CHAPTER 1

Background and Literature Review

1.1 The Metabolic Roles of Fatty Acids

Fatty acids serve multiple functions. They are precursors for membrane biogenesis and lipid mediators, and are the largest energy reserve in the body supplying energy-yielding substrates by \( \beta \)-oxidation in mitochondria and peroxisomes. Mitochondrial fatty acid \( \beta \)-oxidation (FAO) generates acetyl-CoA and reducing equivalents (NADH and FADH\(_2\)), which are linked to the Krebs cycle and the mitochondrial respiratory chain, leading to ATP production by oxidative phosphorylation in aerobic tissues.

During fasting, FAO provides 80 to 90% of cellular energy requirements, with ketone bodies generated from acetyl-CoA being an excellent energy source for peripheral tissues, especially the brain (Mitchell et al 1995). Whilst almost all tissues depend essentially on FAO for their energy supply during prolonged fasting, heart and skeletal muscle derive most of their required energy from long-chain fatty acid oxidation at all times (Neely et al 1972). Mitochondrial FAO is thus a crucial process, particularly in newborns, as they have limited glycogen reserves and rely heavily on fatty acids for energy (Wolf et al 1974).

1.2 The Mitochondrial Fatty Acid \( \beta \)-Oxidation Pathway

A schematic representation of the mitochondrial FAO pathway from cellular uptake of fatty acid to generation of acetyl-CoA, and its association with the respiratory chain, TCA cycle and ketogenesis, is outlined in Figure 1.1.
Figure 1.1: Schematic representation of fatty acid oxidation pathway

1.2.1 Plasma Membrane Cellular Uptake and Activation of Fatty Acids

During catabolic episodes, fatty acids within adipose cells are mobilised, transported and distributed to various tissues via the blood-stream as lipoprotein complexes. The mechanisms of cellular uptake and intracellular trafficking of fatty acids are complex and not clearly understood (Glatz et al. 1997; McArthur et al. 1999). The short-chain and medium-chain fatty acids (4 to 6 and 8 to 12 carbon atom chain lengths respectively) diffuse freely across the plasma and mitochondrial membranes into the mitochondrial matrix. In contrast, there is growing evidence that the mechanism of cellular uptake of long-chain fatty acids (14 to 20 carbon atoms) depends on both passive diffusion and active carrier-mediated transmembrane translocation (Berk and Stump 1999; Stump et al. 2001). Several different proteins shown to facilitate transport of long-chain fatty acids have been characterised in different species at the protein and/or gene levels. These include the long-chain fatty acid transporter protein (FATP; with six different proteins, designated FATP1 to 6, identified in humans) (Schaffer and Lodish 1994; Hirsch et al. 1998), the fatty acid translocase (FAT) (Abumrad et al. 1993), and the membrane-associated plasmalemmal fatty acid-binding protein (FABPpm) (Stremmel et al. 1985). Co-expression of genes coding for FATP, FAT and FABPpm in rat heart and muscle cells (Van Nieuwenhoven et al. 1999) and human skeletal muscle (Bonen et al. 1999) suggests that the uptake processes may involve the co-operation of a number of proteins.

Fatty acids are converted to their respective acyl-CoA esters by different fatty acyl-CoA synthetases. The imported long-chain fatty acids are esterified by long-chain acyl-CoA synthetase (LACS) located at the plasma membrane of adipocytes (Gargiulo et al. 1999), the outer mitochondrial membrane of skeletal muscle and microsomes in the liver, whereas the short- and medium-chain fatty acids are activated within the mitochondrial matrix (Trevisan and DiMauro 1983).
1.2.2 The Intracellular Transport of Fatty Acids and Derivatives

Following their uptake, fatty acids and their derivatives translocate through the aqueous cytoplasm to the mitochondria, possibly with the aid of cytoplasmic fatty acid-binding proteins (FABPc) (Glatz et al 1998). FABPc form a family of 14–15 kDa proteins which show a high affinity for the non-covalent binding of long-chain fatty acids that facilitate the solubility and intracellular transport of fatty acids. At least eight different types of human FABPc occur, each with a specific tissue distribution and possibly with a distinct function (Zimmerman et al 2001).

1.2.3 The Trans-Mitochondrial Membrane Carnitine Cycle

Long-chain fatty acyl-CoA esters are transported across the mitochondrial membrane by the carnitine cycle shuttle mechanism, involving the enzymes carnitine palmitoyltransferase I (CPT1), carnitine palmitoyltransferase II (CPT2), and carnitine acylcarnitine translocase (CACT), each with different sub-mitochondrial localisations, and with carnitine as a co-factor (McGarry and Brown 1997; Kerner and Hoppel 2000). Free L-carnitine crosses the plasma membrane against a high concentration gradient with the aid of the plasma membrane carnitine transporter (CT) encoded by the OCTN2 gene (Tamai et al 1998). CPT1 protein, which exists in two genetically distinct isoforms – a liver type (CPT1A) and a muscle type (CPT1B) – is located on the outer mitochondrial membrane, and catalyses the formation of long-chain acylcarnitine from acyl-CoA ester and free L-carnitine. CACT in the inner mitochondrial membrane carries the acylcarnitine into the mitochondrial matrix in exchange for free L-carnitine, and CPT2, situated in the inner mitochondrial membrane, re-esterifies the fatty acylcarnitine to fatty acyl-CoA ester, the substrate for β-oxidation.

1.2.4 The Intra-Mitochondrial β-oxidation Spiral

Within the mitochondria, the fatty acyl-CoA esters undergo repeated cycles of four sequential reactions, catalysed by enzymes with overlapping chain length
specificities. This begins with flavoprotein-linked (FAD) dehydrogenation catalysed by the acyl-CoA dehydrogenases, followed by hydration by the 2-enoyl-CoA hydratases, NAD\(^+\)-linked dehydrogenation by the L-3-hydroxyacyl-CoA dehydrogenases, and lastly, thiolytic cleavage by the 3-ketoacyl-CoA thiolases, generating an acetyl-CoA and an acyl-CoA ester two carbon atoms shorter at end of each cycle (Eaton et al 1996a).

The membrane-bound mitochondrial trifunctional protein (MTP) comprises the last three consecutive steps (Kamijo et al 1994). The electrons generated during the FAD-linked dehydrogenation are transferred via the electron transfer flavoprotein (ETF) and ETF dehydrogenase (ETFDH) to ubiquinone, and those from NADH-linked dehydrogenation are passed to complex I in the respiratory chain leading to production of ATP.

Unsaturated fatty acids with \textit{cis} double bonds are also degraded by mitochondrial \(\beta\)-oxidation, with the pre-existing double bonds being catalysed by auxiliary enzymes such as enoyl-CoA isomerase and dienoyl-CoA reductase (Wanders et al 1999). Long-chain acyl-CoA dehydrogenase serves an important function in the mitochondrial \(\beta\)-oxidation of unsaturated fatty acids (Lea et al 2000).

\subsection*{1.2.5 Hepatic Ketogenesis}

The liver is the only tissue that can synthesise ketone bodies. Acetyl-CoA is transformed to ketone bodies by the enzymes 3-hydroxy-3-methylglutaryl-CoA synthetase (HMGCS2) expressed mainly in liver, and 3-hydroxy-3-methyl-glutaryl-CoA lyase (HMGCL) (McGarry and Foster 1980).

Overall, the pathway requires the concerted action of more than 20 individual steps involving transporter proteins and catalytic enzymes. Our knowledge of the cellular uptake mechanism and intracellular fatty acid trafficking is incomplete, and the pathway is likely to include other enzymes yet to be discovered, as indicated by the
many patients with clinical symptoms strongly suggestive of fatty acid oxidation disorders in whom investigation of all known enzymes failed to reveal any abnormality.

1.3 The Mitochondrial Fatty Acid ß-Oxidation Disorders

1.3.1 Clinical Phenotypes

Mitochondrial fatty acid ß-oxidation (FAO) disorders are a group of metabolic defects mainly associated with intolerance to catabolic stress (Pollitt 1995; Bonnet et al 1999; Brivet et al 1999; Saudubray et al 1999; Wanders et al 1999; Bennett et al 2000; Treem 2000; Roe and Ding 2001; Rinaldo et al 2002). At least 21 different enzyme deficiencies have been characterised and gene defects have been identified in 15 of these enzyme deficiencies. Selected enzymes involved in FAO, their corresponding genes and biochemical abnormalities associated with each defect are listed in Table 1.1. Patients are often well between episodes of acute metabolic decompensation, which are precipitated by catabolic stress such as prolonged fasting, strenuous exercise, intercurrent illness or exposure to certain medications such as sodium valproate, that may occur at any time in life. Affected patients may have a wide variety of symptoms, such as hepatic encephalopathy with hypoketotic hypoglycaemia and coma similar to Reye syndrome, cardiomyopathy, arrhythmia, progressive myopathy, peripheral neuropathy, retinopathy, fulminant hepatic failure, sudden death, or may even have an asymptomatic course. There are overlapping phenotypes between the various enzyme defects of FAO, and the same defective enzyme may be associated with considerable clinical heterogeneity.

1.3.2 Inheritance and Incidence

All the FAO defects described exhibit autosomal recessive inheritance. The overall incidence is at least 1 in 12,000 live births, as estimated by the NSW Newborn Screening Program, Australia. The most common defect is medium chain acyl-CoA
Table 1.1: Mitochondrial fatty acid oxidation disorders – genetic and biochemical features

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Symbolb</th>
<th>OMIM numberb</th>
<th>Gene map locusb</th>
<th>Defect described (year)</th>
<th>Mutation identified (year)</th>
<th>DBS / plasma free carnitine acylcarnitine profiles</th>
<th>Urine organic acid profile</th>
<th>Urine acylglycine</th>
<th>Plasma C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;16&lt;/sub&gt; free fatty acids</th>
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<td>603377</td>
<td>5q33.1</td>
<td>1975</td>
<td>1998</td>
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<tr>
<td>Long-chain fatty acid transporter protein&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>19p13.1</td>
<td>1998&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>—</td>
<td>—</td>
<td>N</td>
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<td>Long-chain fatty acyl-CoA synthetase&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>152425</td>
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<td>Carnitine palmitoyltransferase I&lt;sup&gt;a&lt;/sup&gt; (liver)</td>
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<td>600528</td>
<td>11q13</td>
<td>1981</td>
<td>1998</td>
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<td>Carnitine palmitoyltransferase I&lt;sup&gt;a&lt;/sup&gt; (muscle)</td>
<td>CPT1B</td>
<td>601987</td>
<td>22qter</td>
<td>NC</td>
<td>NC</td>
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<td>?</td>
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<tr>
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<td>212138</td>
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<td>1992</td>
<td>1997</td>
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<td>1p32</td>
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<td>1992</td>
<td>↑C&lt;sub&gt;16&lt;/sub&gt;, C&lt;sub&gt;16:1&lt;/sub&gt;, C&lt;sub&gt;18&lt;/sub&gt;, C&lt;sub&gt;18:1&lt;/sub&gt;</td>
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<td>Intra-mitochondrial β-oxidation spiral</td>
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<td>Very long chain acyl-CoA dehydrogenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VLCAD</td>
<td>201475</td>
<td>17p11.2–11.1</td>
<td>1993</td>
<td>1995</td>
<td>↑C&lt;sub&gt;14:1&lt;/sub&gt;, C&lt;sub&gt;14&lt;/sub&gt;, C&lt;sub&gt;16&lt;/sub&gt;, C&lt;sub&gt;18:1&lt;/sub&gt;</td>
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<td>2q34–q35</td>
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<td>?</td>
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<td>Medium chain acyl-CoA dehydrogenase</td>
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<td>201450</td>
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<td>1982</td>
<td>1990</td>
<td>↑C&lt;sub&gt;8&lt;/sub&gt;, C&lt;sub&gt;8:1&lt;/sub&gt;, C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>+/−</td>
<td>↑</td>
<td>↑</td>
</tr>
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<td>Short chain acyl-CoA dehydrogenase</td>
<td>SCAD</td>
<td>201470</td>
<td>12q22–qter</td>
<td>1984</td>
<td>1990</td>
<td>↑C&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>Urine acylglycine</td>
<td>Plasma C(<em>6)–C(</em>{16}) free fatty acids</td>
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<td>Long-chain 2-enoyl-CoA hydratase (MTP (\alpha)-subunit)</td>
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<td>143450</td>
<td>2p23</td>
<td>See MTP</td>
<td>See MTP</td>
<td>(\mathrm{C}<em>{16}-\mathrm{OH}, \mathrm{C}</em>{18}-\mathrm{OH}) (\pm) N</td>
<td>N</td>
<td>↑</td>
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<td>Short chain 2-enoyl-CoA hydratase</td>
<td>ECHS1, SCEH</td>
<td>602292</td>
<td>10q26.2-q26.3</td>
<td>NC</td>
<td>NC</td>
<td>?</td>
<td>?</td>
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<td>?</td>
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<td>Isolated long-chain L-3-hydroxyacyl-CoA dehydrogenase(^a) (MTP (\alpha)-subunit)</td>
<td>HADHA, LCHAD</td>
<td>600890</td>
<td>2p23</td>
<td>1988</td>
<td>1994</td>
<td>(\mathrm{C}<em>{14}-\mathrm{OH}, \mathrm{C}</em>{16}-\mathrm{OH}) (\pm) N</td>
<td>N</td>
<td>↑</td>
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<tr>
<td>Medium/short chain 3-hydroxyacyl-CoA dehydrogenase (liver)</td>
<td>HADHSC, M/SCHAD</td>
<td>601609</td>
<td>4q22-q26</td>
<td>1996(^e)</td>
<td>NR</td>
<td>(\mathrm{C}_4)-OH, (\mathrm{C}_8)-OH (\pm) N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Short-chain 3-hydroxyacyl-CoA dehydrogenase (fibroblasts)</td>
<td>HADHSC, SCHAD</td>
<td>601609</td>
<td>4q22-q26</td>
<td>2001</td>
<td>2001</td>
<td>(\mathrm{C}_4)-OH (\pm) N</td>
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<td>↑</td>
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<td>Short-chain 3-hydroxyacyl-CoA dehydrogenase (muscle)</td>
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<td>601609</td>
<td>4q22-q26</td>
<td>1991(^f)</td>
<td>NR</td>
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<td>Long-chain 3-ketoacyl-CoA thiolase(^a) (MTP (\beta)-subunit)</td>
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<td>2p23</td>
<td>See MTP</td>
<td>See MTP</td>
<td>(\mathrm{C}<em>{16}-\mathrm{OH}, \mathrm{C}</em>{18}-\mathrm{OH}) (\pm) N</td>
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<td>602199</td>
<td>NR</td>
<td>1997(^g)</td>
<td>NR</td>
<td>Probably (\mathrm{C}_4) to (\mathrm{C}_8) (\pm) N</td>
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<td>Mitochondrial trifunctional protein (\alpha)-subunit (LCEH &amp;LCHAD)(^a) (\beta)-subunit (LCKAT)(^a)</td>
<td>MTP, HADHA, HADHB</td>
<td>2p23</td>
<td>1992</td>
<td>1994</td>
<td>(\mathrm{C}<em>{16}-\mathrm{OH}, \mathrm{C}</em>{18}-\mathrm{OH}) (\pm) N</td>
<td>N</td>
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Table 1.1 continued:
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<th>Mutation identified (year)</th>
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<th>Urine organic acid profile</th>
<th>Urine acylglycine</th>
<th>Plasma C₆-C₁₆ free fatty acids</th>
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<td>Electron transfer flavoprotein dehydrogenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ETFDH</td>
<td>231675</td>
<td>4q32 – qter</td>
<td>1985</td>
<td>1993</td>
<td>C₄, C₅, C₆, C₈, C₁₀, C₁₂</td>
<td>+/−</td>
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<td>↑</td>
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<td>2,4-Dienoyl-CoA reductase</td>
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<td>8q21.3</td>
<td>1990&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>2-trans,4-cis-decadienoylcarnitine</td>
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**Hepatic ketogenesis**

- 3-Hydroxy-3-methylglutaryl-CoA synthetase (mitochondrial)  
  | HMGCS2                  | 600234                   | 1p13–p12                 | 1997                     | 2001                      | —                                        | —                        | —              | N                           |
- 3-Hydroxy-3-methylglutaryl-CoA lyase                                 | HMGCL                    | 246450                   | 1pter–p33                | 1976                     | 1993                      | C₅-OH, methylglutaryl-carnitine               | +                        | N              | N                           |

**Table 1.1 continued:**

- DBS: whole blood dried on filter paper sample, NR: not reported, NC: no case known, N: normal concentration
- ?: not known, ↑: increased concentration, ↓: reduced concentration
- <sup>a</sup>: membrane-associated protein, <sup>b</sup>: from the Online Mendelian Inheritance in Man database, http://www.ncbi.nlm.nih.gov/Omim/<sup>c</sup>, <sup>c</sup>: Cases described only with biochemical characterizations, <sup>d</sup>: abbreviation
- —: no abnormality detected, +/−: informative findings may be detected in samples collected during crisis only, +: abnormal findings may be detected most of the time,  C₄₀: free carnitine,  C<sub>number</sub>: acylcarnitine species with the corresponding carbon chain length, -OH: hydroxyl
dehydrogenase (MCAD), occurring in at least 1 in 20,000 babies in New South Wales. Initial studies suggest that as a group, FAO defects probably represent one of the most common groups of inborn errors of metabolism (Stanley 1998) and their association with sudden infant death leads to a considerable demand for laboratory investigation (Pollitt 1993).

1.3.3 Association of Sudden and Unexpected Death with Fatty Acid β-Oxidation Disorders

Evidence that FAO defects may be related to sudden infant death syndrome (SIDS) comes from case reports (Howat et al 1984; Wanders et al 1989; Chalmers et al 1997), metabolic studies on siblings of SIDS victims and 'near-miss' infants (Harpey et al 1990), and autopsy studies. Systematic pathological investigation of postmortem samples collected at autopsy from infants with sudden and unexpected death including histological and biochemical analyses of liver (Boles et al 1998), acylcarnitine profiling in bile (Rashed et al 1995a) and dried blood specimens (Chace et al 2001), organic and fatty acid studies in urine and blood (Bennett and Powell 1994a), and fatty acid oxidation rate study in cultured fibroblasts (Lundemose et al 1997), indicate that FAO defects play a small but significant role in the cause of unexpected death in infants and young children. Sudden death after a relatively short prodromal illness is the first manifestation of MCAD deficiency in 18% of patients (Wang et al 1999), and has also been reported in adulthood (Raymond et al 1999; Losty et al 2001), which is potentially preventable (Keppen and Randall 1999).

1.3.4 Association of Fetal Fatty Acid β-Oxidation Disorders with Severe Complications During Pregnancy

The association of severe complications during pregnancy such as pre-eclampsia, the syndrome of haemolysis, elevated liver enzymes and low platelet counts (HELLP) and acute fatty liver of pregnancy (AFLP) in women carrying a fetus
with long chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency has been well documented (Schoeman et al 1991; Wilcken et al 1993; Treem et al 1996; Tyni et al 1998; Ibdah et al 1999; Strauss et al 1999). It is possible that long-chain 3-hydroxyacyl metabolites produced by the LCHAD deficient fetus or placenta are highly toxic to the maternal liver (Ibdah et al 1999; Matern et al 2001b). However, fetuses affected with other FAO defects including hepatic carnitine palmitoyltransferase I (CPT1A) (Innes et al 2000), MCAD (Nelson et al 2000) and short chain acyl-CoA dehydrogenase (SCAD) (Matern et al 2001a) born to mothers who developed liver disease during their pregnancies have also been reported. Whilst the causes of pregnancy complications with fetal FAO disorders are not clear, these cases suggest that derangement of mitochondrial fatty acid metabolism may play a role in the pathogenesis of maternal liver disease. Hence, investigation of FAO disorders other than LCHAD deficiency should be considered in all infants born after a pregnancy complicated by HELLP syndrome or AFLP.

1.3.5 Prognosis

MCAD deficiency was reported to have an overall mortality of approximately 25% (Touma and Charpentier 1992; Iafolla et al 1994; Wilcken et al 1994). Investigation of the outcome of MCAD deficient children after diagnosis and initiation of appropriate management showed that there were no additional deaths (Iafolla et al 1994; Wilson et al 1999). The prognosis of most diagnosed FAO disorders is generally favourable (Pons and De Vivo 2001), even in patients with two null mutations in very long chain acyl-CoA dehydrogenase (VLCAD) gene (Touma et al 2001).

1.3.6 Treatment

The primary treatment strategy for FAO defects is the strict avoidance of fasting, prevention of catabolism or other exacerbating factors such as infection. Treatment options for long-chain fat disorders are dietary modifications including maintaining a
very low intake of natural fats with supplementary medium-chain-triglyceride, a high carbohydrate intake, adequate essential fatty acids and L-carnitine or riboflavin in some cases. Prompt medical intervention during intercurrent illness is necessary to prevent catastrophic metabolic decompensation. The benefits of other therapies including creatine (Shortland et al 2001), and 3-hydroxybutyrate administration (Van Hove et al 2001) under some circumstances have been reported.

1.3.7 Newborn Screening of Mitochondrial Fatty Acid β-Oxidation Disorders

Most of the FAO defects are treatable, but potentially fatal if undetected. Early recognition is important to prevent morbidity and mortality. It is now possible through the expanded newborn screening program, to test for some FAO deficiencies using tandem mass spectrometric analysis of acylcarnitines (Millington et al 1990; Naylor and Chace 1999; Rashed et al 1999; Wiley et al 1999). This service is available in several centres around the world, and has been established in the NSW Newborn Screening Program, NSW, Australia, since 1998.

1.3.7.1 What is the prognosis for asymptomatic (pre-symptomatic) fatty acid β-oxidation deficient neonates detected by newborn screening?

Where newborn screening of FAO defects has been used as part of an established program, a number of FAO-affected neonates have been identified in whom the risk of developing clinical symptoms is not known (Wiley et al 1999; Carpenter et al 2001, Zytkovicz et al 2001). Family studies and genetic counselling are required. The prognosis for these asymptomatic (pre-symptomatic) neonates is not clear. A test that could indicate the severity of disease would be clinically relevant, assisting in medical treatment decisions and accurate genetic counselling. The correlation of disease phenotypes with residual enzymatic activity, DNA mutational analysis and metabolite accumulation as a means of prognostic / prediction study of FAO defects is discussed in Section 1.10.
1.4 Pathophysiology of Mitochondrial Fatty Acid β-Oxidation Disorders

The biochemical manifestations of FAO disorders include deficient production of the energy yielding substrates, acetyl-CoA and ketone bodies, and accumulation of free fatty acids and toxic acyl-CoA intermediates upstream of the enzymatic block. This leads to the formation of dicarboxylic and hydroxy-dicarboxylic acids from fatty acids via ω- and ω-1 oxidation, and the conversion of acyl-CoA esters to the corresponding acylglycines and acylcarnitines (mechanisms for detoxification and replenishing the free CoA) with secondary L-carnitine depletion. The accumulated intermediary products may further impair the FAO pathway by substrate/product feedback inhibition (Eaton et al 1996a; Baillet et al 2000). Other pathways including the Krebs cycle, the urea cycle and the mitochondrial oxidative phosphorylation may be affected (Ventura et al 1995; Ventura et al 1996), resulting in ATP deficit, lactic acidosis and hyperammonemia.

1.5 Biochemical Investigation of Mitochondrial Fatty Acid β-Oxidation Disorders

Biochemical investigation of suspected defects in FAO is difficult primarily because flux through this pathway is low when there is metabolic stability, and the diagnostic metabolites in physiological fluids may be absent or be undetectable by many systems. Numerous methodologies have been explored to find sensitive and specific tests to indicate likely disorders, an important aspect since confirmatory assays may be laborious and require special expertise. The strategy is a staged procedure, and commonly begins with a search for key metabolites in physiological fluids including urine, plasma and whole blood dried on filter paper samples (DBS), followed by the in vitro functional studies if the initial findings are inconclusive. Confirmation of defects is by enzyme activity measurements and/or DNA mutational analyses.
1.6 *In vivo* Metabolic Screening in Physiological Fluids

Compounds identified by urinary analyses of organic acids and indicative of FAO defects include the saturated and unsaturated dicarboxylic acids, hydroxy dicarboxylic acids, and acylglycines (Kimura and Yamaguchi 1999; Bonafe et al 2000; Costa et al 2000). Measurement of free fatty acids (Martinez et al 1997), total fatty acids (Lagerstedt et al 2001), 3-hydroxy fatty acids in plasma (Costa et al 1998; Jones et al 2000), acylcarnitines in plasma (Costa et al 1997; Vreken et al 1999) and DBS (Millington et al 1989; Van Hove et al 1993; Rashed et al 1995b; Chace et al 1997; Wiley et al 1999; Sim et al 2001) are mostly informative. Table 1.1 summarises some of the characteristic biochemical findings associated with individual FAO deficiencies detected in plasma, urine and DBS.

Whilst *in vivo* analyses of intermediate metabolites in body fluids are convenient and informative, results are influenced by numerous factors. From the sampling standpoint, it is necessary to know the clinical status, age, diet and medications of the patient when the sample was collected. From the technical standpoint, analytical expertise and considerable interpretational experience are required (Downing et al 1999). Very often, a knowledge of not only the actual analyte concentrations, but also the profile pattern, the metabolite ratio studies (Van Hove et al 1993; Fingerhut et al 2001) or additional investigations to identify analytes are required for interpretation of assay results. Moreover, some defects, such as HMGCS2 (Thompson et al 1997; Morris et al 1998; Aledo et al 2001) and plasma membrane long-chain fatty acid uptake (Odaib et al 1998), apparently never show specific metabolite profiles. Hence, findings may be difficult to interpret. When a FAO defect is clinically suspected but the metabolite profiles of body fluids are inconclusive, as is often the case, *in vitro* metabolic studies are the appropriate follow up investigations.
1.7 **In Vitro Metabolic Studies in Cells**

These include fatty acid oxidation rates and acylcarnitine profile studies, described in the 1980s and early 1990s respectively.

1.7.1 **Fatty Acid Oxidation Rate Study**

The fatty acid oxidation rate assay assesses the overall flux and integrity of the pathway by determination of end products after incubating intact cells with radio-labelled fatty acids (Saudubray et al 1982; Rhead et al 1985; Manning et al 1990). Cells including cultured skin fibroblasts, lymphocytes (Brivet et al 1995) and whole blood (Seargeant et al 1999) may be employed and fatty acids of different chain lengths are used to differentiate short, medium or long-chain defects.

The differential diagnosis of FAO defects derived from the oxidation of \([9,10-^3\text{H}]\text{myristate (M)}\) and \([9,10-^3\text{H}]\text{oleate (O)}\) is summarised Table 1.2. Analytically, the ranges of oxidation rates from fibroblasts of normal control subjects are wide and there are considerable inter-assay variations (Manning et al 1990; Olpin et al 1992). While FAO deficient cells generally show reduced oxidation rates, normal results have also been obtained, as shown by the adult presentation of CPT2 deficiency (Bonnefont et al 1996) and some other milder forms of FAO deficiencies.

Impaired oxidation rates have been demonstrated in some cell strains from patients with primary mitochondrial respiratory chain disorders (Venizelos et al 1998; Seargeant et al 1999), malonyl-CoA decarboxylase deficiency (Bennett et al 2001) and cell lines infected with mycoplasma or bacterial contamination (unpublished observations). Hence, a reduced oxidation rate does not conclusively indicate a FAO disorder and a normal oxidation rate does not exclude a FAO deficiency. Nonetheless, the O/M ratio is a sensitive indicator of medium or long-chain oxidation defects.
Table 1.2: Differential diagnosis of mitochondrial fatty acid oxidation disorders and other defects based on the \textit{in vitro} fatty acid oxidation rate study analysing tritium labelled water production

<table>
<thead>
<tr>
<th>O:M ratio (0.7-1.3)</th>
<th>O &lt; 50%</th>
<th>M Normal to ↓</th>
<th>M &lt; 50%</th>
<th>O: Normal to ↓</th>
<th>O:M ratio (&gt;1.3)</th>
<th>O:M ratio (0.7-1.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>VLCAD</td>
<td>MCAD</td>
<td>CPT2 (late onset)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT1A</td>
<td>LCHAD</td>
<td></td>
<td>SCAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACT</td>
<td>MTP</td>
<td>HMGCS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT2 (neonatal onset)</td>
<td>FATP</td>
<td>HMGCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETFDH</td>
<td></td>
<td>Potentially other milder FAO disorders</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ETF</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Respiratory chain defects

O: oxidation rate of [9,10-\textsuperscript{3}H] Oleate, M: oxidation rate of [9,10-\textsuperscript{3}H] Myristate

Results expressed as % of intra-batch controls

O:M ratio (0.7-1.3): Observed lower and upper limits of O to M ratio of normal controls

↓: reduced

CT: Plasma membrane carnitine transporter, CPT1A: Carnitine palmitoyltransferase I (liver isoform), CACT: Carnitine acylcarnitine translocase, CPT2: Carnitine palmitoyltransferase II, ETFDH: Electron transfer flavoprotein dehydrogenase, ETF: Electron transfer flavoprotein, VLCAD: Very long chain acyl-CoA dehydrogenase, LCHAD: Isolated long-chain L-3-hydroxyacyl-CoA dehydrogenase, MTP: Mitochondrial trifunctional protein, FATP: Long-chain fatty acid transporter, MCAD: Medium chain acyl-CoA dehydrogenase, SCAD: Short chain acyl-CoA dehydrogenase, HMGCS2: 3-Hydroxy-3-methylglutaryl-CoA synthetase, HMGCL: 3-Hydroxy-3-methylglutaryl-CoA lyase, FAO: fatty acid oxidation
(Olpin et al 1999), and is useful for the recognition of a defect in the cellular uptake of long-chain fatty acids where no characteristic metabolite is available (Odaib et al 1998).

**1.7.2 Acylcarnitine Profile Study**

**1.7.2.1 Reaction principle**

Acylcarnitines are derived from the acyl-CoA esters. Their formation is catalysed by the carnitine acyltransferases (van der Leij et al 2000) in the presence of sufficient L-carnitine to drive the equilibrium (Ramsay and Arduini 1993). Metabolic disorders including defects of FAO and branched-chain amino acid catabolism, the congenital lactic acidoses and organic acidurias accumulate acyl-CoA esters upstream of the enzymatic block resulting in elevation of corresponding acylcarnitines which are therefore important diagnostic markers.

**1.7.2.2 The in vitro acylcarnitine profile assay**

In *in vitro* acylcarnitine profiling, cells are incubated in a medium supplemented with precursors, a long-chain fatty acid plus excess free L-carnitine, which results in elevation of acylcarnitine species derived from the FAO pathway, and the accumulated acylcarnitine species analysed. Studies using this approach in cells from patients with FAO deficiencies have been reported to provide more specific information about the probable site(s) of defects in comparison to oxidation rate studies (Kler et al 1991; Pourfarzam et al 1994; Schaefer et al 1995; Nada et al 1995b; Schmidt-Sommerfeld et al 1998; Ventura et al 1999).

**1.7.2.3 Overview of the in vitro acylcarnitine profile assays**

Kler et al described the *in vitro* analysis of acylcarnitines and acyl-CoA esters utilising mitochondria isolated from human fibroblasts (Kler et al 1991). The mitochondria were incubated with radio-labelled [U-14C]hexadecanoate and L-carnitine
for one hour, and the products, $^{14}$C-labeled acylcarnitines and acyl-CoA intermediates, were detected by radio-high-pressure liquid chromatography (radio-HPLC).

Similar methodology but employing permeabilised fibroblasts (Pourfarzam et al 1994), or permeabilised leukocyte-platelets mixtures (Schaefer et al 1995) have been reported. The crude whole cells were either treated with the polycationic protein histone II-AS to permeabilise the cell membrane without disrupting the mitochondria, or were subjected to controlled centrifugation to disrupt the cell membrane before assay.

Results obtained from the methods described above using cell strains form patients with defects of CPT1A, CPT2, CACT, VLCAD, MTP and MCAD produced the unique disease-specific acylcarnitine patterns characteristic for the enzyme defects. However, intact whole cells including cultured fibroblasts and peripheral blood cells, did not produce detectable acylcarnitine intermediates with this method.

Schmidt-Sommerfield et al reported an in vitro assay using intact cells and radio-labelled carnitine instead of labelled fatty acid (Schmidt-Sommerfeld et al 1998). The intact fibroblasts were pre-incubated with L-[methyl-$^{3}$H]carnitine for 3-6 days followed by incubation with unlabelled hexadecanoic acid for two hours producing acyl-$^{3}$H]carnitines, which were extracted by methanol and analysed using radio-HPLC. Cell strains from patients with documented deficiencies of CPT1A, CPT2, VLCAD, LCHAD, MTP, MCAD, SCAD, ETF-α and ETFDH showed disease specific patterns of acylcarnitines in all 16 cases except one, a case of ETF-α deficient cell line, for which there was no abnormality.

As C$_{5}$-acylcarnitine isomers (isovaleryl carnitine and 2-methyl-butyrylcarnitine derived from degradation of leucine and isoleucine respectively), and C$_{4}$-acylcarnitine isomers (butyrylcarnitine and isobutyrylcarnitine, derived from fat and valine
degradation respectively) are some of the markers of ETF deficiency (Nada et al 1995b; Shen et al 2000), the authors suggested that the sensitivity of the method may be improved by the addition of branched-chain amino acids to the medium. One of the disadvantages of Schmidt-Sommerfeld’s method is that all acylcarnitines will be labelled due to preincubation with L-[methyl-\(^3\)H]carnitine. This makes it difficult to trace the origin of the acylcarnitine species, to determine whether they are derived from the FAO or the branched-chain amino acids catabolism pathway.

Ventura and colleagues performed quantitative acylcarnitine profiling by incubating the intact fibroblasts in media without foetal calf serum, in the presence of [U-\(^{13}\)C]palmitic acid (\(C_{16}\)-fatty acid) and L-carnitine for 96 hours (Ventura et al 1999). The resultant acylcarnitines accumulated in cells plus media were extracted with a solid-phase cation-exchange procedure and analysed either by quantitative stable isotope dilution gas chromatography chemical ionisation mass spectrometry (GC-CI-MS) or by fast atom bombardment tandem mass spectrometry (FAB-MS/MS). Two techniques were involved because GC-CI-MS is unable to identify long-chain 3-hydroxy-acylcarnitines, and therefore a second derivatisation and analysis by FAB-MS/MS is required to detect LCHAD and MTP deficiencies. Cultured cells from patients with CPT1A, CACT, CPT2, VLCAD, LCHAD, MTP, MCAD and SCAD deficiencies were investigated, but those with ETF or ETFDH defects were not mentioned. The authors found that reactions in media without foetal calf serum (which contains some branched-chain amino acids) generated higher levels of labelled acylcarnitines. However, this approach may risk missing ETF, ETFDH deficiencies (Schmidt-Sommerfeld et al 1998).

Nada et al (1995a, 1995b; 1996) described the \textit{in vitro} acylcarnitine assay using deuterium stable–isotope labelled long-chain fatty acids including 9,12-[17,17,18,18-\(^2\)H\(_4\)]linoleic acid (\(^2\)H\(_4\)-C18:2 fatty acid), [9,10-\(^2\)H\(_2\)]oleic acid (\(^2\)H\(_2\)-C18:1 fatty acid), [16,16,16-\(^2\)H\(_3\)]palmitic acid (\(^2\)H\(_3\)-C16 fatty acid) and L-carnitine.
Intact fibroblasts were incubated for 72 hours, and the acylcarnitine intermediates produced were detected by liquid secondary ionisation (LSI) tandem mass spectrometry (MS/MS) (Nada et al 1995a; Nada et al 1995b). Deuterium-labelled acylcarnitines derived from the exogenous source of fatty acids, as well as unlabelled acylcarnitines originating from endogenous fatty acids and branched-chain amino acid catabolism, were detected and disease specific acylcarnitine profiles were obtained for SCAD, MCAD, VLCAD, LCHAD and ETFDH deficient cells. Prenatal diagnosis of FAO disorders using cultured amniocytes has been successful (Nada et al 1996).

Roe et al reported their findings following the incubation of intact fibroblasts with \([16,16,16-^{2}\text{H}_3]\)palmitic acid and L-carnitine. The accumulated acylcarnitines in a sample of cells plus media were analysed by MS/MS. Diagnostic results were obtained from FAO deficient fibroblasts including CACT, CPT2, VLCAD, LCHAD, MTP, MCAD and SCAD, but not the CPT1A deficient cells, which showed a profile indistinguishable from that of normal controls (Roe and Roe 1999).

An *in vitro* acylcarnitine assay using the unlabelled fatty acids of different chain lengths and L-carnitine has also been informative (Shen et al 2000; Vianey-Saban et al 1998). The unlabelled fatty acids are less costly and are readily available, but the origin of the resultant acylcarnitines, whether from an exogenous or endogenous source of fatty acids, or from catabolism of branched-chain amino acids, is not traceable.

### 1.7.2.4 Summary: Previous studies of the *in vitro* acylcarnitine assays

Several methodologies have been described, varying from the use of isolated mitochondria, disrupted fibroblast/peripheral blood cells, intact fibroblasts; unlabelled, radioactive-labelled, stable-isotope labelled fatty acids of different chain lengths; labelled or unlabelled L-carnitine, to sophisticated instruments including radio-HPLC, MS/MS and GC-CI-MS. Overall, these different techniques of *in vitro* acylcarnitine
assay in FAO deficient cells show disease-specific acylcarnitine profiles, proving them useful as screening tools for the detection of some FAO disorders that result in elevation of acylcarnitines. Previous studies have also shown that:

- The *in vitro* acylcarnitine assay can be performed in intact cells, in contrast to earlier reports (Kler et al 1991; Pourfarzam et al 1994; Schaefer et al 1995). In fact, reactions in intact cells represent a more physiologically relevant system compared to disrupted cells, allowing the observation of *in vivo* complex intrinsic intracellular substrate trafficking and interactions between organelles (Jakobs and Wanders 1995; Verhoeven et al 1998).

- Assays using L-[methyl-\(^3\)H]carnitine or unlabelled fatty acids have the disadvantage that the origin of the resultant acylcarnitines is not traceable.

- Using labelled fatty acids facilitates the differentiation of acylcarnitines derived from FAO and those from branched-chain amino acid catabolism. This has led to the discovery of a new enzyme in the valine metabolism pathway (Roe et al 1998).

- It was suggested that the sensitivity of detecting ETF and ETFDH deficiencies could be improved by the addition of branched-chain amino acids to the reaction medium (Schmidt-Sommerfeld et al 1998).

- Chromatographic separation (radio-HPLC) may be able to resolve acylcarnitine isomers (e.g. C\(_4\)-, butyrylcarnitine or isobutyrylcarnitine; C\(_5\)-, isovalerylcarnitine or 2-methyl-butyrylcarnitine), but other compounds with different mass and structures could co-elute (e.g. 3-\(\text{OH}\)-palmitate and 3-\(\text{Oxo}\)-palmitate), posing difficulties in peak identification (Schmidt-Sommerfeld et al 1998).
- MS/MS is unable to distinguish isomers based on identification of the molecular mass of compounds alone. This limitation could partly be overcome by the use of labelled precursors.

Based on these reported methods, it is likely that the *in vitro* acylcarnitine assay using intact cells incubating with stable-isotope labelled fatty acid and excess L-carnitine in presence of fetal bovine serum and MS/MS for acylcarnitine detection would provide the most comprehensive information.

### 1.7.2.5 Questions relating to the *in vitro* acylcarnitine assay

The described *in vitro* assay quantitated the accumulating acylcarnitines in samples containing cells plus medium (Ventura et al 1999; Roe and Roe 1999). It is not clear whether the sensitivity of the assay could be improved by analysing the accumulating acylcarnitines in the medium only, rather than medium plus cells, which may better simulate the *in vivo* acylcarnitine status of a patient during a controlled fat loading test.

Furthermore, it appears that the diagnostic specificity of the *in vitro* acylcarnitine assay has not been thoroughly investigated. A review of FAO and respiratory chain (RC) defects showed that these two groups of disorders have some biochemical features in common. *In vitro* fatty acid oxidation rate studies in cultured fibroblasts from patients with RC defects have revealed variable impairment of the oxidation rate (Venizelos et al 1998). Patients with primary RC defects can exhibit urinary organic acid profiles mimicking FAO disorders (Christensen et al 1993; Bennett et al 1994b; Enns et al 2000). In addition, functional enzyme analyses have revealed concomitantly reduced activities of some enzymes in both pathways (Reichmann et al 1992; Tyni et al 1996; Das et al 2000; Diogo et al 2000; Vladutiu et al 2000). It is possible that the
acylcarnitine profiles in fibroblasts from patients with primary RC defects could accumulate abnormal acylcarnitines indistinguishable from FAO disorders.

1.8 Enzymatic Studies

Assays of most of the enzyme activities of the FAO pathway, including catalytic and transporter enzymes are available, but are inherently analytically demanding and subject to certain limitations. Some of the difficulties include:

- The significant overlap in substrate chain length specificity between the different enzymes of the ß-oxidation spiral (Eaton et al 1996a).

- The availability of substrates. Certain compounds such as ETF (the physiological electron acceptor of acyl-CoA dehydrogenases), and 3-ketopalmitoyl-CoA (the substrate for measurement of LCHAD and long-chain 3-keto-acyl-CoA thiolase activity) are not commercially available.

- The interference from enzyme activities of other pathways. Enzymes of the peroxisomal pathway have many catalytic activities similar to those in the mitochondrial FAO pathway. Part of the enzyme activity detected in cell homogenates is potentially the result of the peroxisomal enzymes, leading to difficulties in interpretation.

• The availability of sample types. Estimating catalytic enzyme activities can be performed on tissue biopsies or cultured fibroblasts stored frozen, but measurement of transporter protein activities (eg. CT) requires assays on intact cells in culture.

• The existence of tissue-specific enzyme isoforms and availability of expressing tissues for study. CPT2 is ubiquitously expressed, thus fibroblasts or muscle biopsy tissues may be used. On the other hand, CPT1 exists in two genetically distinct isoforms, a liver-type and a muscle-type (Yamazaki et al 1996; Britton et al 1997). Studies using fibroblasts measure the liver-type activity only and the muscle-type, which is expressed predominantly in muscle, would overlook defects in this enzyme. The mitochondrial HMGCS2 gene is expressed mainly in liver and testes, and is absent in fibroblasts, so that enzymatic study of HMGCS2 is only possible in liver biopsy.

1.9 Molecular Studies

The genes for most of the described enzymes of FAO have been isolated and defined, facilitating molecular analysis of these defects (Table 1.1). However, the occurrence of many disease-causing mutations in each gene, private mutations, polymorphisms, and a high degree of allelic heterogeneity make mutation analysis complicated, and molecular studies may not be completely diagnostic in every case.

The application of mutation analysis is most useful in the context of family studies, prenatal diagnosis when mutations in an index case had been identified. Prevalent mutations are known in only a few of the fatty acid oxidation genes (Gregersen et al 2000). The most common mutations in MCAD deficiency include the
985A>G, (90% of the alleles identified retrospectively (Wang et al 1999); prevalence carriers of 1:86 (Carpenter et al 2001)), and the 199T>C (a carrier frequency of 1/500 identified prospectively (Andresen et al 2001)). The prevalent mutation of LCHAD deficiency involves a single base substitution, 1528G>C, and is seen in 71% of mutant alleles (Ibdah et al 1999). Adult CPT2 deficiency is most commonly due to a missense mutation, 439C>T, and is found in 60% of mutant alleles (Kaufmann et al 1997).

1.10 Research Questions and Aims

Previous researchers have demonstrated that *in vitro* acylcarnitine profiling is a useful test for detecting selected patients suspected of having a FAO defect when the *in vivo* findings are inconclusive.

The aims of this research were to investigate some of the questions posed in this literature review:

**Is it possible to improve the diagnostic sensitivity of *in vitro* quantitative acylcarnitines profiling?**

Under normal physiological conditions the direction of fatty acid flux is into the mitochondrial matrix, catalysed by enzymes of the trans-mitochondrial membrane carnitine cycle (Section 1.2.3). The acylcarnitines exit the mitochondria via the reverse action of the CACT (Pande 1975), and leave the cell cytosol, possibly with the aid of CT (Wu et al 1999), as apparent by the appearance of pathognomonic acylcarnitines in the blood of FAO-deficient patients during metabolic stress. Previous researchers analysed the acylcarnitines in a sample containing medium plus cells. It was proposed that analysing the acylcarnitines accumulating in medium only would better simulate the *in vivo* acylcarnitine status of a patient during a controlled fat loading test, and possibly improve the diagnostic sensitivity.
This question was first examined by comparing the acylcarnitines accumulating in samples containing medium plus cells with that containing medium only, followed by validation and optimisation of methods (Chapter 3). The possible improvement in sensitivity was assessed in cultured fibroblasts from 38 patients with eight different enzyme deficiencies of FAO (Chapter 4).

**Would in vitro acylcarnitine profiling studies be of prognostic value?**

FAO disorders are clinically heterogenous. The same defective enzyme can be associated with considerable variation in the severity of symptoms (Pollitt 1995; Saudubray et al 1999; Roe and Ding 2001). The expanded newborn screening program has identified a number of asymptomatic or perhaps pre-symptomatic FAO deficient neonates in whom the prognosis is not known (Section 1.3.7.1). A test that could predict prognosis would facilitate selection of therapeutic strategies and accurate genetic counselling.

Available data show that correlations of clinical phenotypes with residual enzyme activities (Vianey-Saban et al 1998; Parini et al 1999) are usually poor. Correlation of genotype with phenotype for the different enzyme defects of FAO is also variable. Studies in patients with CT deficiency revealed that there is no correlation of genotype with phenotype, residual enzyme activity nor age at presentation (Wang et al 2000). A similar finding was reported in MCAD deficiency (Andresen et al 1997), where identical genotypes occur with clinical features ranging from asymptomatic to lethal within affected members of a single family (Heptinstall et al 1995). To some extent, there are genotype-phenotype correlations for CPT2 (Bonnefont et al 1999) and LCHAD deficiencies (Ibdah et al 2000); however, similar mutations causing variable phenotypes can also be found with CPT2 deficiency (Handig et al 1996). Although there is a clear correlation of genotype with phenotype in the VLCAD defect, in which
differences in clinical disease specific to allelic variants have been documented, the mutational spectrum is very wide with no prevalent pathogenic mutation (Andresen et al 1999), making phenotype-genotype correlation studies difficult.

Studies of *in vitro* acylcarnitine concentrations in fibroblasts from patients with VLCAD deficiency correlated with the clinical phenotype (Vianey-Saban et al 1998; Roe and Roe 1999; Roe et al 2001). Other FAO enzyme deficiencies have not been thoroughly investigated.

The association of *in vitro* accumulation of acylcarnitine concentrations with phenotypes of a particular enzyme defect was investigated in fibroblasts from patients with the same defective enzyme but variable degree of clinical severities, and is reported in Chapter 4.

**What is the diagnostic specificity of *in vitro* acylcarnitine profiling studies?**

β-Oxidation is linked to the respiratory chain at two stages, that of the 3-hydroxyacyl-CoA dehydrogenase via NAD+/NADH and complex I, and the acyl-CoA dehydrogenases via the FADH_2 linked ETF and ETFDH to ubiquinone, Figure 1.1 (Eaton et al 1996a). The *in vivo* and *in vitro* biochemical differentiation between patients with FAO disorders and those with RC defects can be difficult (discussed in Section 1.7.2.5). Whether the findings of *in vitro* acylcarnitine profiling are exclusively indicative of FAO disorders is not clear.

The results of acylcarnitine profiling in fibroblasts from patients with RC defects are reported in Chapter 5.