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Molecular Genetics and Metabolism xxx (2007) xxx–xxx

Molecular Genetics  
and Metabolism[www.elsevier.com/locate/ymgme](http://www.elsevier.com/locate/ymgme)

## Choice of oils for essential fat supplements can enhance production of abnormal metabolites in fat oxidation disorders

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Received 10 May 2007; received in revised form 13 July 2007; accepted 13 July 2007

### Abstract

Patients with mitochondrial long-chain fat oxidation deficiencies are usually treated with diets containing reduced fat and increased carbohydrate, at times via gastrostomy feeding. To ensure adequate intake of essential fatty acids, supplements are provided to their diets using commercially available oils. These oils contain large quantities of non-essential fats that are preferentially oxidized and produce disease-specific metabolites (acyl-CoA intermediates) due to the genetic defect. This study describes the concentrations of these intermediates as reflected by acylcarnitines as well as the % contribution from each of four fatty acids: palmitate, oleate, linoleate, and  $\alpha$ -linolenate when incubated with fibroblasts from patients with VLCAD, LCHAD, and trifunctional protein (TFP) deficiencies. Palmitate and oleate produce the majority of disease-specific acylcarnitines with these defective cell lines (79–94%) whereas linoleate and linolenate produced less (6–21%). On average, the amount of acylcarnitines decreased with increasing unsaturation (C18:1 > C18:2 > C18:3:34% > 11% > 3%, respectively). This relationship may reflect the “gatekeeper” role of carnitine palmitoyltransferase I (CPT I). A diet comparison between Canola and a combination of Flax/Walnut oils revealed that the latter, containing the least amount of non-essential fats, reduced blood acylcarnitine levels by 33–36%. The etiology of the severe peripheral neuropathy of TFP deficiency may result from the unique metabolite, 3-keto-acyl-CoA, after conversion to a methylketone via spontaneous decarboxylation. Essential fatty acid supplementation with oils should consider these findings to decrease production of disease-specific acyl-CoA intermediates. © 2007 Elsevier Inc. All rights reserved.

**Keywords:** Fatty acid oxidation disorders; Diet supplements; Essential fats; Methylketones

Patients affected with mitochondrial long-chain fat oxidation disorders such as the very long-chain acyl-CoA dehydrogenase (VLCAD), trifunctional protein (TFP), and L-3-hydroxy-acyl-CoA dehydrogenase (LCHAD) deficiencies all require diets with reduced fat and increased carbohydrate composition sometimes involving gastrostomy feedings. The necessary reduction in dietary fat often requires an additional source of the essential fats, linoleate (C18:2) and  $\alpha$ -linolenate (C18:3). These are essential since they are required for synthesis of important compounds such as docosahexenoate (DHA), prostaglandins, thromboxanes, etc. [1]. To provide the essential fatty acids, diets

are supplemented with commercially available oils [2]. Each of these contain substantial amounts of non-essential fats such as palmitate (C16), stearate (C18:0), and oleate (C18:1). These fatty acids are non-essential since they can be endogenously synthesized and are not involved in the synthesis of the important lipids mentioned above. However, they do enter  $\beta$ -oxidation and contribute substantially, to the accumulation of the abnormal acyl-CoA intermediates observed as acylcarnitines in the long-chain fat oxidation disorders.

This study examines the relative contributions of these essential and non-essential fatty acids to the accumulation of abnormal metabolites, in defective fibroblasts. Then, two patients received diets of either canola or a combination of Flax and Walnut oils to observe the impact on blood acylcarnitine levels.

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## 53 Materials and methods

### 54 Media and reagents

55 All tissue culture reagents and media were obtained from Gibco/BRL  
56 (Gaithersburg, MD) or Atlanta Biologicals (Norcross, GA). Dulbecco's  
57 Modified Eagle Medium (DMEM), 1X, low glucose (25 mM), with L-glu-  
58 tamine (4 mM) was used as the basic growth media. L-Carnitine and fatty  
59 acid precursors were purchased from Sigma Chemical (St. Louis, MO). All  
60 other chemicals were of analytical grade.

### 61 Fibroblast cell lines

62 These were obtained from four patients with specific fat oxidation dis-  
63 orders: VLCAD (1), TFP (1), and LCHAD (2). Diagnosis was confirmed  
64 on each by performing the established "in vitro probe" technique using  
65  $16\text{-}^2\text{H}_3$ -palmitic acid in intact fibroblasts and direct enzyme assay.

### 66 Patient diet comparisons

67 Two patients (VLCAD and TFP deficient), both receiving tube feed-  
68 ings containing provimin, polycose, and triheptanoin (C7), agreed to diet  
69 modifications of essential fatty acid supplements. The TFP patient's diet  
70 with Canola oil supplement contained protein 14%, carbohydrate 30%,  
71 fat (canola oil) 21%, and C7 35%. Linoleate and linolenate were 3% and  
72 1.3% of total Kcal, respectively. After 3 days of the Canola supplemented  
73 diet, the fat was changed to Flax/Walnut providing linoleate and linole-  
74 nate at 3.1% and 1.6% of total Kcal, respectively. The VLCAD patient's  
75 initial diet consisted of protein 14%, carbohydrate 30%, fat (canola)  
76 15%, and C7 39%. Linoleate and linolenate were 3% and 1.4% of total  
77 Kcal, respectively. The Flax/Walnut supplemented diet provided linoleate  
78 and linolenate at 3.0% and 1.4% of total Kcal, respectively. Blood for  
79 acylcarnitine analysis was obtained on the third day of the Canola diet  
80 and again 3 days after changing to the Flax/Walnut diet.

81 All studies were approved by the Baylor University Medical Center  
82 Institutional Review Board.

### 83 Enzyme activity

84 Activity was measured in cultured skin fibroblasts using established  
85 methods previously described for VLCAD [3], LCHAD [4], and TFP [5].

### 86 Fibroblast cultures

87 Fibroblasts from all patients and controls were maintained in complete  
88 DMEM at 37 °C in a humidified 5% CO<sub>2</sub>/95% air incubator. Approx-  
89 imately 1–3 days before initiation of incubation studies, the cells were  
90 sub-cultured into duplicate T-25 flasks and grown to within 90–95% con-  
91 fluency (approx 0.15–0.40 mg protein).

### 92 Protein measurement

93 Protein concentration was determined by a modification of the Brad-  
94 ford method [6] using the Bio-Rad protein assay (Bio-Rad, Hercules,  
95 CA) with BSA as standard.

## Incubation studies

97 Fibroblast cell lines from patients with suspected or proven VLCAD,  
98 TFP, LCHAD deficiencies and control cell lines were incubated for 72 h  
99 with 3.5 ml complete DMEM containing 0.4 mM L-carnitine plus the  
100 0.2 mM of the fatty acid—palmitate (C16), oleate (C18:1), linoleate  
101 (C18:2), and linolenate (C18:3)—bound to fatty acid free bovine serum  
102 albumin (BSA) as previously described [7]. In simultaneous experiments,  
103 the deficient and control cell lines were incubated *without* the labeled fatty  
104 acids in 3.5 ml complete DMEM containing 0.4 mM L-carnitine in BSA to  
105 serve as blank levels for the resulting acylcarnitine metabolites.

### Sample preparation and acylcarnitine analysis

106 Cells were incubated for 72 h at 37 °C in humidified 5% CO<sub>2</sub>/95% air.  
107 After the incubation period, media and cells were collected and the acyl-  
108 carnitines from 67 μl media and 33 μl cell homogenate were extracted  
109 and derivitized in 100 μl of 3 N HCl in *n*-butanol (Regis, Morton, Grove,  
110 IL) and analyzed as butylesters on a QUATTRO II tandem mass spec-  
111 trometer (Waters Micromass, Beverly, MA) equipped with electrospray  
112 ionization using a precursor scan of *m/z* 85 [8]. All acylcarnitines were  
113 quantified; however, only the following were found to be elevated and per-  
114 tinent to this study: from palmitate incubations: C16, C14, C12, 3-OH-  
115 C16, 3-OH-C14, and 3-OH-C12; from oleate incubations: C18:1, C16:1,  
116 C14:1, 3-OH-C18:1, 3-OH-C16:1, and 3-OH-C14:1; from linoleate incu-  
117 bations: C18:2, C16:2, C14:2, 3-OH-C18:2, 3-OH-C16:2, and 3-OH-  
118 C14:2; from  $\alpha$ -linolenate incubations: C18:3, C16:3, C14:3, 3-OH-C18:3,  
119 3-OH-C16:3, and 3-OH-C14:3.

120 The above acylcarnitines were expressed in nanomoles per mg protein  
121 per 72 h after subtracting "blank" levels determined in cultures without a  
122 precursor fatty acid. Statistical analysis of this data included the mean and  
123 standard deviation as well as *t*-test.  
124

## Results

125 The fatty acid composition in 100 g of five commercially  
126 available oils was obtained from the USDA Nutrient Data-  
127 base [9] (Table 1). The non-essential fats, palmitate, stea-  
128 rate, and oleate, account for 31.0–67.7% of the fatty acid  
129 composition of these oils with the largest amount found  
130 in Canola oil. Conversely, the essential fats, linoleate and  
131  $\alpha$ -linolenate, ranged from a low level of 32.3% in Canola  
132 to the highest concentrations in Walnut and Flaxseed oil  
133 (67.0–69.0%, respectively).  
134

135 Cell lines from normal and four patients with VLCAD  
136 (1), TFP (1), and LCHAD (2) were studied in vitro. The  
137 results of the enzyme assays (nmol/min/mg protein) docu-  
138 menting the deficiencies from the four defective cell lines  
139 were as follows: VLCAD: 0.02 (mean 1.57; range: 1.26–  
140 1.88); TFP: LCHAD: 19.6 (mean 89.9; range 74.3–105.5);  
141 Thiolase: 0.9 (mean 28.7; range 23.3–34.1); LCHAD-1:  
142 LCHAD: 18.8 (mean 89.9; range 74.3–105.5); LCHAD-2:

Table 1  
Comparison of fatty acid composition of commercial oils (g/100 g oil)

Oils	C16 + C18	C18:1	C18:2	C18:3	Non-essential	Essential
Canola	5.8	56.1	20.3	9.3	61.9	29.6
Soy	14.1	22.8	51.0	6.8	36.9	57.8
Corn	12.4	27.3	53.5	1.2	39.7	40.9
Flaxseed	9.4	20.2	12.7	53.3	29.6	66.0
Walnut	9.0	22.2	52.9	10.4	31.2	63.3

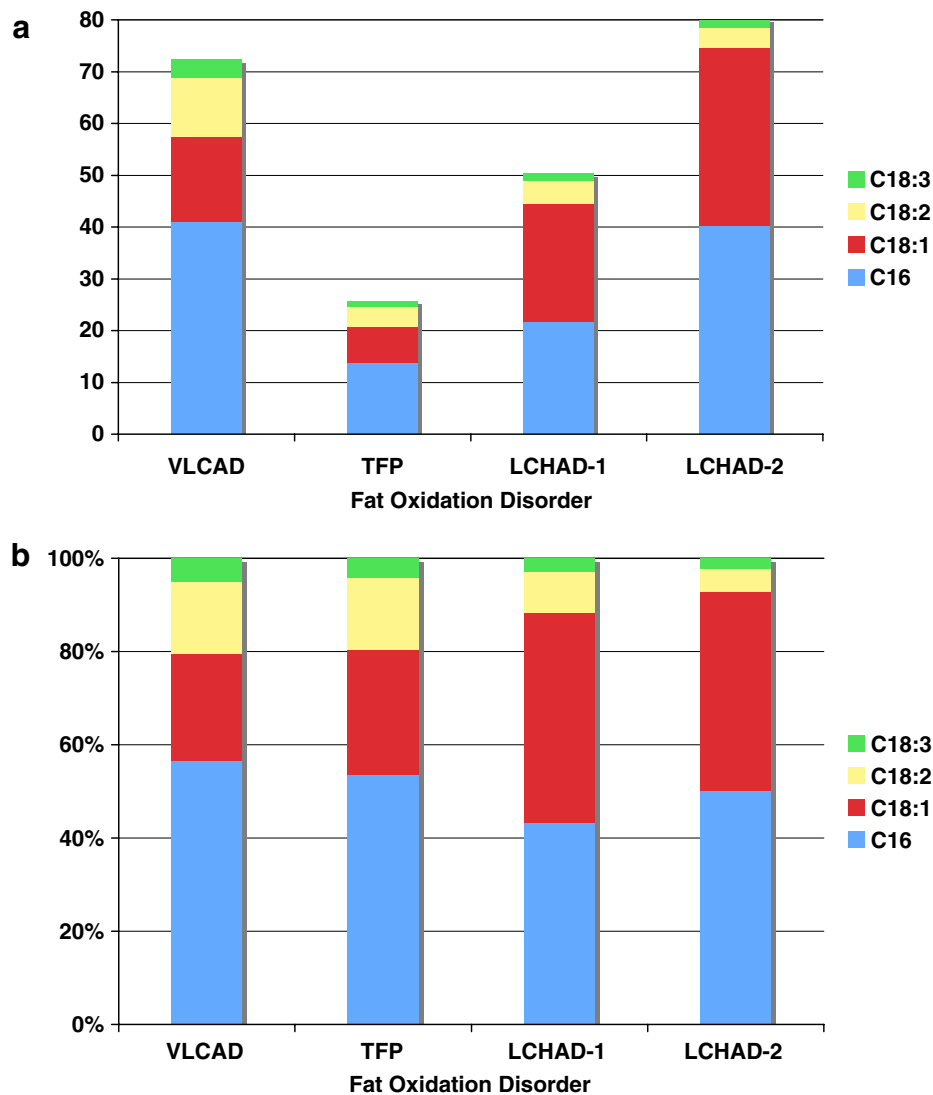


Fig. 1. (a) Contribution of fatty acids to disease-specific acylcarnitines in fibroblasts from long-chain fat oxidation disorders following 72 h incubations. (b) Percent of disease-specific acylcarnitines derived from individual fatty acid precursors, in vitro. C18:3 ( $\alpha$ -linolenate), C18:2 (linoleate), C18:1 (oleate), and C16 (palmitate).

LCHAD: 30.4 (mean 88.3; range 68.5–108.1), and Thio-  
lase: 21.8 (mean 25.0; range 17.3–32.7).

Each cell line was incubated separately for 72 h with equimolar (0.2 mM) concentrations of palmitate, oleate, linoleate and  $\alpha$ -linolenate to determine their relative contributions to abnormal metabolites. The total and individual long-chain acylcarnitines produced from these in vitro incubations are represented in Fig. 1a. The TFP cell line produced the least amount of acylcarnitines from these fatty acid substrates (25.7 nmol/mg protein/72 h) compared to VLCAD (72.3), LCHAD-1 (50.3), and LCHAD-2 (80.3). Normal cells produced only 4.4 nmol/mg protein/72 h.

Despite the relative differences in total acylcarnitines, it is obvious that for any individual defect, the majority are derived from the non-essential fats, palmitate and oleate (C16 and C18:1, respectively) with lesser amounts derived from linoleate (C18:2) and the least from  $\alpha$ -linolenate

(C18:3). This was also observed with normal cells even with the considerably lower levels. The percent contribution for each of the fatty acid substrates to these disease related acylcarnitines is presented in Fig. 1b and Table 2. In each defect, the non-essential fats (palmitate and oleate) produced the largest contributions to the acylcarnitines—between 80 and 94% with an average of 86%. Palmitate's contribution ranged from 46% for LCHAD to 57% for VLCAD while oleate ranged from 23% in VLCAD to 43% in LCHAD. The combined contributions from both

Table 2  
Percent of total abnormal acylcarnitines

Cell line	VLCAD	TFP	LCHAD-1	LCHAD
Palmitate	57	56	46	51
Oleate	23	26	43	43
Linoleate	16	14	8	5
$\alpha$ -Linolenate	5	3	3	2

linoleate and  $\alpha$ -linolenate (essential fats) to their total metabolites ranged from 7% to 21%. Linoleate's individual contributions ranged from 5% for LCHAD to 16% for VLCAD. The range for  $\alpha$ -linolenate was only 2% for LCHAD with a maximum of 5% with the VLCAD cell line. There appeared to be a progressively decreased contribution to acylcarnitine metabolites for all cell line incubations as the number of double bonds in the fatty acid increased (C18:1–C18:3) Table 2.

Two of the patients studied (VLCAD and TFP) consented to an evaluation of essential fatty acid supplements comparing Canola oil to a combination of Flaxseed and Walnut oils. Both patients received the bulk of their daily caloric requirements via gastrostomy. The diets were designed to provide equivalent amounts of linoleate and  $\alpha$ -linolenate (3% and 1% of daily caloric intake, respectively). Due to its composition, the amount of Canola oil required to meet the essential fatty acid needs contained much greater quantities of non-essential fats compared to the Flax/Walnut supplement. The patients received these supplemented diets for 3 days at which time the blood acylcarnitines were measured to determine if there was any difference in their levels with these two oil supplements. Table 3 reveals that the use of the Flax/Walnut oil with the least non-essential fats produced a decrease in circulating acylcarnitines from 33% to 36%. This decrease paralleled the results observed with the fibroblasts from these two patients.

## Discussion

Dietary supplementation of essential fatty acids for patients requiring fat restricted diets has relied on commercially available oils for both convenience and reducing expense. Currently, for patients with long-chain fat oxidation defects the diet is either restricted fat and increased carbohydrate or supplemental medium-chain triglyceride oil. In either case, supplementation of essential fats is routinely provided [2]. These oil supplements often contain large quantities of non-essential fats (palmitate, stearate, and oleate) that are not effectively oxidized due to the genetic defect (Table 1). As illustrated in Fig. 1b and Table 2, these non-essential fatty acids are responsible

for 80–94% (average = 86%) of abnormal acylcarnitines compared to only 7–21% (average = 14%) derived from the essential fats in vitro. The acylcarnitine contributions from the *unsaturated* fatty acids decreased in each of the disorders with increasing unsaturation of the 18 carbon fatty acids: cis-9- > cis-9,12- > cis-9,12,15-C18. On average, the contributions were 34%, 11%, and 3%, respectively, Table 2. After entry into the cell, all fatty acids must become substrates for the acyl-CoA synthetase to either enter into either cytosolic synthesis or mitochondrial  $\beta$ -oxidation. For the latter, they must be converted to acylcarnitines by action of carnitine palmitoyltransferase I (CPT I). CPT I is considered to be the “gatekeeper” that controls both further oxidation or synthesis. The substrate specificity and kinetics of CPT I with the different unsaturated C18 compounds has not been systematically studied. Since the cis- double bond creates a rigid 30° angle in the carbon chain, it is likely that these configurational changes due to increasing unsaturation found in oleate (cis-9), linoleate (cis-9,12), and  $\alpha$ -linolenate (cis-9,12,15) could reflect a decreasing affinity of CPT I for these fatty acids. This possibility may be responsible for directing the essential fatty acids more to cytosolic synthesis after activation.

These disease related acylcarnitines are all derived from mitochondrial acyl-CoA intermediates. Due to the high concentration of L-carnitine (0.4 mM) in the incubations, acylcarnitines are preferentially formed thus preventing any marked increase in the intra-mitochondrial acyl-CoA to free Co-enzyme A (CoA) ratio. This ratio is increased, in vivo, when fasting or infection stimulates lipolysis in patients with these disorders. This may produce toxic effects on citric acid cycle function, compromised energy production via the electron transport chain, and activation of AMP-mediated protein Kinase (AMPK) [10]. Enhanced acyl-CoA:CoA ratio is also known to inhibit pyruvate dehydrogenase and  $\alpha$ -keto-glutarate dehydrogenase complexes [11].

The relative amounts of non-essential fats included in the diet supplements are clearly not of the same magnitude as experienced in vivo during lipolysis but it is clear that disease-specific metabolites are consistently present as blood acylcarnitines in patients. However, it is not known if these compounds, at those levels, exert any kind of chronic pathologic effect. The trials with 2 patients alternating with Canola versus Flax/Walnut oils as essential supplements to their gastrostomy feedings did support the notion that excessive non-essential fats in the supplement should be avoided. The 33–36% reduction in disease-specific blood acylcarnitines with the Flax/Walnut supplement reflects this and suggests that improved diet therapy can be achieved Table 3. It is unfortunate that an economical preparation is not available that contains only linoleate and  $\alpha$ -linolenate in the appropriate ratio.

Consideration of the clinical course of these disorders suggests that even low levels of some metabolites may have a pathologic role. Peripheral neuropathy is a severe feature of TFP deficiency and less severe with LCHAD deficiency. Peripheral neuropathy is not observed in the other long-

Table 3  
Effect of reducing non-essential long-chain fatty acids on blood spot acylcarnitine levels ( $\mu$ M)

	Limit	Canola	Flax/Walnut	Change (%)
<i>VLCAD patient</i>				
C14:1	<0.14	2.74	1.78	(–35)
C16	<1.97	2.30	1.80	(–22)
C18:1	<2.64	3.53	2.15	(–39)
<i>TFP Patient</i>				
C14:1	<0.14	0.45	0.03	(–93)
C16	<1.97	1.38	0.95	(–31)
C16–OH	<0.06	0.18	0.06	(–67)
C18:1	<2.64	2.14	1.64	(–23)
C18:1–OH	<0.05	0.15	0.06	(–60)

268 chain disorders. The acylcarnitines observed with TFP and  
 269 LCHAD deficiencies appear identical by blood acylcarni-  
 270 tine analysis and in vitro incubation of fibroblasts with  
 271  $16\text{-}^2\text{H}_3$ -palmitate. In both cases, one observes elevation of  
 272 palmitoylcarnitine and to a lesser extent its 3-OH deriva-  
 273 tive. TFP can now be distinguished reliably from LCHAD  
 274 deficiency by in vitro incubation with pristanate that is oxi-  
 275 dized first in the peroxisome followed by final oxidation in  
 276 the mitochondrion. The ratio of dimethylnonanoylcarni-  
 277 tine (C11) to dimethylheptanoylcarnitine (C9) is consider-  
 278 ably greater in TFP deficiency than with LCHAD cell  
 279 lines [12]. The enzyme assay results from the patient in this  
 280 study revealed the greatest deficiency in thiolase activity as  
 281 opposed to the isolated LCHAD deficient cell lines.  
 282 LCHAD deficiency prevents the conversion of L-3-OH-  
 283 acyl-CoA to the 3-keto-acyl-CoA intermediate, normally  
 284 a reversible reaction. In TFP deficiency due to absent thio-  
 285 lase activity, the 3-keto-acyl-CoA intermediate can be  
 286 reduced to the L-3-OH-acyl-CoA by the LCHAD enzyme  
 287 or, as the free keto-acid, it can be spontaneously decarbox-  
 288 ylated to a methylketone (e.g. 3-keto-palmitate to 2-keto-  
 289 pentadecane) (Fig. 2). Methylketones are not observed by  
 290 organic acid analysis since there is no hydroxyl- or car-  
 291 boxyl- group to derivatize. Only in 2-methyl-acetoacetyl-  
 292 CoA thiolase deficiency ( $\beta$ -ketothiolase), in the isoleucine  
 293 pathway, has a methyl ketone, 2-butanone been detected.  
 294 It is the decarboxylated product of 2-methyl-acetoacetate  
 295 [13]. Severe peripheral neuropathy has been previously  
 296 studied in workers in chemical plants manufacturing hexane.  
 297 It was shown that the metabolism of hexane produces a  $\gamma$ -  
 298 diketone (2,5-hexanedione) that forms covalent bonds with  
 299 lysyl residues of myelin. This interrupts axonal flow resulting  
 300 in a peripheral neuropathy and myopathy [14,15]. Since the  
 301 etiology of the more severe peripheral neuropathy in TFP  
 302 deficiency remains unknown, we hypothesize that the neu-  
 303 ropathy may be related to the fate of a unique metabolite  
 304 such as the 3-keto acids that accumulate. For these reasons  
 305 as well as the observations that the 3-OH-acylcarnitines are  
 306 always increased in blood acylcarnitine analysis from these  
 307 patients, it may be useful to further reduce their occurrence

308 by substantial reduction in dietary non-essential fatty acids.  
 309 The reduced production of abnormal acylcarnitines, in vitro,  
 310 by the essential fatty acids and decreased blood levels of all  
 311 acylcarnitines resulting from the Flax/Walnut supplement,  
 312 compared to Canola, seem to support this strategy.

## Acknowledgments

The Summers-Courtwright fund for metabolic research  
 and the Baylor Research Foundation, Dallas, Texas.

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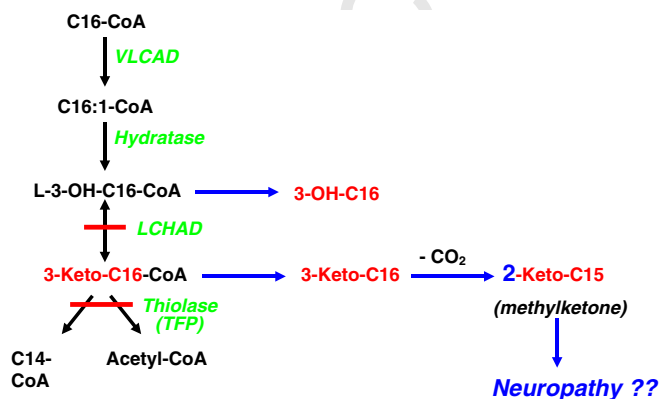


Fig. 2. Metabolites of palmitoyl-CoA (C16-CoA) produced by deficiencies of LCHAD and the thiolase portion of the trifunctional protein. Proposed relationship of methylketones and peripheral neuropathy.