Genetic testing for FH-the options

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Familial Hypercholesterolaemia (FH)

- Common genetic disorder
  - Heterozygous form 1:500 in Caucasian population
  - Severe homozygous form 1:1,000,000
  - Approximately 110,000 affected individuals affected in UK, majority of whom are undiagnosed
Clinical features

- Elevated total and LDL cholesterol
- Tendon Xanthomas
- Xanthelasma
Clinical features

- Atherosclerosis
- Premature coronary heart disease
  - Seen in up to 50% of men by age 55
  - Seen in women with slightly later age of onset
Genetics of FH

- Cholesterol is an important biological molecule
- Transported through the circulation in lipoprotein (LDL) particles
- Synthesised de novo and also ingested
- Cholesterol levels have to be carefully controlled
- Excess cholesterol is removed from the circulation by interaction of the LDL particle with the LDL receptor in the cell surface
Genetics of FH

- Majority of cases of FH are caused by mutations in the gene for the LDL receptor
- Mutations also known in the APOB gene and the PCSK9 gene
**LDLR gene**

From: www.ucl.ac.uk/ldlr/LOVDv1.1.0
Genetics of FH

- 1122 mutations described on the *LDLR* mutation database (on 18/8/10). ([www.ucl.ac.uk/ldlr](http://www.ucl.ac.uk/ldlr))
  - >200 known in UK

- *APOB* – 1 common mutation (p.Arg3527Gln)
  - ~5% of UK patients
  - Other variants also known

- *PCSK9* – One common mutation (p.Asp374Tyr)
  - ~2% of UK patients
  - 161 variants on database
Types of mutations

Summary of sequence variants in the LDLR

*64% of these variants are unique: From: www.ucl.ac.uk/ldlr/LOVDv1.1.0
Diagnostic testing for FH mutations

- **Options**
  - Screen the entire gene by sequencing
    - Highest pick up rate
    - Costly?
  - Target screening to the most common mutations
    - ARMs – simple and familiar to labs
    - MLPA - simple and familiar to labs
    - LIPOchip – more complex but higher pick up rate
ARMs

Target DNA Sequence

Non-target DNA Sequence

Template sequence
Allele specific primer
Common primer

Extension by Taq polymerase
No extension
Preliminary studies

- ARMs for 13 mutations
  - 11 in *LDLR*, 1 in *APOB* and 1 in *PCSK9*

- Panel of 400 cases of definite or possible FH
  - Mutations were known in 141 cases and 38% of these detectable by ARMs

Multiplex ARMS analysis to detect 13 common mutations in familial hypercholesterolaemia Taylor et al
Clinical Genetics 2007: 71: 561-568
ARMs kit FH20

- Gel based assay
  - 3 tubes
    - Upper and lower PCR controls
    - Mutation specific
  - 20 most common mutations in UK population

<table>
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<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tbody>
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<td>Upper C</td>
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<tr>
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<td>400bp</td>
<td>R3500Q</td>
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<td>P664L</td>
<td>330bp</td>
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<td>D461H</td>
<td>225bp</td>
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<td>R329X</td>
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<tr>
<td>D200G</td>
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<td>E80K</td>
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<td>D461N</td>
<td>103bp</td>
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<td>Lower C</td>
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<td>150</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

DNA ladder in basepairs (bp).
## Mutation Table

<table>
<thead>
<tr>
<th>Traditional</th>
<th>HGVS Nomenclature Nucleotide (protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E80K</td>
<td>c.301G&gt;A (p.Glu101Lys)</td>
</tr>
<tr>
<td>IVS3+1G&gt;A</td>
<td>c.313+1G&gt;A</td>
</tr>
<tr>
<td>ΔG197</td>
<td>c.654_656delTGG (p.Gly218del)</td>
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<tr>
<td>D200G</td>
<td>c.662A&gt;G (p.Asp221Gly)</td>
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<tr>
<td>fs206</td>
<td>c.680_681delAC (p.Asp227GlyfsX12)</td>
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<tr>
<td>E207X</td>
<td>c.682G&gt;T (p.Glu228X)</td>
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<td>R329X</td>
<td>c.1048C&gt;T (p.Arg350X)</td>
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<td>Q363X</td>
<td>c.1150C&gt;T (p.Gln384X)</td>
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<tr>
<td>L458P</td>
<td>c.1436T&gt;C (p.Leu479Pro)</td>
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<tr>
<td>D461H</td>
<td>c.1444G&gt;C (p.Asp482His)</td>
</tr>
<tr>
<td>P664L</td>
<td>c.2054C&gt;T (p.Pro685Leu)</td>
</tr>
<tr>
<td>C163Y</td>
<td>c.551G&gt;A (p.Cys184Tyr)</td>
</tr>
<tr>
<td>D461N</td>
<td>c.1444G&gt;A (p.Asp482Asn)</td>
</tr>
<tr>
<td>W66G</td>
<td>c.259T&gt;G (p.Trp87Gly)</td>
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<tr>
<td>V408M</td>
<td>c.1285G&gt;A (p.Val429Met)</td>
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<tr>
<td>D206E</td>
<td>c.681C&gt;G (p.Asp227Glu)</td>
</tr>
<tr>
<td>C656R</td>
<td>c.2029T&gt;C (p.Cys677Arg)</td>
</tr>
<tr>
<td>K290RfsX20</td>
<td>c.932_933delAA (p.Lys311ArgfsX20)</td>
</tr>
<tr>
<td>R3500Q (APOB)</td>
<td>c.1058G&gt;A (p.Arg3527Gln)</td>
</tr>
<tr>
<td>D374Y (PCSK9)</td>
<td>c.1120G&gt;T (p.Asp374Tyr)</td>
</tr>
</tbody>
</table>
FH20 ARMs Kit

- FH20 kit from Tepnel has 3 mixes (A, B and C)
- Doesn’t require specialised equipment, completed in 1-2 days
- Efficient and cost effective screen for FH testing
- Used as initial screen – Detection rate of 44%

Mutation detection rate and spectrum in familial hypercholesterolaemia patients in the UK pilot cascade project. Taylor et al, Clinical Genetics 2010, 77, 572
Can this detection rate be improved?

- ~5% of cases of FH are caused by large rearrangements of the *LDLR* gene
- Can be detected by MLPA
  - Kit based assay looking at all 18 exons of the gene
  - Quantitative so can tell if there are 1 or 2 copies of the gene or part of the gene
MLPA analysis

Courtesy Lucy Jenkins, NE Thames Regional Genetics Service
How can we target a larger proportion of mutations?
Microarray analysis - Lipochip

- Microarray based technology to examine larger number of mutations including copy number changes
- Manufactured by Progenika
  - Kit based assay
- May require purchase of specific equipment depending on laboratory
- 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
# Mutation composition of LIPOchip

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR</td>
<td>242</td>
</tr>
<tr>
<td>APOB</td>
<td>3</td>
</tr>
<tr>
<td>PCSK9</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>251</td>
</tr>
</tbody>
</table>

- All types of mutations can be detected:
  - Small insdel
  - DNA substitutions
  - CNC

- Mutations’ pathogenicity verified by literature or validation studies

## Point mutations %

<table>
<thead>
<tr>
<th>Location</th>
<th>Point mutations %</th>
<th>CNC %</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>83.90</td>
<td>7.65</td>
<td>91.55</td>
</tr>
<tr>
<td>Netherlands</td>
<td>78.24</td>
<td>5.17</td>
<td>83.41</td>
</tr>
<tr>
<td>Italy</td>
<td>70.24</td>
<td>6.48</td>
<td>76.72</td>
</tr>
<tr>
<td>Norway</td>
<td>61.53</td>
<td>2.82</td>
<td>64.35</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>47.62</td>
<td>4.77</td>
<td>52.39</td>
</tr>
</tbody>
</table>
MOLECULAR BASIS OF HYBRIDIZATION

Patient DNA

3’

5’

ATCG

TAGC

5’

3’

AMPLIFICATION

(PCR)

(region of interest)

LABELLING

Biotin: Indirect labelling

HYBRIDIZATION

DNAchip

With mutation specific probes
**Analysis**

- **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

<table>
<thead>
<tr>
<th></th>
<th>Normal Sample</th>
<th>HTZ Mutated Sample</th>
<th>HMZ Mutated Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{\text{normal oligo}}$ ($I_n$)</td>
<td>1000</td>
<td>500</td>
<td>≈0</td>
</tr>
<tr>
<td>$I_{\text{mutated oligo}}$ ($I_m$)</td>
<td>≈0</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>Ratio</td>
<td>$I_n$</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

- Automatic detection of heterozygous or homozygous mutants by the software
- SNPs in the LDLR gene used as controls for
  - capacity of genotyping
  - samples identification
Workflow for LIPOchip

0 Extraction
Sample
2 hours

1 Amplification
PCR mixes 1, 2, 3, and 4
7.5 μL DNA (20 ng/μL)
2 hours

2 Fragmentation
DNAse + Alkaline Phosphatase
24 μL
45 minutes

3 Labelling
TdT + Biotin-dUTP
56 μL
60 minutes

4 Hybridization
Tecan 4800 HS Pro
3 hours and 30 minutes

5 Results analysis

[Image with workflow stages and corresponding images]
LIPOchip EVOLUTION

LIPOCHIP VERSIONS DETECTED MUTATIONS

- **V1.0**: 118 (Dec 2002)
- **V2.0**: 154 (Dec 2003)
- **V3.0**: 181 (July 2004)
- **V4.0**: 207 (July 2005)
- **V5.0**: 234 (March 2007)
- **V6.0**: 238 + CNV (March 2007)
- **V7.0**: 251 + CNV (June 2008)
- **V8.0**: 220 + CNV (March 2009)
- **V9.0**: 190 + CNV (March 2010)
- **V10.0**: (July 2010)

- **European market**
- **UK market**

Map showing the distribution of LIPOchip versions across Europe with detected mutation percentages:
- **91.3%**
- **80.5%**
- **83.8%**
- **80.5%**
- **76.7%**
- **91.3%**
- **75.7%**
Limitations of targeted screening

- What is the pick up rate in other ethnic populations
  - ARMs designed around the SE of England population
    - 3/10 Asian patients were detected by ARMs using FH 20
  - LIPOchip initially on Spanish population but now including “UK” mutations
If no mutation is found using either ARMs or L1POchip what are the options?
Analysis of entire gene(s)

- Exact composition of the entire gene can be determined by sequencing
  - PCR amplification of each exon of the gene
  - sequencing reaction
  - sequence analysis
- Whole process semi-automated
- Still much more time consuming than a targeted approach
Sequencing
DNA sequencing

Multistep process carried out by robots

Semi automated analysis by Mutation Surveyor software
DNA sequencing

- Sequencing still quite a costly process
- Next generation of sequencing now on horizon
- Will significantly reduce the cost of sequencing
Cascade screening

- Once a mutation has been identified in the index case, relevant family members can be tested
- Methodology used will depend on the mutation and how it was detected
  - ARMs – same method
  - MLPA – same method
  - Sequencing – same method but just one fragment
  - LIPOchip – probably sequencing of one fragment
# Test costs and turnaround times

<table>
<thead>
<tr>
<th>Test method</th>
<th>Cost for diagnostic samples</th>
<th>Cost for cascade screening</th>
<th>TaT for diagnostic screening</th>
<th>TaT for Known mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMs (+ MLPA)</td>
<td>£50-£160 (£130-£140)</td>
<td>£50-£160 (£130-£140)</td>
<td>10 days</td>
<td>10 days</td>
</tr>
<tr>
<td>LIPOchip</td>
<td>150 euros (reagents only – no staffing and overheads)</td>
<td>N/A</td>
<td>(10 days)</td>
<td>N/A</td>
</tr>
<tr>
<td>Mutation scanning or Sequencing (may include MLPA at this stage)</td>
<td>£325-£650</td>
<td>£75-£100</td>
<td>40 days</td>
<td>10 days</td>
</tr>
</tbody>
</table>
Summary

- A number of molecular tests are available for the diagnosis of FH
- Vary in complexity and number of mutations which can be detected
- Possible to design a 2/3 stage screening strategy with excellent pick up rates
- Once a mutation is identified, cascade screening is possible for that family using a simple test for a specific mutation