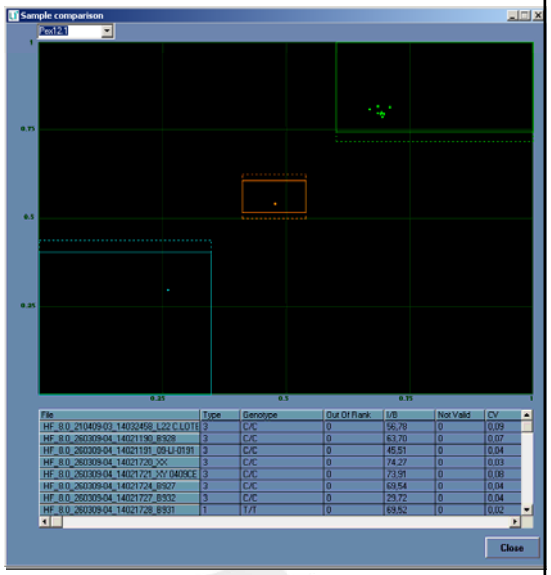
GENOTYPES COMPUTING

Based on intensity values of normal and mutated probes:

- 2 sets of probes specific of the mutated and normal allele
- Normal and mutated ranges computed with at least 100 normal samples and 7 mutated samples

	Normal Sample	HTZ Mutated Sample	HMZ Mutated Sample
$I_{normal\ oligo}$ (I_n)	1000	500	≈0
$I_{mutated\ oligo}$ (I_m)	≈0	500	1000
Ratio $\frac{I_n}{I_n + I_m}$	1	0.5	0



→ Automatic detection of heterozygous or homozygous mutants by the software

→ SNPs in the LDLR gene used as controls for
- capacity of genotyping
- samples identification

DETECTION OF COPY NUMBER CHANGES

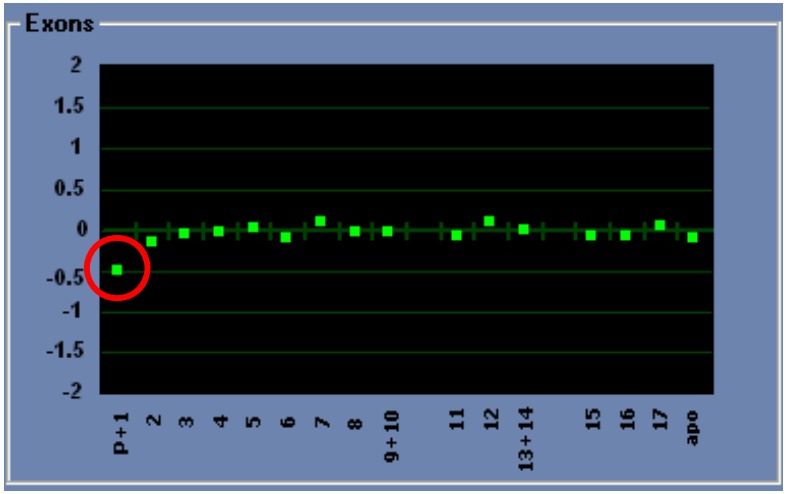
- **Specific controls included in the chip and in each PCR group**:
  - Normalization : Chromosome 21
  - Copy number change detection : Chromosome X

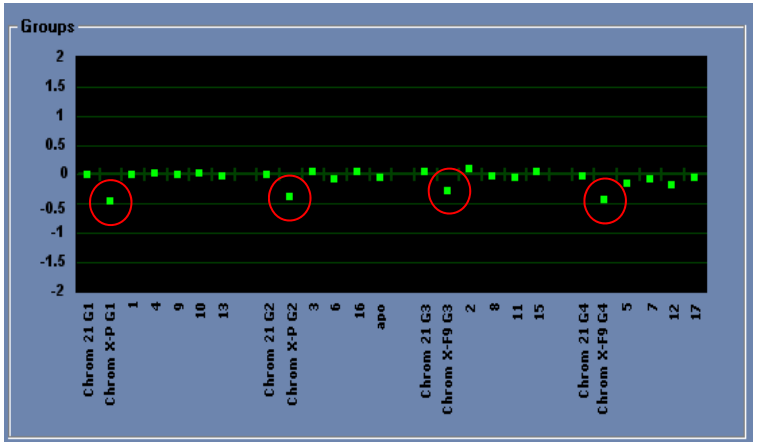
- **In each batch of hybridization, male and female controls are processed**

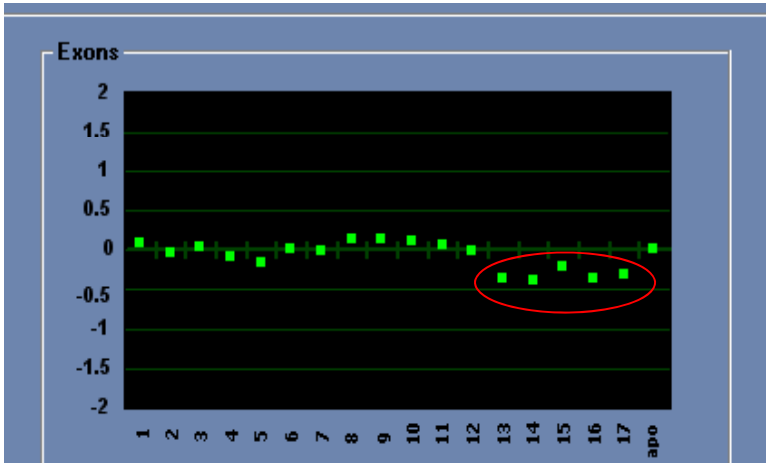
- **Based on ratio of intensities of hybridization**

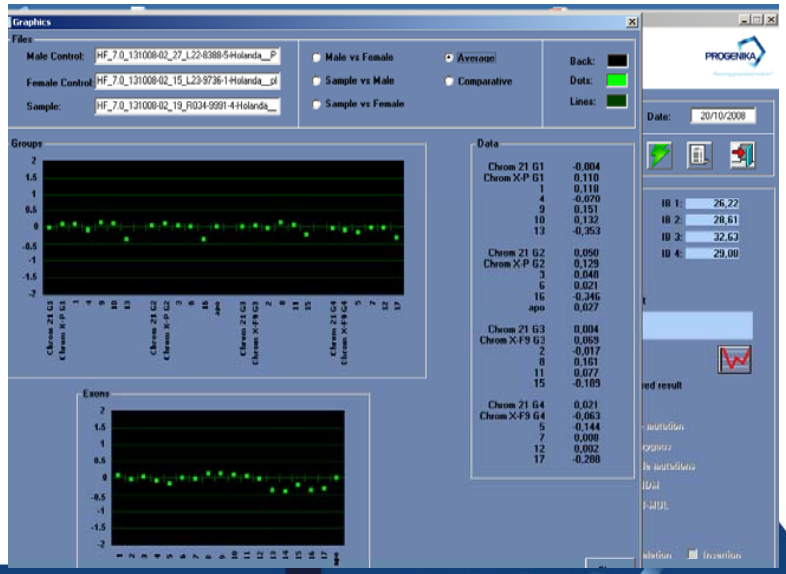
	Chr 21	Chr X	Normal exon	Deleted exon
$I_{sample\ (male)}$	1000	500	1000	500
$I_{control\ (female)}$	1000	1000	1000	1000
Ratio	1	0.5	1	0.5

DETECTION OF COPY NUMBER CHANGES









MUTATIONAL PATHOGENICITY ASSESSMENT

- Bioinformatical analysis
  - Amino-acid conservation
  - Nucleotide conservation
  - Physico-chemical distance between AA
  - Confirmation with 3 softwares (Polyphen/SIFT/Align GVGD)
  - Splicing prediction (3 softwares)

- Familial studies
  - Co segregation mutation and FH

- Protein modeling (in collaboration with Zaragoza Laboratory)
  - Modeling of AA change in binding domain

- Promotor mutations (in collaboration with Zaragoza Laboratory)
  - Electrophoretic mobility shift assay

- Patients' receptor activity
  - Real-time PCR, Western blot and LDLR activity assay in cultured lymphocytes

- Daily update of specific literature

LIPOchip+ REPORTS

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Date	00-00-00
Sample Code	000000
Doctor	

Hospital
Unit/Department
Address

Result of the Familial Hypercholesterolemia genetic analysis[1,2]
MUTATIONAL PATHOGENICITY PENDING VALIDATION STUDIES

Mutation reference: M129

In the sample labelled with the code indicated above, a mutation was identified with the following characteristics:

Gene: **LDL Receptor**

Mutation Reference No: **M129**

Genetic identifier: **c.1186+5G>A**
Mutation Class: **Splicing**
Classification of the Mutation: **Class B**

Class B Mutations

Since no validation of the pathogenicity of class B mutations has been performed, **it is necessary to establish the association of the genotype with the phenotype of the illness.** For example, a familial genetic analysis is recommended to determine whether the mutation is present in members of the family with high level of cholesterol.

Pathogenicity clues:
Highly conserved nucleotide among species (8/9)

Splicing prediction[3]
SpliceSiteFinder score (normal/mutated): 76.77/64.62 [0-100]
MaxEntScan score (normal/mutated): 7.23/0 [0-12]
GeneSplicer score (normal/mutated): 8.64/4.08 [0-15]

COMMENTS

Mutational pathogenicity pending validation studies

REFERENCES

[1]The analysis has been performed as described in the technical specifications, which are available upon request: services@progenika.com
[2]Tejedor et al., *Clinical Chemistry* 51(7):1137-144 (2005); Alonso et al., *Clinical Biochemistry* 42:899-903 (2009)
[3]Yeo et al., *J Comput Biol* 11:377-394 (2004); Shapiro et al., *Nucleic Acids Res* 15:7155-7174 (1987); Pertea et al., *Nucleic Acids Res* 29:1185-1190 (2001)

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Signed Dr. Diego Tejedor

→ Pathogenecity clues

LIPOchip+ REPORTS

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Date	00-00-00
Sample Code	000000
Doctor	

Hospital
Unit/Department
Address

Result of the Familial Hypercholesterolemia genetic analysis[1,2]
POSITIVE

Mutation reference: M006

In the sample labelled with the code indicated above, a mutation was identified with the following characteristics:

Gene: **LDL Receptor**

Mutation Reference No: **M006**

Genetic identifier: **c.1054_1060+4delTGCGAAGGTGA**
Protein identifier: **p.Cys331IlefsX16 (HGVS nomenclature p.Cys352IlefsX16)**
Mutation Class: **Null Allele**
Classification of the Mutation: **Class A**

Class A Mutations

These **mutations are directly associated with Familial Hypercholesterolemia**, since their pathogenicity has been validated.

Validation study: Neu-Yilik et al., *Adv Genet* 62:185 (2008)

Null Allele Mutations

The mutations that result in a null allele are usually **associated with more severe phenotypes**, including advanced atherosclerosis, as indicated in the literature[3]. Null mutations have been associated with a high risk of cardiovascular disease, high levels of cholesterol, and the need for intensive treatment to achieve a therapeutic response (a decrease in LDL-cholesterol levels). A close follow-up of the patients that carry this type of mutation is recommended.

COMMENTS

REFERENCES

[1]The analysis has been performed as described in the technical specifications, which are available upon request: services@progenika.com
[2]Tejedor et al., *Clinical Chemistry* 51(7):1137-144 (2005); Alonso et al., *Clinical Biochemistry* 42:899-903 (2009)
[3]Junyent et al., *Arterioscler Thromb Vasc Biol* 28(3):580-586 (2008); Alonso et al., *Atherosclerosis* 200(2):315-21 (2008); Tejedor et al., *Clinical Chemistry* 51(7):1137-144 (2005)

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Signed Dr. Diego Tejedor

Highlighting the pathogenicity of Null Allele / Receptor negative mutations

VALIDATION STUDIES

	Total of point mutations	Total of CNVs	Total of negative samples	% of match
Spanish validation	67	6	65	100
Italian validation	36	3	58	100
Dutch validation	65	28	11	99.03

<u>One discrepancy during Dutch validation:</u>

Duplication of exon 9, which can't be detected by the chip because of exons 9 and 10 are amplified together (as well as promoter + exon1 and exon13 +14)

<u>Spanish services:</u>

Versions v7, v8 and v9 used by Spanish services since July 2008: **2663 samples**

77 CNC detected (21 random MLPA verification)
1072 samples with point mutations detected by the chip (150 random fully sequenced)
1369 negative samples (all sequenced, 100 random MLPA verification)

Mutation composition of LIPOchip

Gene	Mutation Number
LDLR	242
APOB	3
PCSK9	6
Total	251

- **All types of mutations can be detected:**
  - **Small insdel**
  - **DNA substitutions**
  - **CNC**
- **Mutations' pathogenicity verified by literature or validation studies**

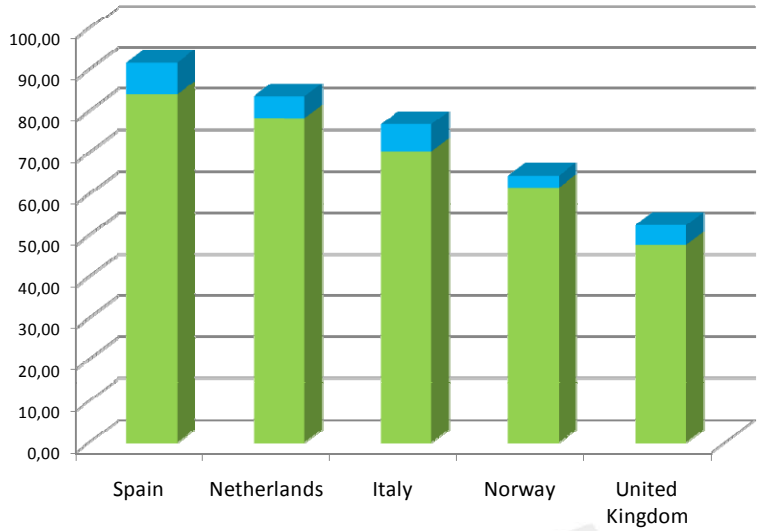
D151N	c.514G>A	p.Asp151Asn	ES NL NO
C371X	c.1176C>A	p.Cys371X	ES NL NO
W556R	c.1729T>C	p.Trp556Arg	ES NL NO
R723Q	c.2231G>A	p.Arg723Gln	ES NL NO
T740M	c.2282C>T	p.Thr740Met	ES NL NO
2393del9bp	3_2401delTCCTC	p.Lys778_phe780del	ES NL NO
1359-1G>A	c.1359-1G>A	N/A	ES NL NO
S156L	c.530C>T	p.Ser156Leu	ES NL NO UK
C152X	c.519C>A	p.Cys152X	ES NL UK
P587L	c.1823C>T	p.Pro587Leu	ES NL UK
D200G	c.662A>G	p.Asp200Gly	ES NL UK IT NO

Mutation composition of LIPOchip

Gene	Mutation Number
LDLR	242
APOB	3
PCSK9	6
Total	251

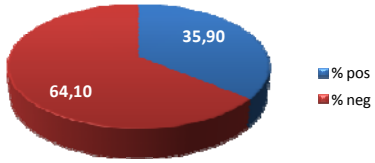
- All types of mutations can be detected:
  - Small insdel
  - DNA substitutions
  - CNC
- Mutations' pathogenicity verified by literature or validation studies

	Point mutations %	CNC %	Total %
Spain	83.90	7.65	91.55
Netherlands	78.24	5.17	83.41
Italy	70.24	6.48	76.72
Norway	61.53	2.82	64.35
United Kingdom	47.62	4.77	52.39



BRITISH STUDIES

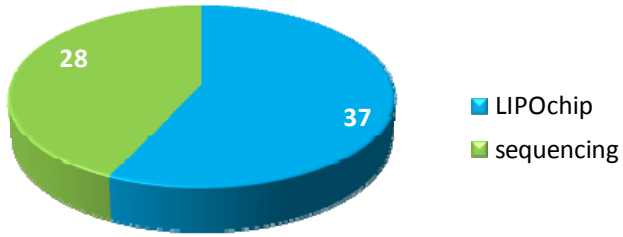
- Newcastle samples : cohort of 126 samples of previously known mutational status
  - 6 samples' DNA quality not meeting requirements, CNVs not analyzable
  - 120 samples analyzed with v8.0
- Wales samples: continuous sample delivery: 45 samples to date
  - 1 sample still in process (negative in chip, being sequenced)
  - 39 samples analyzed with v8.0 or v9.0



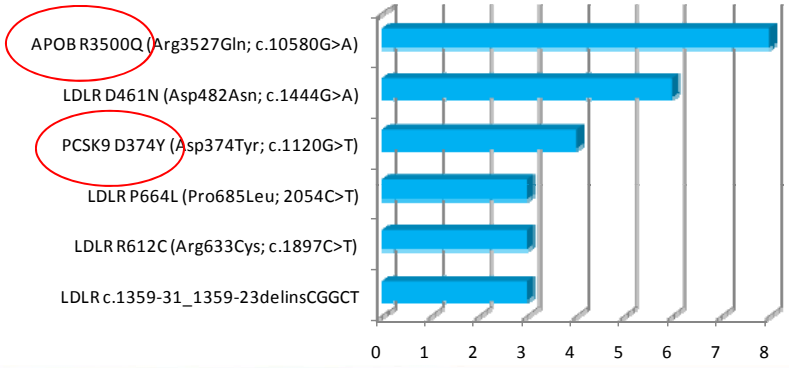
Wales samples

BRITISH STUDIES
Among positives samples

- Newcastle samples :
  - 65 positive samples:
    - 52 LDLR mutations
    - 8 APOB mutations
    - 4 PCSK9 mutations
    - 1 LDLR CNV

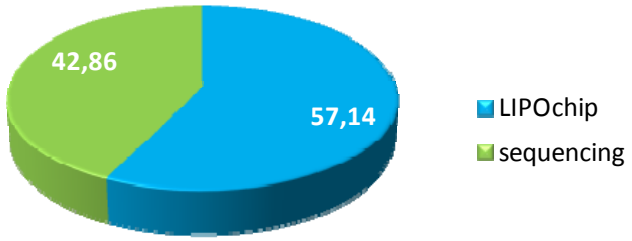


➢ All results consistent with previous studies (Tepnel kit + MLPA + sequencing)

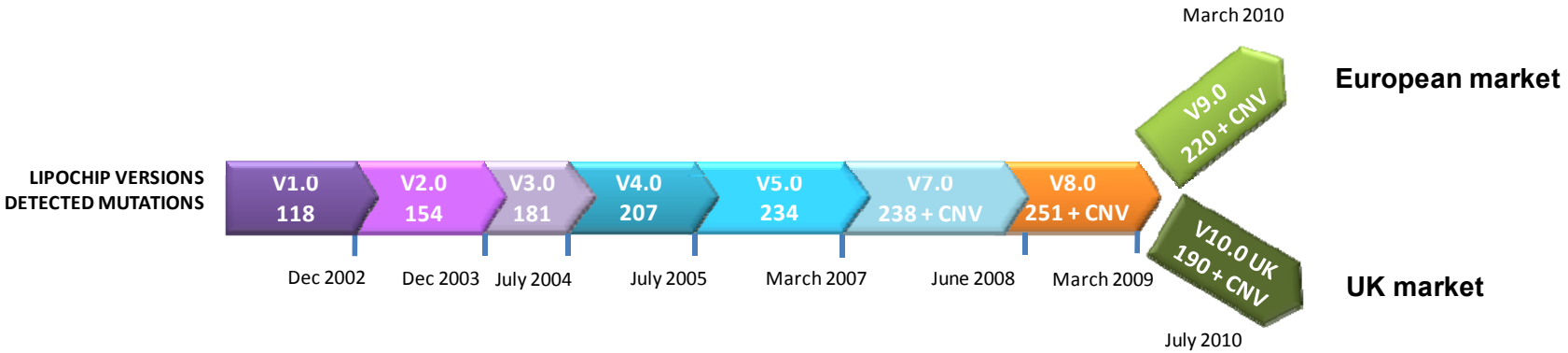


BRITISH STUDIES
Among positives samples

- Wales samples :
  - 14 positive samples:
    - 12 LDLR mutations
    - 1 PCSK9 mutation
    - 1 LDLR CNV
  - All mutations different



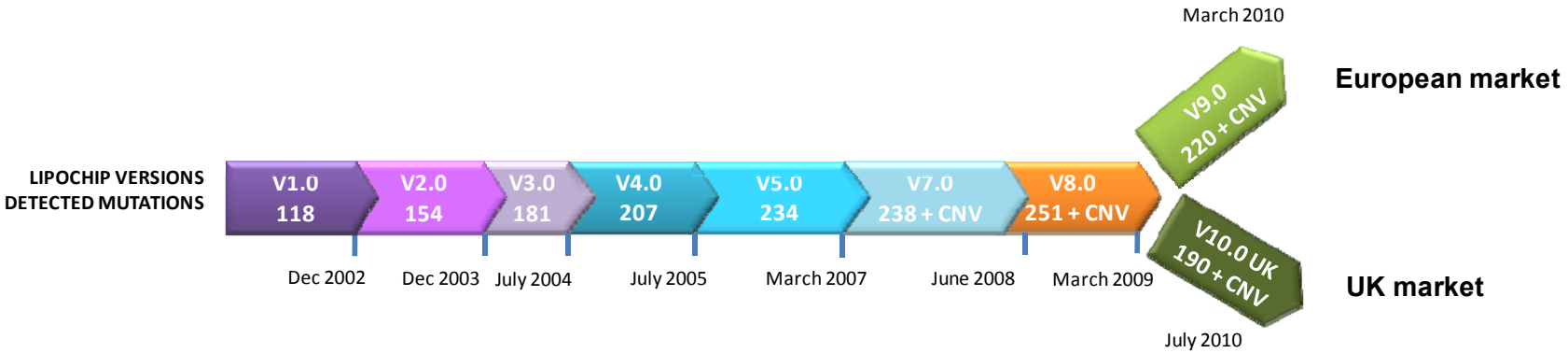
LIPOchip EVOLUTION

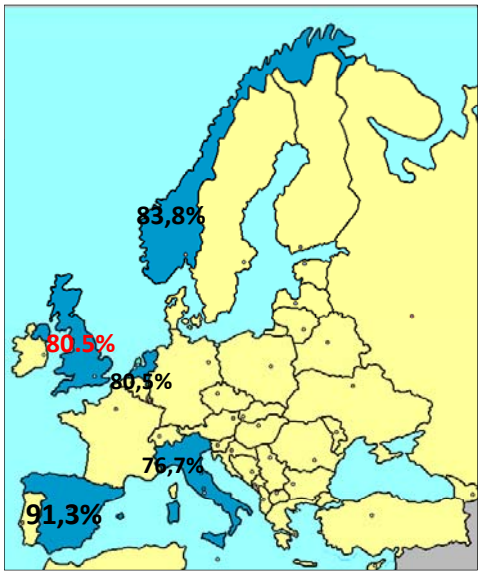


- LIPOchip v9: Based on frequencies provided by European specialists

- LIPOchip v10: Based on frequencies provided by University College of London

LIPOchip EVOLUTION





CONCLUSION

- Capacity for one tool to detect point mutations and CNC
- Reproducibility, sensitivity and specificity > 99.5%
- Results in less than one week with the chip
- LIPOchip UK expected pick up rate around 80%
  - confirming negatives by sequencing may only be required in special cases
- Can be applied to any kind of illness linked to both point mutations and CNVs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