MITOCHONDRIAL FATTY ACID OXIDATION AND ITS DISORDERS : THE CARNITINE CYCLE

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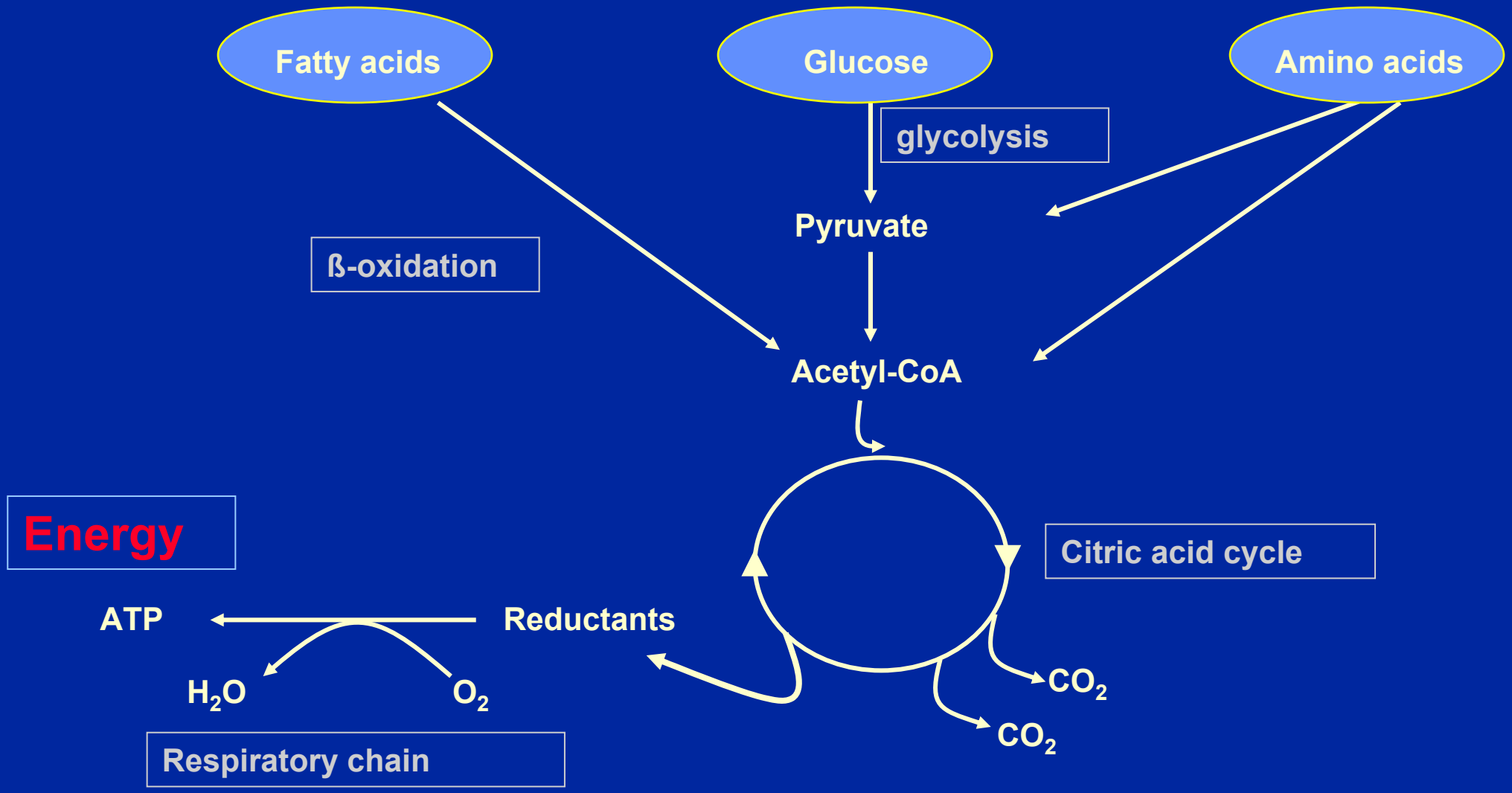
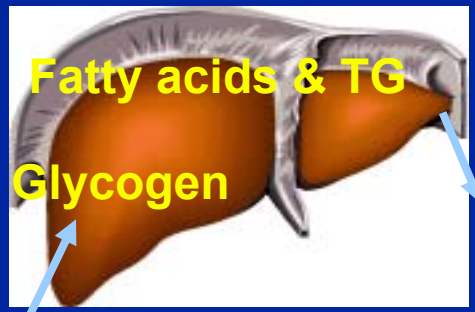
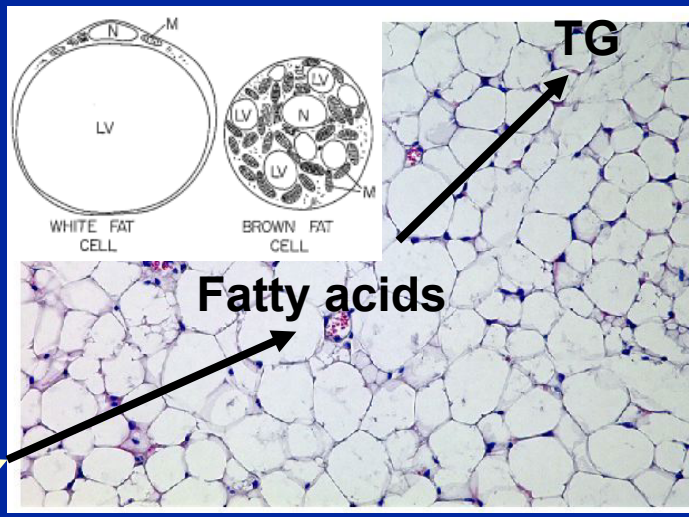
BIOCHEMISTRY BASICS



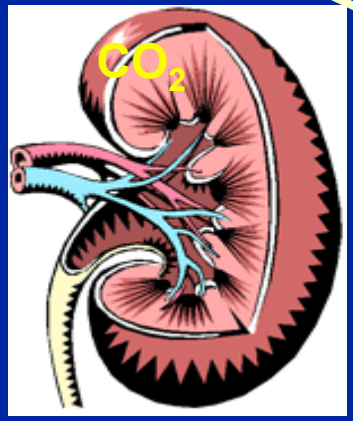
THE FED STATE
GLYCOGEN SYNTHESIS & LIPID BIOSYNTHESIS

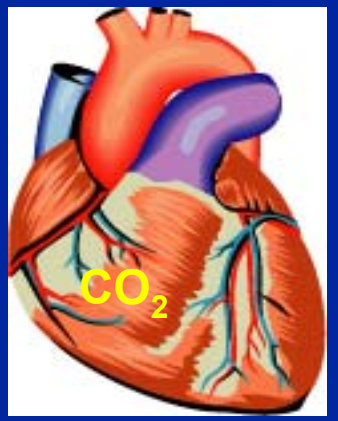




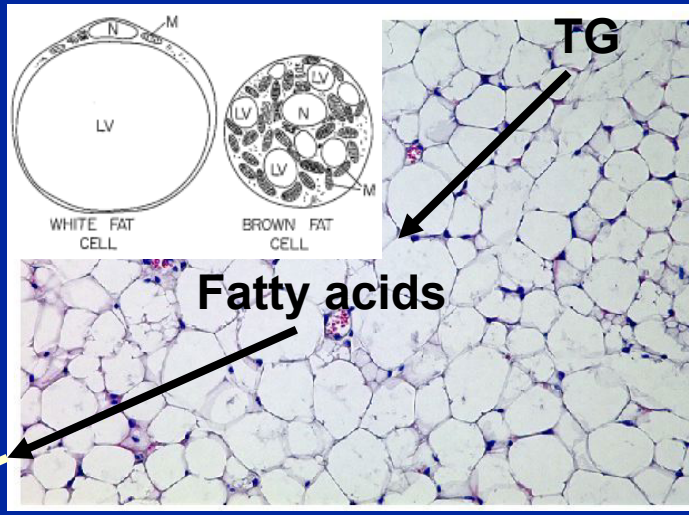
Glucose

Fatty acids



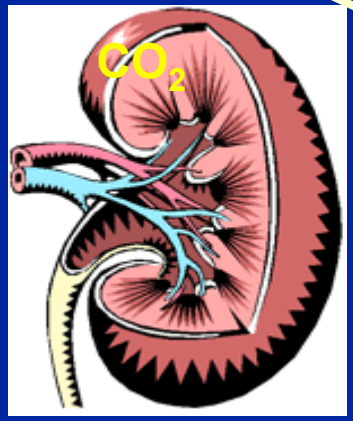


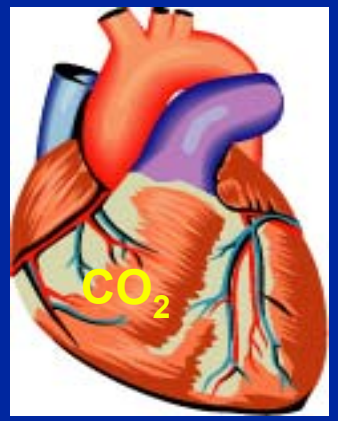
THE FASTED STATE (EARLY PHASE)
GLUCONEOGENESIS



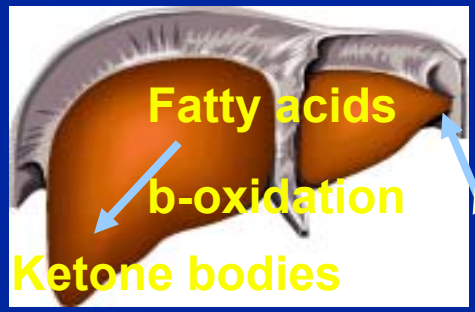
Glucose

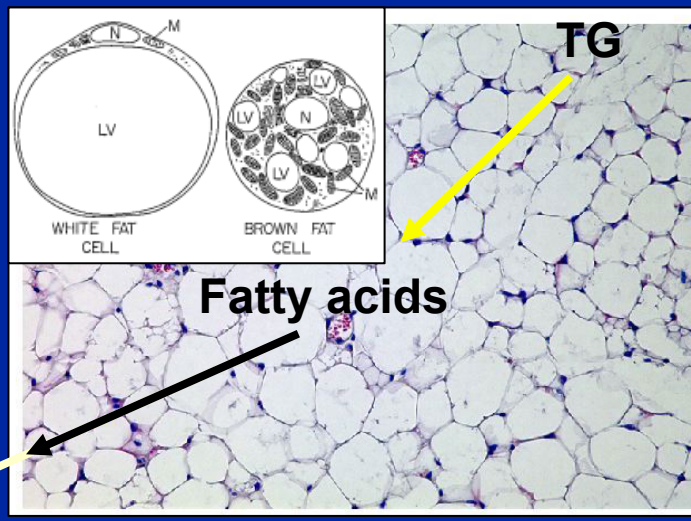
Fatty acids





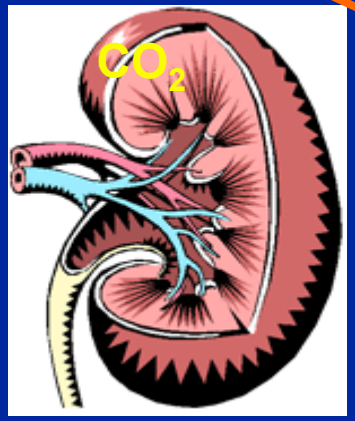
THE FASTED STATE (PROLONGED FASTING)
B-OXIDATION & KETOGENESIS

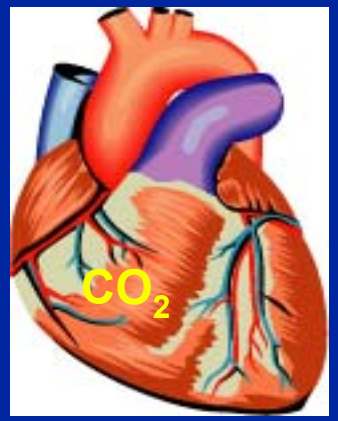




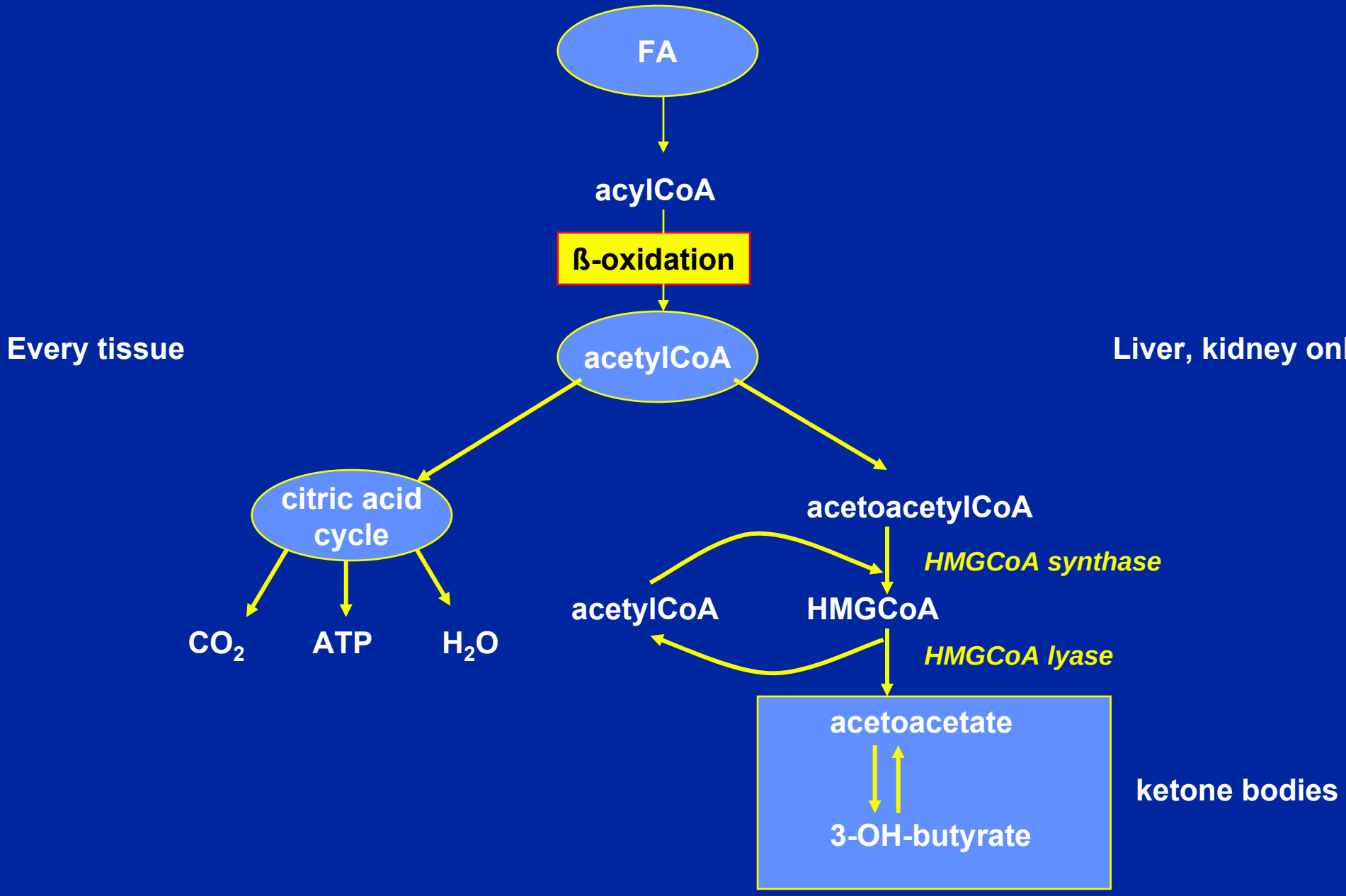
Ketone bodies

Fatty acids

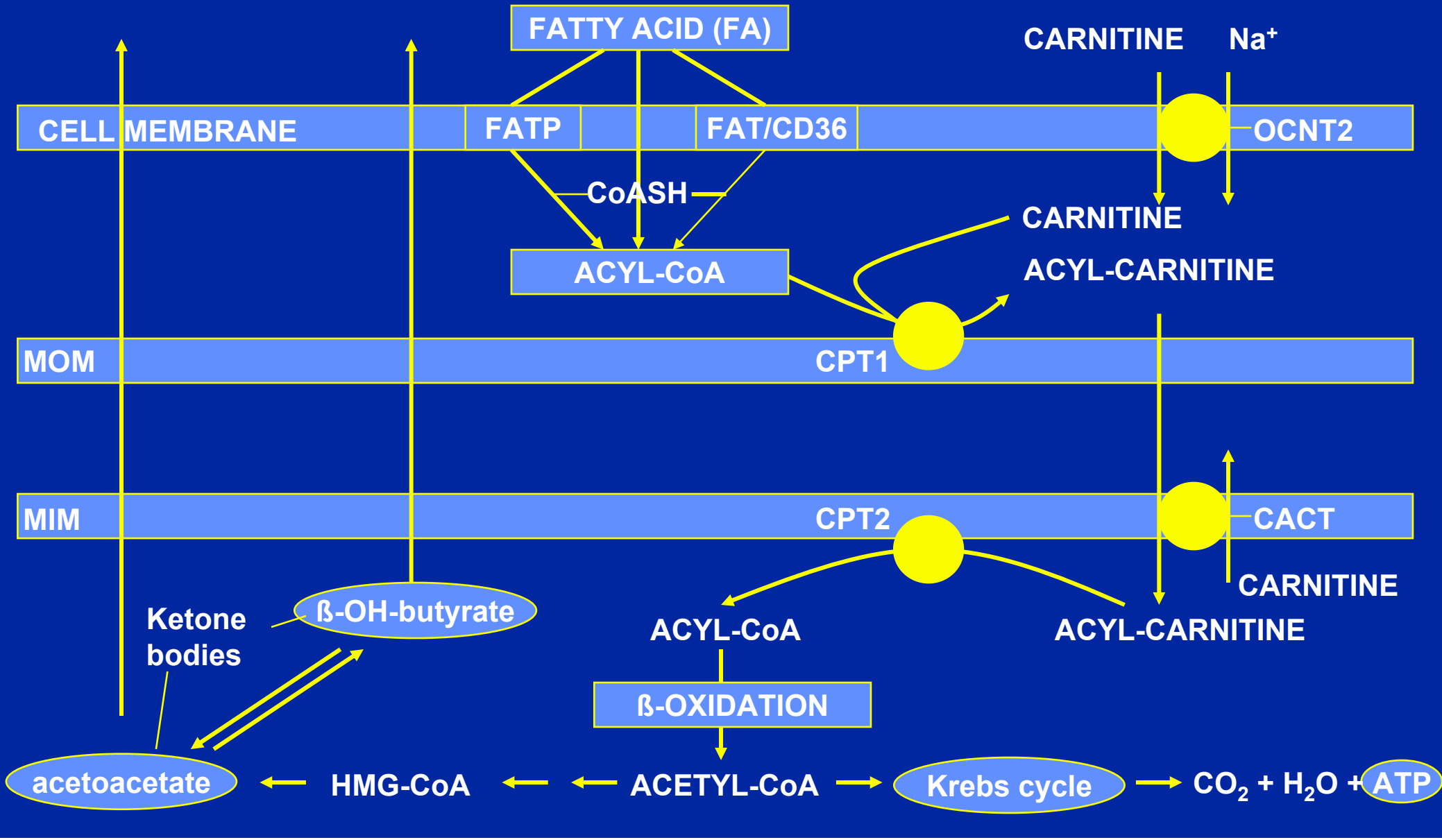




SIMPLIFIED SCHEME OF MITOCHONDRIAL FATTY ACID ß-OXIDATION



ENZYMOLOGY OF THE MITOCHONDRIAL ß-OXIDATION SYSTEM



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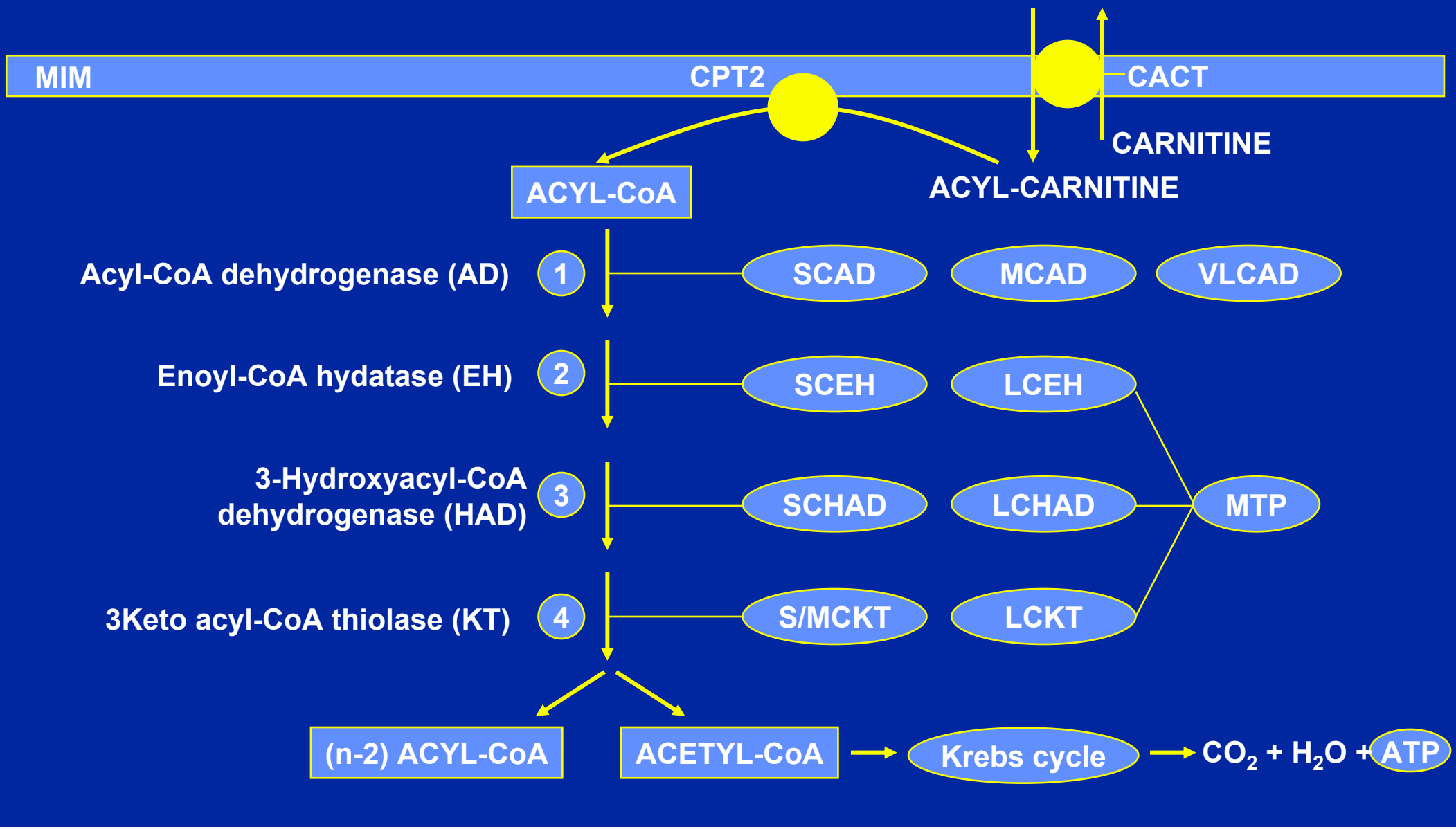
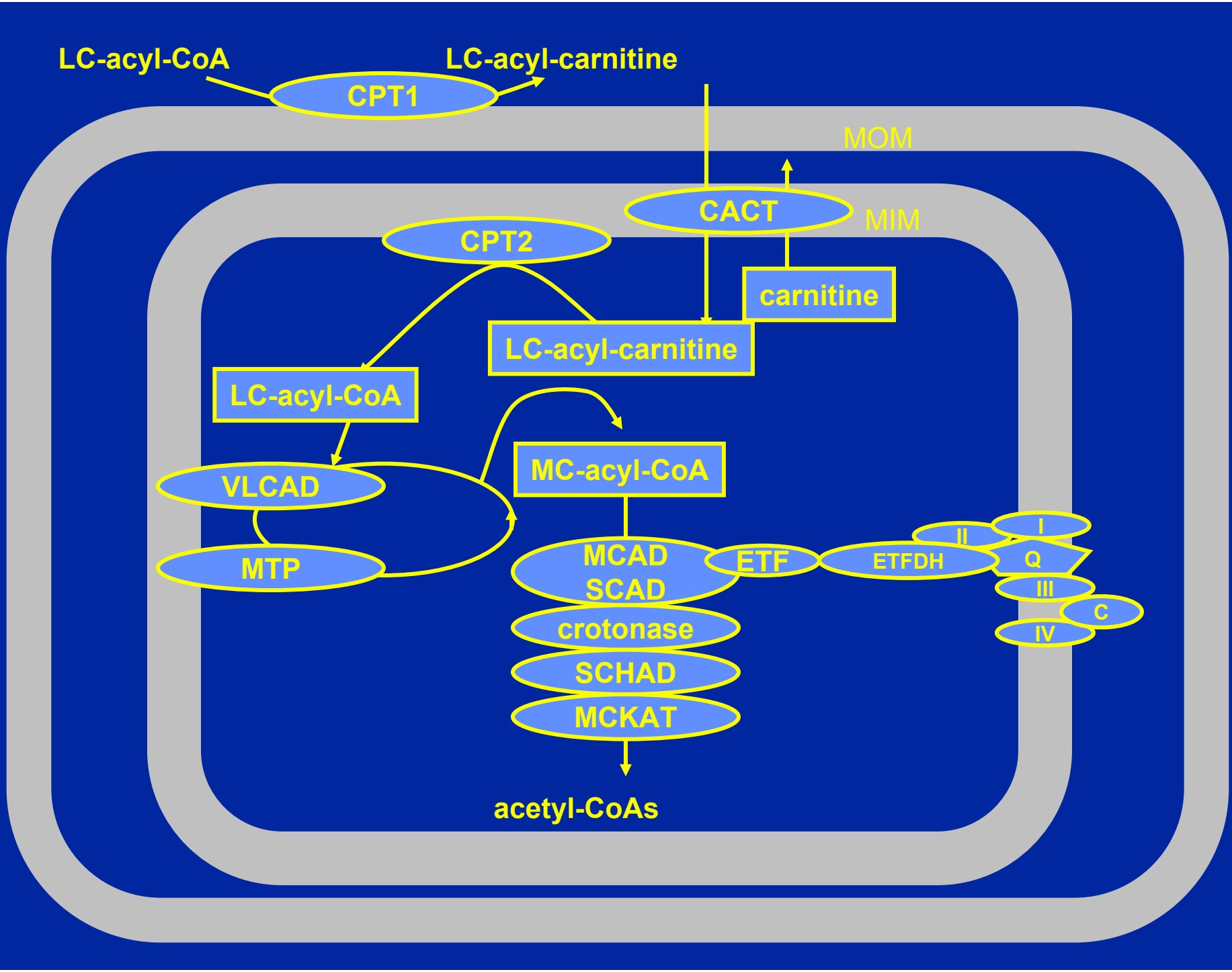



LC-acyl-CoA
LC-acyl-carnitine
CPT1
MOM
MIM
CACT
CPT2
carnitine
LC-acyl-carnitine
LC-acyl-CoA
MC-acyl-CoA
VLCAD
MTP
MCAD
SCAD
ETF
ETFDH
I
II
Q
III
C
IV
crotonase
SCHAD
MCKAT
acetyl-CoAs

DEFECTS OF THE CARNITINE CYCLE

Deficiency	Plasma carnitine (free)	Plasma acylcarnitines	Cardiomyopathy
CPT1	N - ↑	(C0/C16+C18) ↑	-
CACT	↓	C16, C18 ↑	+/-
CPT2	↓ - N	C16, C18 ↑	+/-

OVERVIEW OF THE MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASES (CPT)

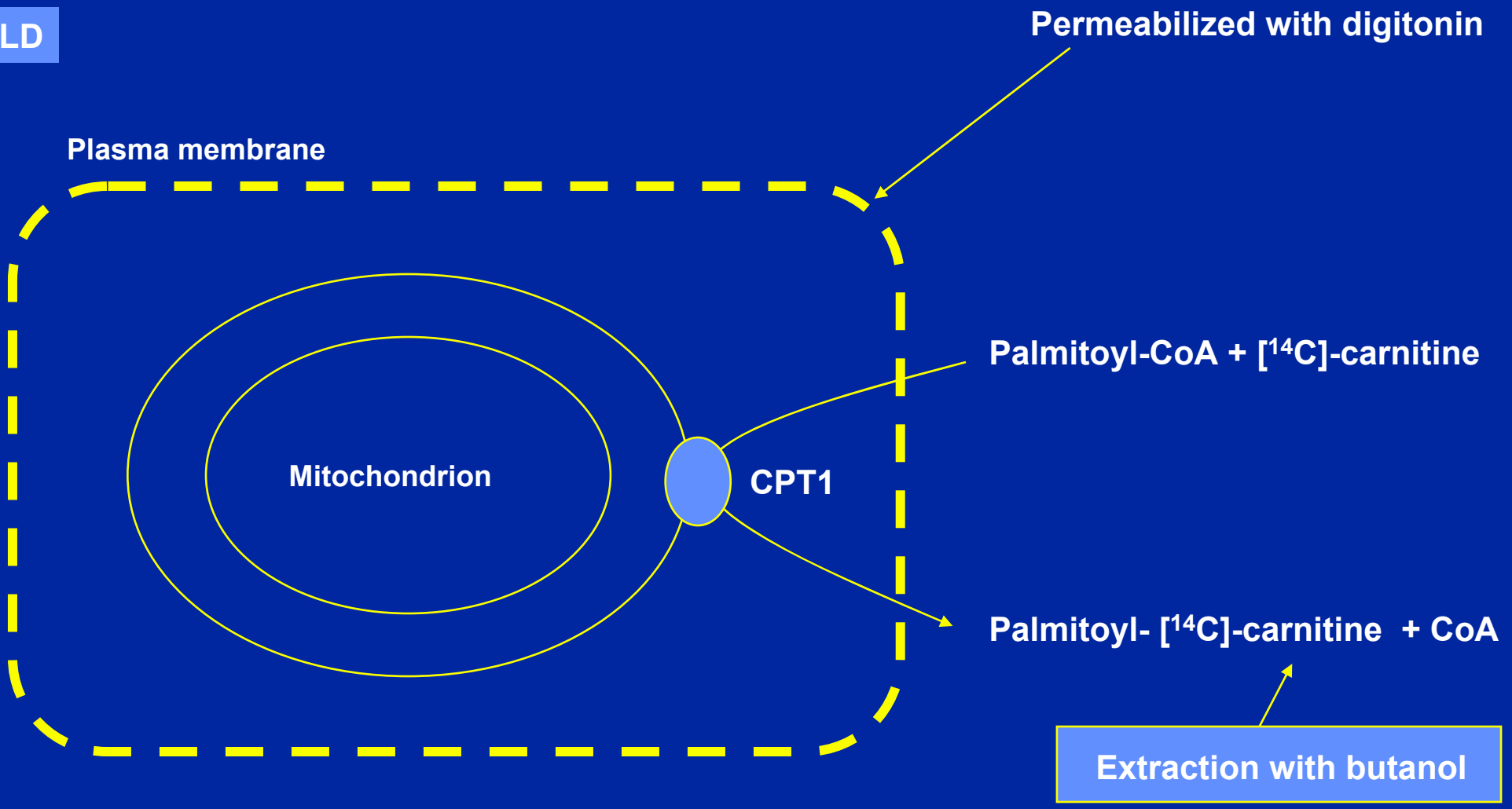
Feature	L-CPT1	M-CPT1	CPT2
Mass (kDa)	88	88	70
Malonyl-CoA (IC_{50})	2.5 µM	0.03 µM	-
Carnitine (K_m)	30 µM	500 µM	120 µM
Chromosome	11q13	22q13.3	1p32
Tissue expression			
• Liver	++++	-	+
• Skeletal muscle	(+)	++++	+
• Heart	(+)	+++	+
• Kidney	++++	(+)	+
• Lung	++++	(+)	+
• Spleen	++++	-	+
• Intestine	++++	-	+
• Pancreas	++++	-	+
• Brown adipose tissue	(+)	++++	+
• White adipose tissue	+	+++	+
• Ovary	++++	(+)	+
• Testis	(+)	++++	+
• Fibroblasts	++++	-	+
Human deficiency known	Yes	No	Yes

OVERVIEW OF THE CLINICAL AND BIOCHEMICAL FEATURES OF CPT1-DEFICIENCY IN A SERIES OF 25 PATIENTS

Feature	Abnormal/total	%
• Hypoketotic hypoglycemia	21/23	91
• Renal tubular acidosis	5/10	50
• Seizures	18/20	90
• Hepatomegaly	20/20	100
• Hemiplegia	4/4	100
• Coma	12/21	57
• Early death	3/21	14
• Cardiomyopathy	0/6	0
• Elevated free carnitine (>55)	14/21	67

CARNITINE PALMITOYLTRANSFERASE 1 (CPT1) ASSAY

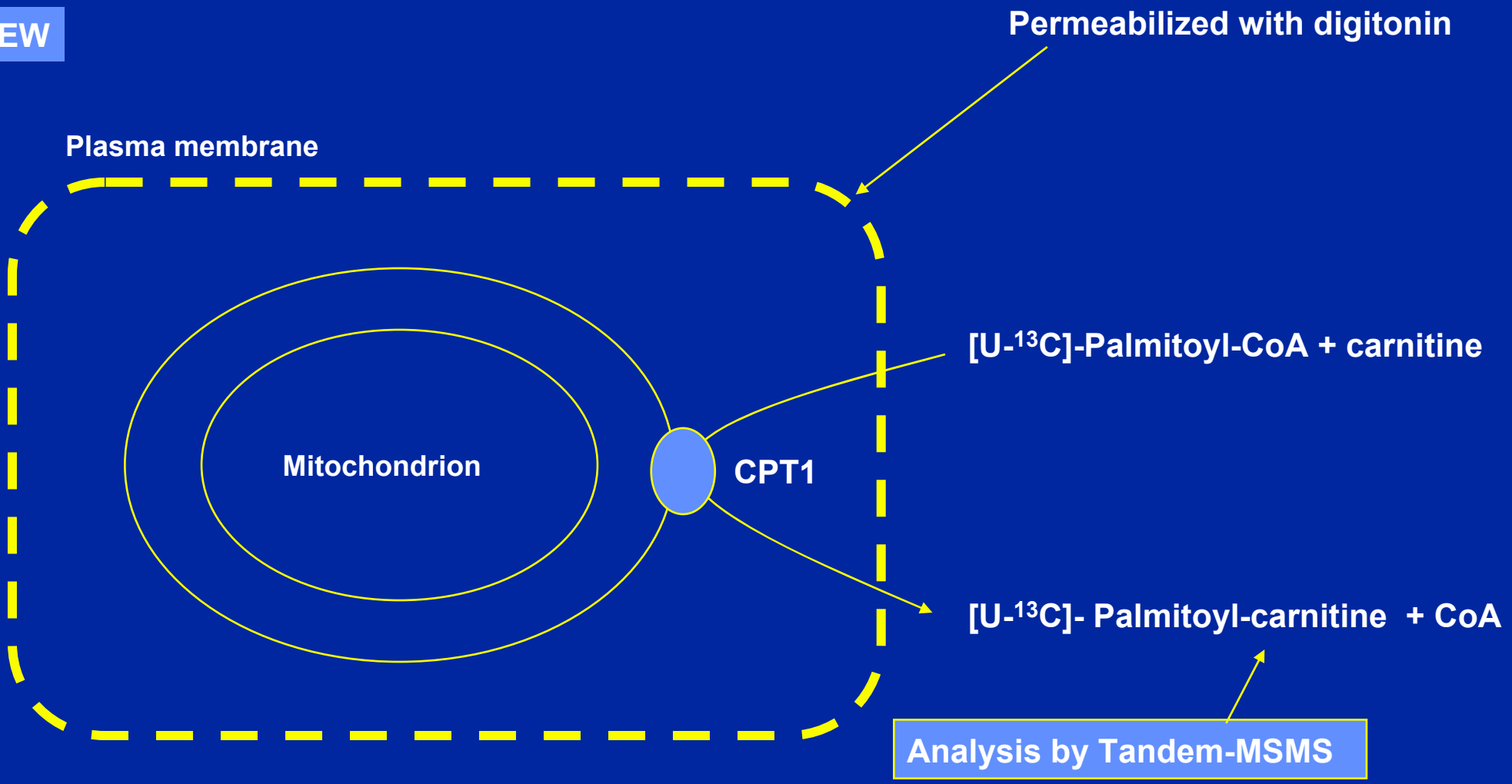
OLD



Works in fibroblasts as well as in lymphocytes
Performed in the absence and presence of malonyl-CoA, a specific inhibitor of CPT1

CARNITINE PALMITOYLTRANSFERASE 1 (CPT1) ASSAY

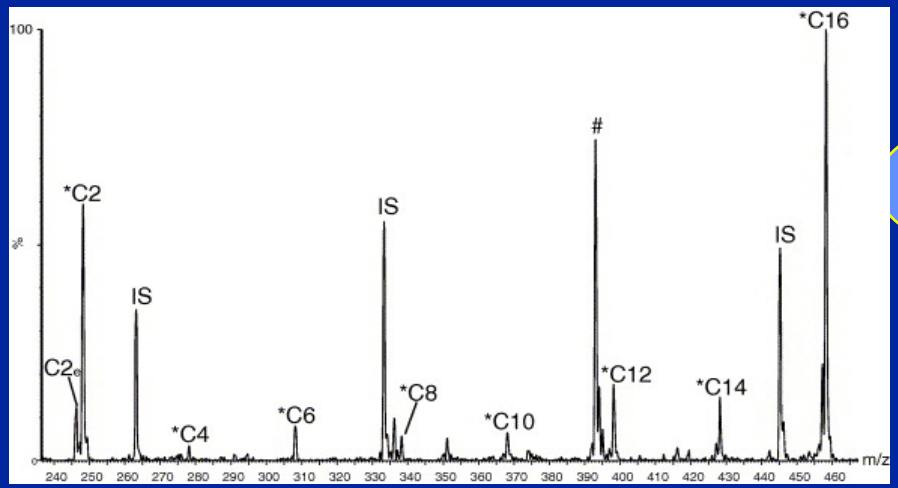
NEW



van Vlies etal Mol Genet Metab. 2007 Jan;90(1):24-9.

Works in fibroblasts as well as in lymphocytes
Performed in the absence and presence of malonyl-CoA, a specific inhibitor of CPT1

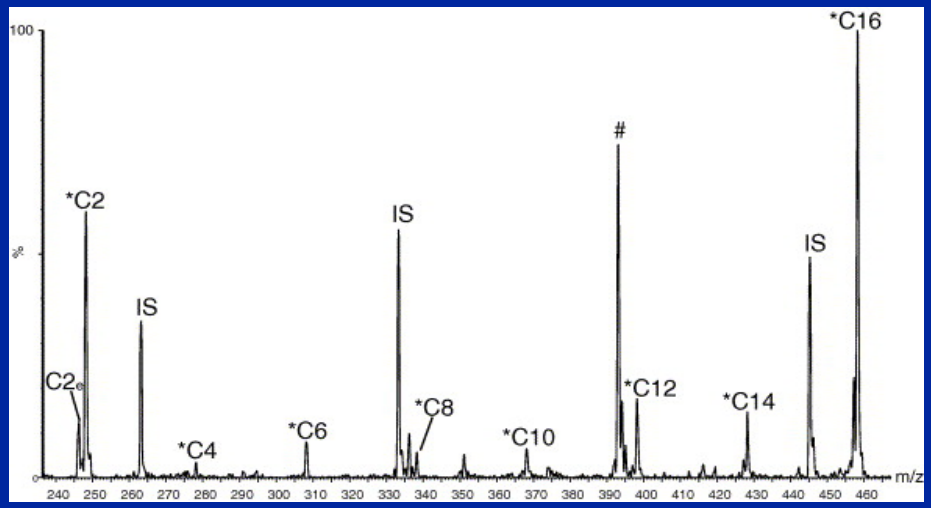
A NOVEL SENSITIVE CPT1 ASSAY

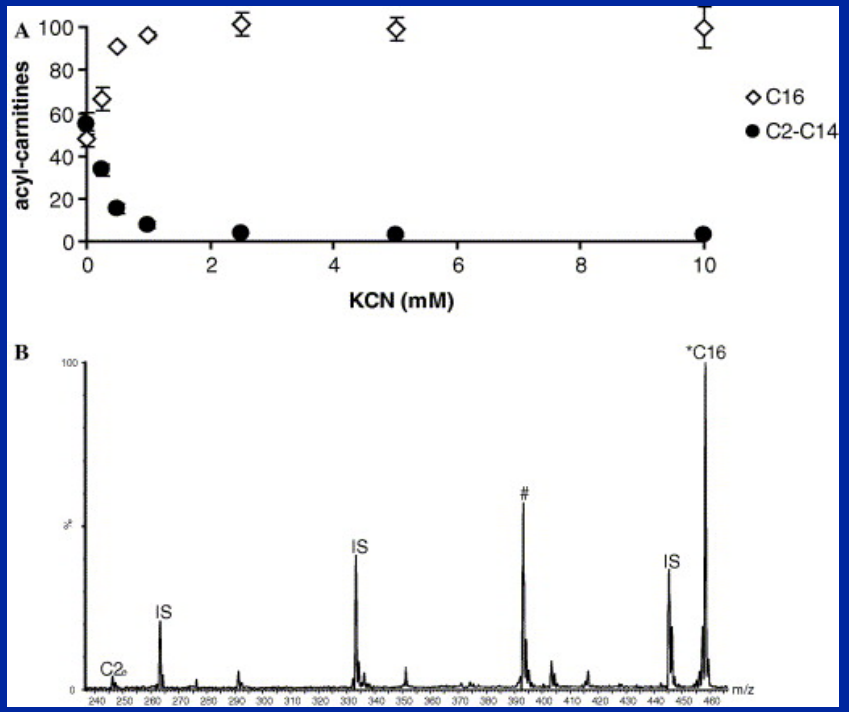


In the classic CPT1 assay, C16-carnitine is not the final endproduct!

Van Vlies et al, Mol Genet Metab. 90, 2007, 24

A NOVEL SENSITIVE CPT1 ASSAY

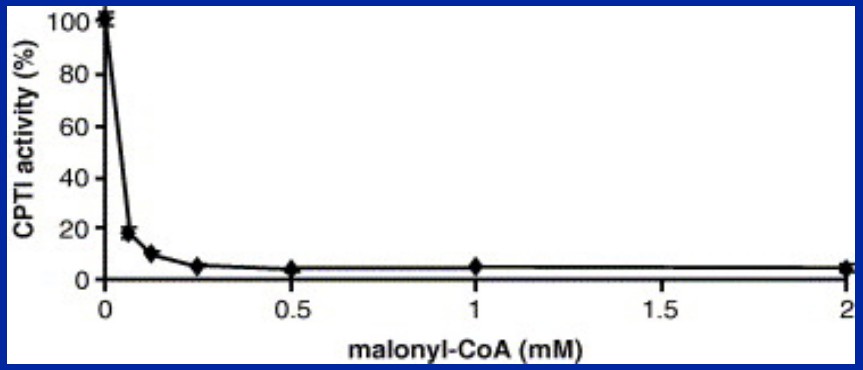




In the new CPT1 assay, which now includes 5 mM KCN, C16-carnitine is the final endproduct!

Van Vlies et al, Mol Genet Metab. 90, 2007, 24

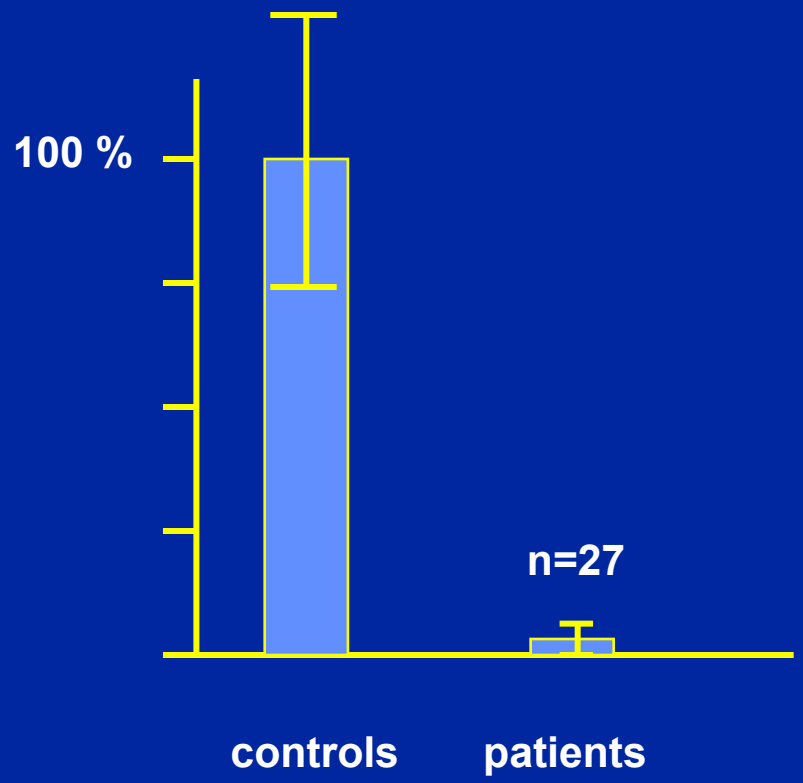
A NOVEL SENSITIVE CPT1 ASSAY



- Malonyl-CoA sensitivity of CPT1 using the new modified assay

Van Vlies et al, Mol Genet Metab. 90, 2007, 24

CARNITINE PALMITOYLTRANSFERASE 1 (CPT1) ASSAY



Patient U

- Girl, born January 2003
- Consanguineous parents (nephew-niece)
- December 2008: upper airway infection, food refusal
- Lethargy progressing into coma
- Hypoglycemia (1.8 and 2.2 mmol/l)
- ASAT, CRP and leukocytes ↑
- Complete and rapid recovery upon i.v. glucose.

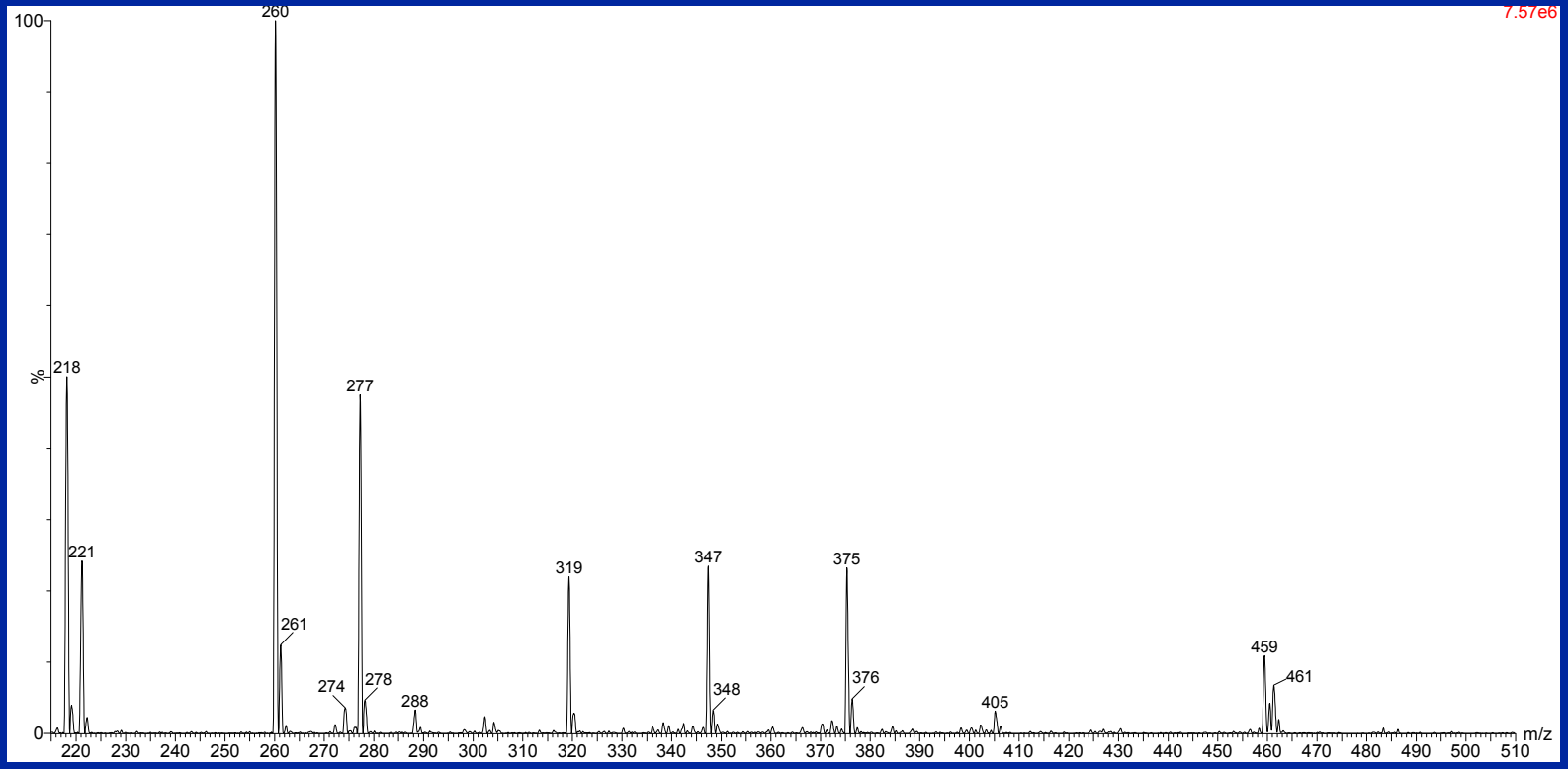
Patient U

METABOLITE INVESTIGATIONS

- Plasma (crisis) :
  - Acylcarnitines
  - Amino acids
- Urine (crisis) :
  - Acylcarnitines
  - Organic acids

Patient U

PLASMA ACYLCARNITINE PROFILE

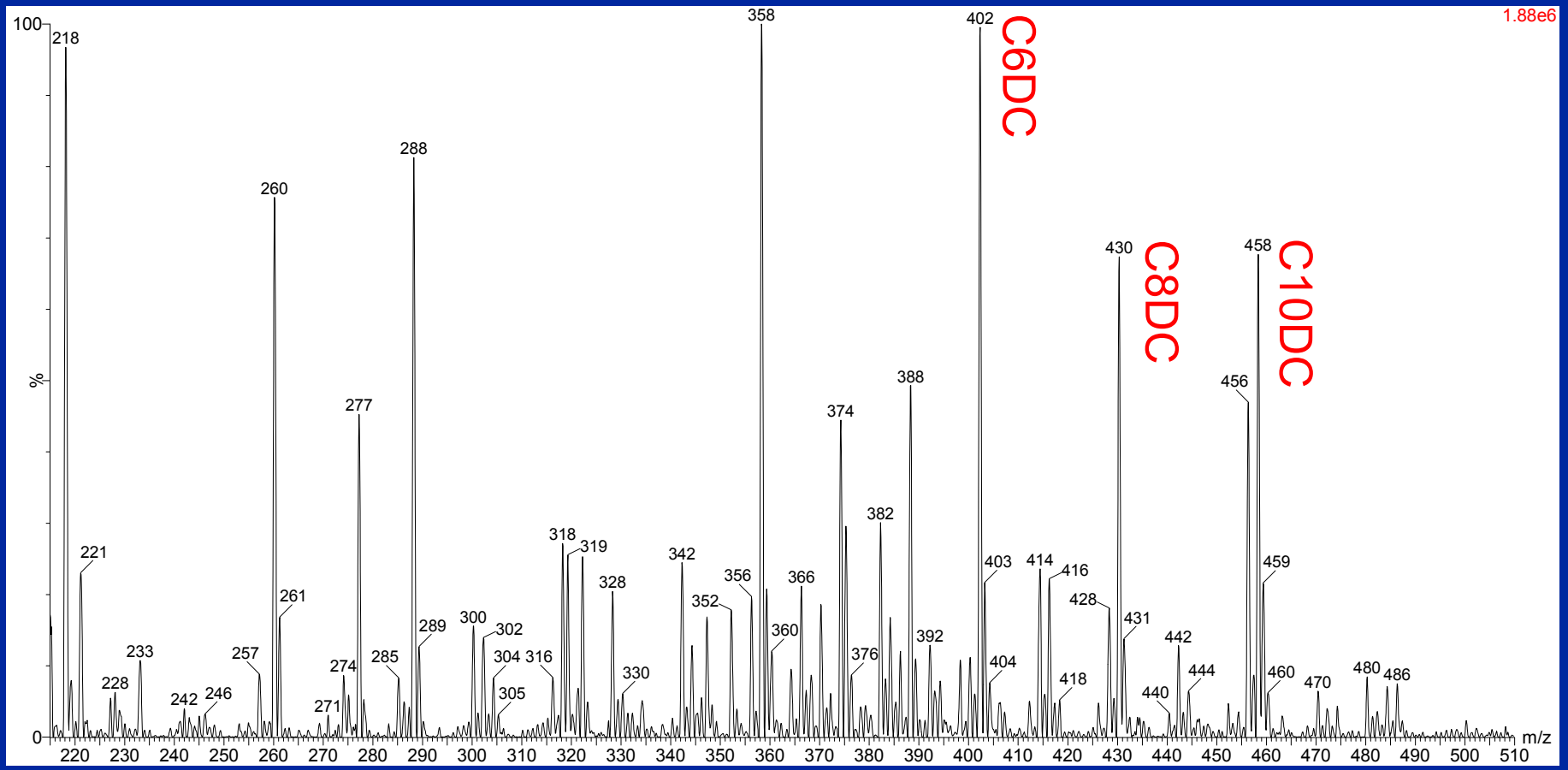


free carnitine	54.05
C2-carnitine	10.51
C3-carnitine	0.37
C4-carnitine	0.34
C5:1-carnitine	0.02
C5-carnitine	0.2
C4-3-OH-carnitine	0.16 +
C6-carnitine	0.03
C5-OH-carnitine	0.01
C8-carnitine	0.09
C3-DC-carnitine	0.08
C10:1-carnitine	0.11
C10:0-carnitine	0.14
C4-DC-carnitine	0.05
C5-DC-carnitine	0.05
C12:1-carnitine	0.06
C12:0-carnitine	0.07
C6-DC-carnitine	0.1 +
C12:1-OH-carnitine	0.02
C12-OH-carnitine	0.03
C5-3M-3OH-carnitine	0
C14:2-carnitine	0.07
C14:1-carnitine	0.04
C14:0-carnitine	0.04
C8-DC-carnitine	0.1 +
C14:1-OH-carnitine	0.02
C14-OH-carnitine	0
C16:1-carnitine	0.03
C16-carnitine	0.08
C10-DC-carnitine	0.11 +
C16:1-OH-carnitine	0.03 +
C16-OH-carnitine	0.01
C18:2-carnitine	0 -
C18:1-carnitine	0 -
C18:0-carnitine	0.03

Relatively normal profile but high carnitine with low long-chain acylcarnitines

Patient U

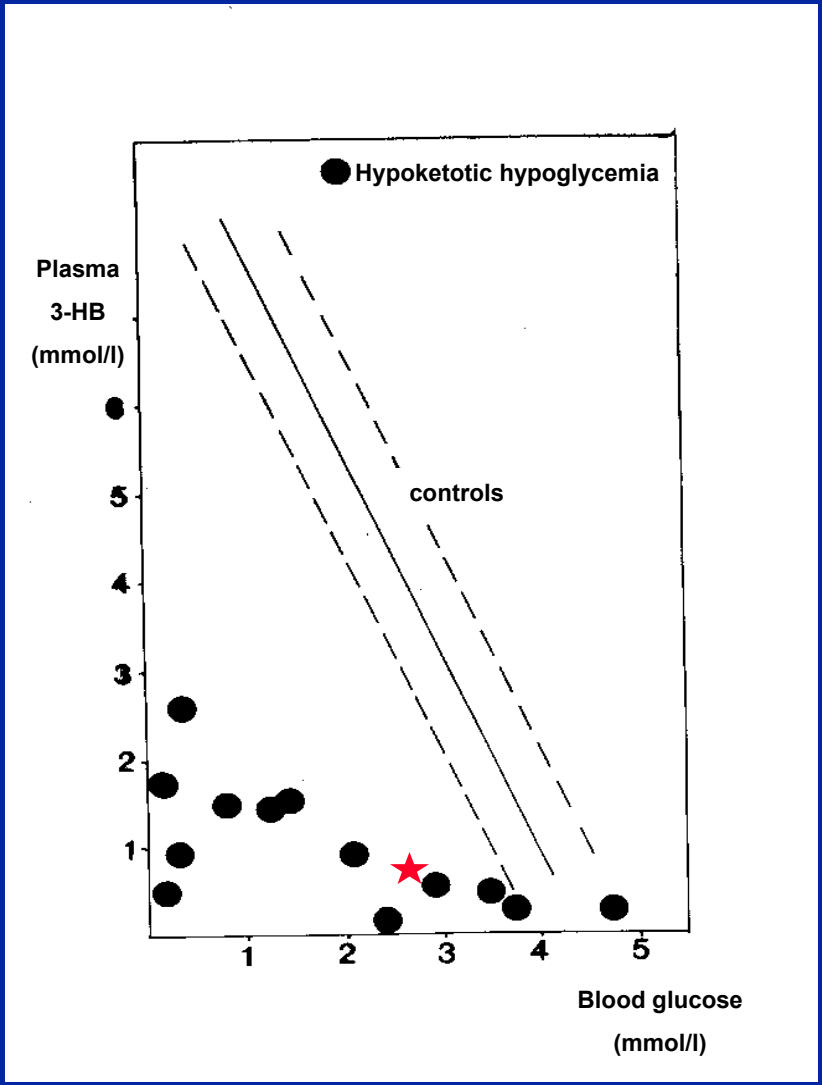
URINARY ACYLCARNITINE PROFILE



Dicarboxylic acylcarnitines

Patient U

FASTING TEST



Patient U

ENZYME AND DNA STUDIES

- CPT1-activity in fibroblasts : 0.08 nmol/(min.mg protein)

  Controls : 1.34 ± 0.57 nmol/(min.mg protein)

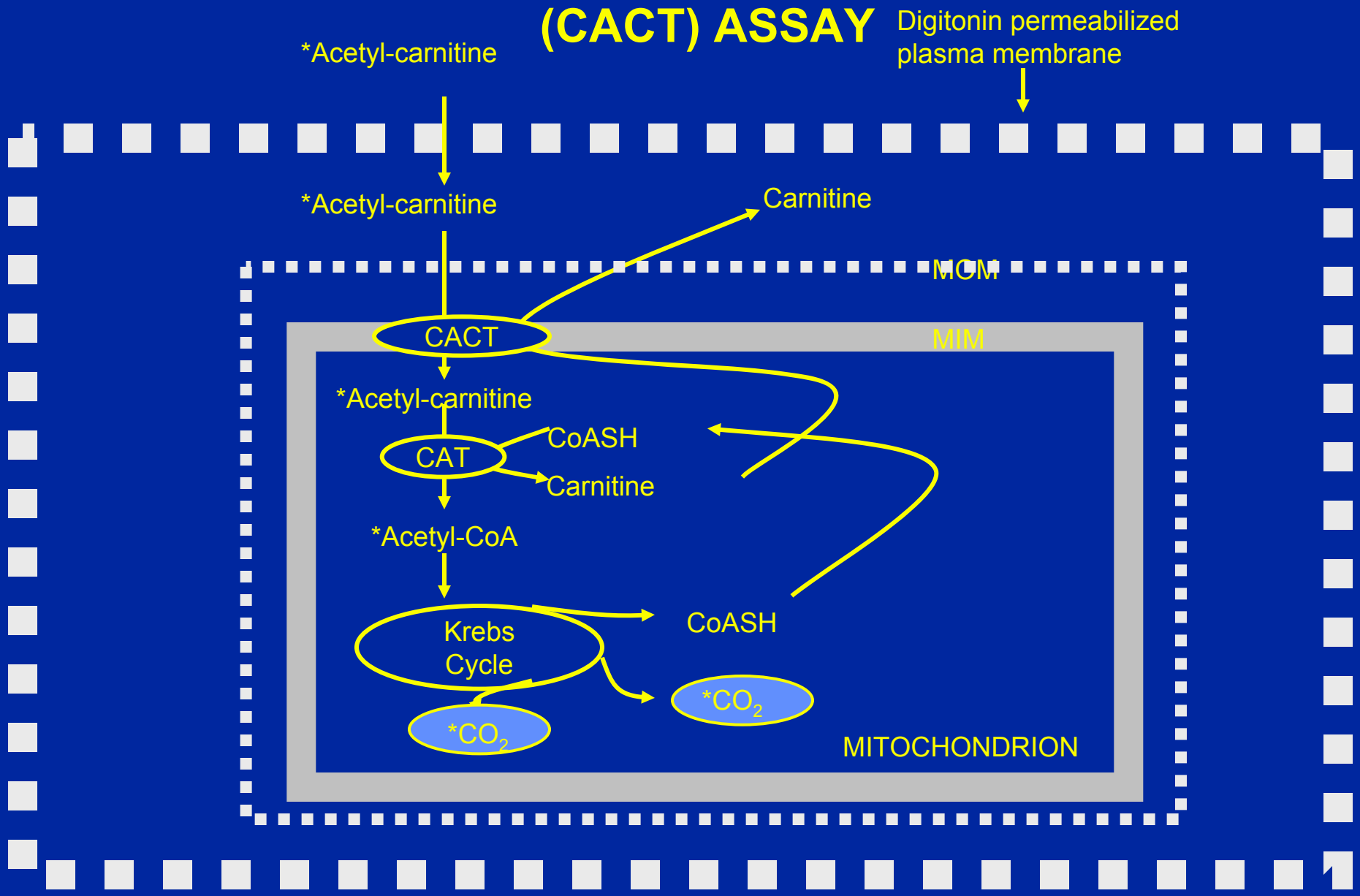
- Homozygous 1318G>A (A440T)

Brother and sister of patient both homozygous for the same mutation !!

KEY FEATURES OF THE MITOCHONDRIAL CARNITINE / ACYLCARNITINE TRANSLOCATOR

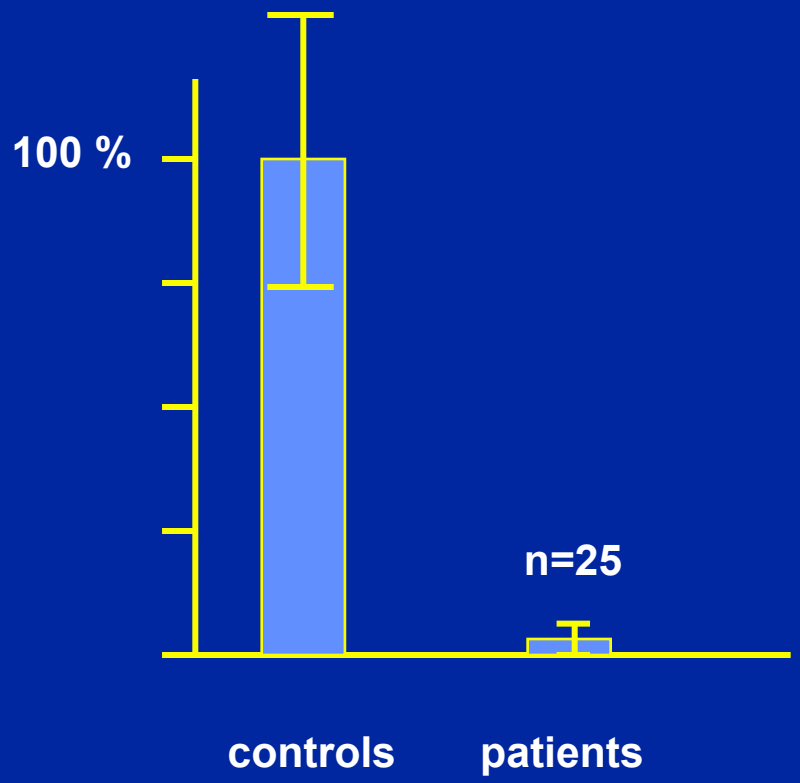
1. Belongs to the family of mitochondrial solute carriers
2. 33 kDa integral membrane protein
3. Six transmembrane spanning elements
4. Single protein present in mitochondria of *all* tissues
5. Official gene name : *SLC25A20*

MITOCHONDRIAL CARNITINE/ACYLCARNITINE TRANSPORTER (CACT) ASSAY



Works in fibroblasts as well as in lymphocytes

MITOCHONDRIAL CARNITINE/ACYLCARNITINE TRANSPORTER (CACT) ASSAY



CLINICAL AND BIOCHEMICAL FEATURES OF CACT DEFICIENCY

1. Most patients present in the neonatal period with seizures, heart problems (arrhythmias, cardiomyopathy, heart block) and apnea.
2. Often triggered by fasting or infections
3. Primary organs involved : heart, liver, skeletal muscle, and brain
4. Patients with presentations later in life also described
5. Most patients show hypoketotic hypoglycemia, hyperammonemia with elevated CK and liver enzymes
6. Acyl-carnitine profile : C16:0, C18:0, C18:1 and C18:2

   PS Pattern indistinguishable from CPT2 deficiency

Molecular basis of carnitine acyl-carnitine translocase (CACT) deficiency

in a patient with severe presentation

and with mild presentation

Patient 1: severe presentation

Presented at 36 hours of age:

- sudden cardiorespiratory insufficiency
- extreme hypoglycaemia
- hyperammonaemia

carnitine and low fat diet

died at 24 months of age

(hypotrophic cardiomegaly)

Patient 2: mild presentation

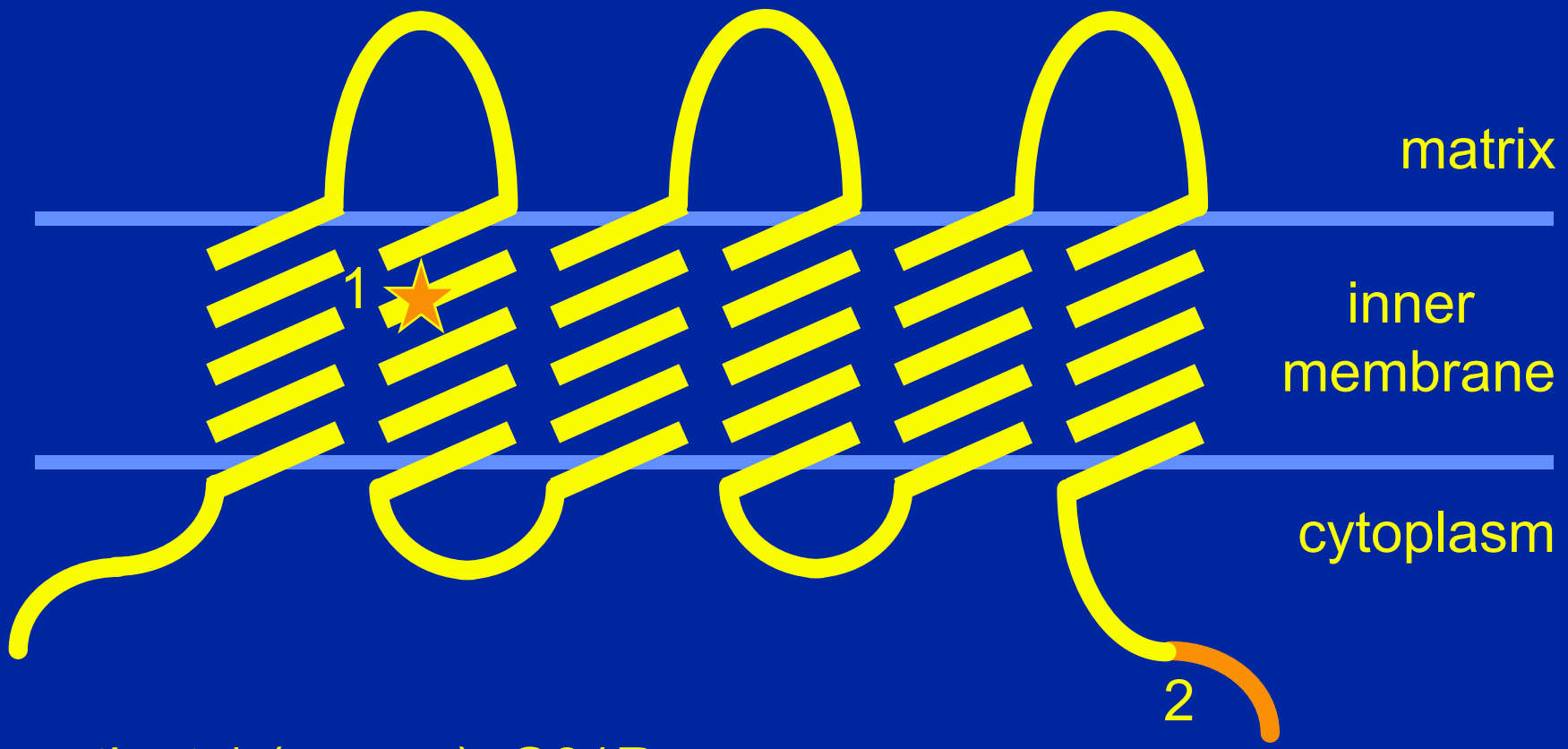
Severe neonatal condition with:

- hypoglycaemia
- cardiac arrest
- hepatomegaly and hepatic disfunction
- lethargic during mild viral infections

At present (9y), physical and neurophysiological developement essentially normal

Biochemical investigations in fibroblasts

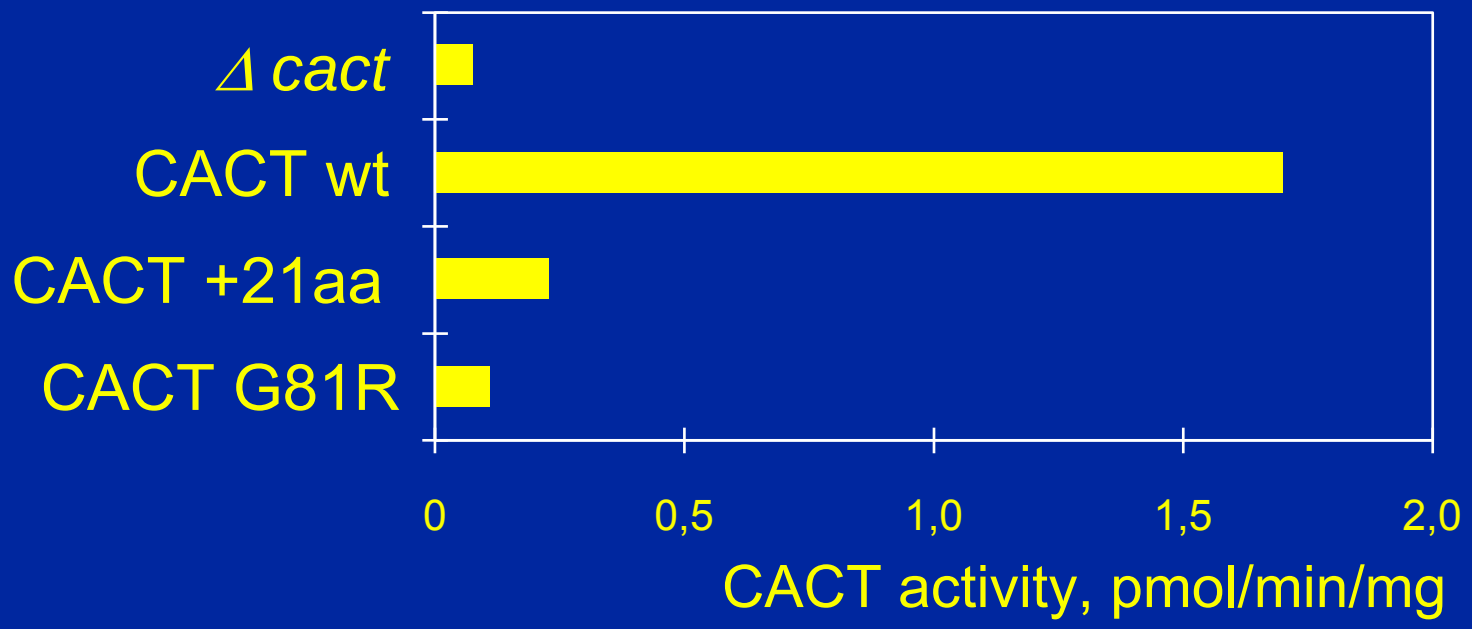
	fatty acid oxidation	CACT activity
controls	7.2 ± 3.2	51 ± 15
patient 1 (severe)	< 0.1	not detectable
patient 2 (mild)	2.4	< 0.1



patient 1 (severe): G81R

patient 2 (mild): 21 aa extension

Expression of human CACT in *S.cerevisiae*



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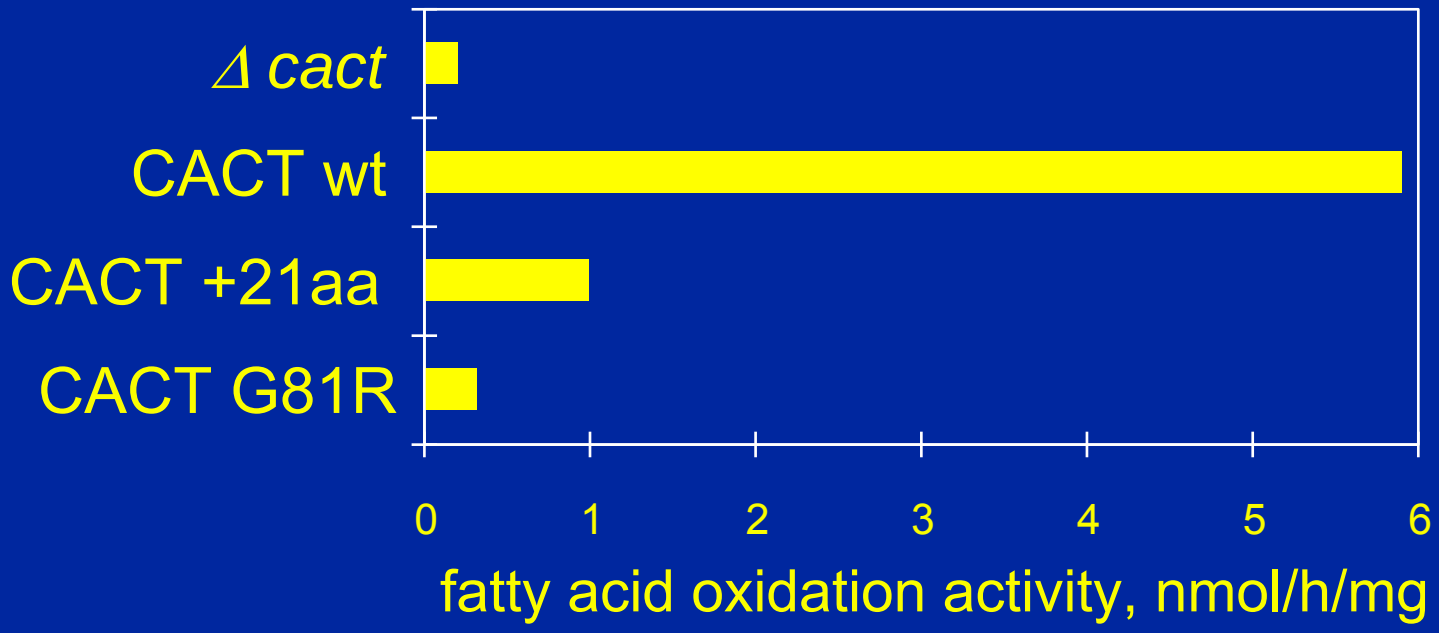


Conclusions -1-

Patient 1 (severe phenotype)

Findings in fibroblasts:

- no CACT activity
- very low fatty acid oxidation rate

Molecular findings:

- G81R mutation
- expressed protein shows no activity

Conclusions -2-

Patient 2 (mild phenotype)

Findings in fibroblasts:

- very low CACT activity
- fatty acid oxidation partly impaired

Molecular findings:

- C-terminal 21 amino acid extension
- expressed protein has residual activity

Conclusions -3-

The activity of both mutant CACTs as determined by expression in the *Δcact* yeast mutant, reflects the findings in the corresponding patients.

KEY FEATURES OF THE MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE 2

1. 80 kDa peripheral membrane protein
2. No transmembrane spanning elements
3. Single CPT2 present in mitochondria of *all* tissues
4. Catalyzes the reversible reaction between free carnitine and acyl-CoAs

CARNITINE PALMITOYLTRANSFERASE 2 (CPT2) ASSAY

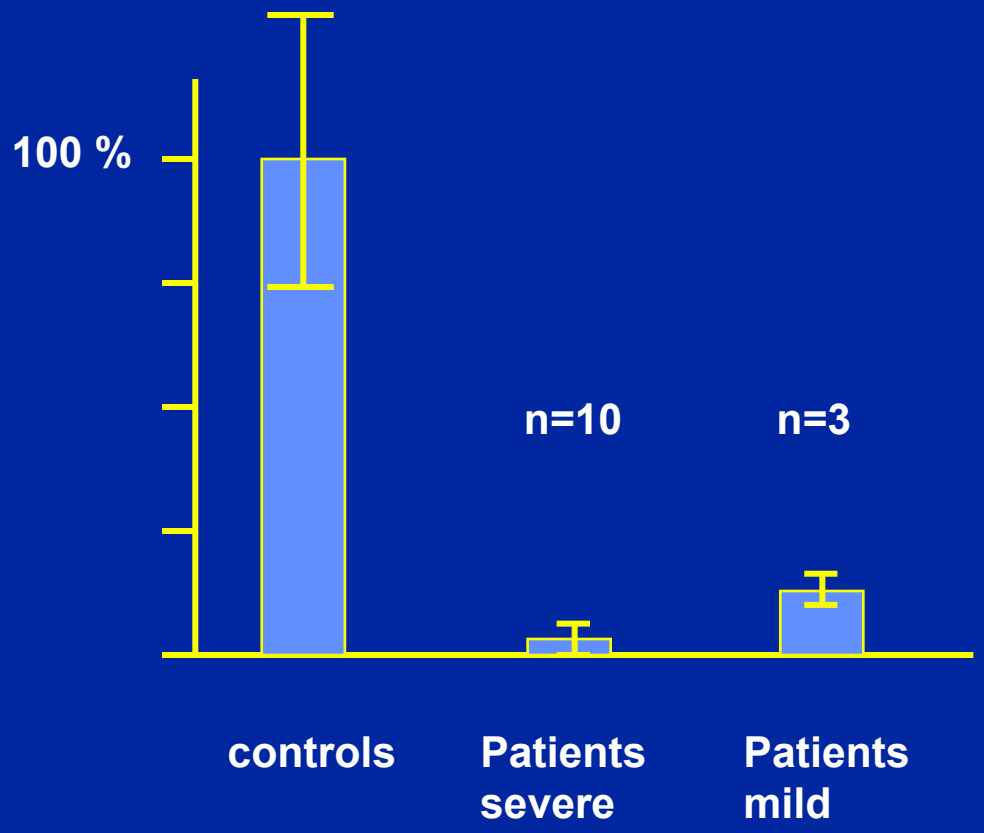
- Orginally measured in the reverse direction

$[^{14}C]$ palmitoyl-CoA + carnitine $\longrightarrow$ $[^{14}C]$ palmitoyl-carnitine + CoASH

- Now modified and measured in the physiological direction using HPLC with or without tandem MS

palmitoyl-carnitine + CoASH $\longrightarrow$ palmitoyl-CoA + carnitine

CARNITINE PALMITOYLTRANSFERASE 2 (CPT2) ASSAY



CLINICAL AND BIOCHEMICAL FEATURES OF CPT2 DEFICIENCY

1. Three distinct phenotypes described although intermediate forms have also been published (CPT2 spectrum).
2. Classical muscular form: onset in teenagers/young adults, recurrent episodes of rhabdomyolysis triggerd by (prolonged) exercise, fasting or febrile illness.
3. Infantile, hepato-cardio-muscular form: hypoketotic hypoglycemia, liver failure, cardiomyopathy and periferal myopathy often with sudden death.
4. Neonatal form: resembles the infantile form but in addition: facial dysmorphia, cerebral malformations, and renal cysts.

   PS Reminiscent of Zellweger syndrome and GA2.
5. Acylcarnitine profile: elevated C16:0, C18:0, C18:1, and C18:2

FATTY ACID OXIDATION DEFICIENCIES IN THE MOUSE

Several mouse models with specific defects in the ß-oxidation have been generated

Disease	Human deficiency	Mouse model	Reference
OCTN2 / Primary carnitine deficiency	+	+	Koizumi et al. 1988; Kuwajima et al. 1991
CPT1a	+	†	Nyman et al. 2005
CPT1b	?	†	Ji et al. 2008
LCHAD / MTP	++	++	Ibdah et al. 2001
VLCAD	++	+/−	Cox et al. 2001; Exil et al. 2003
LCAD	?	+	Kurtz et al. 1998
MCAD	−(++)	+/−	Tolwani et al. 2005
SCAD	−(?)	+/−	Wood et al. 1989; Schiffer et al. 1989
DCI	?	+/−	Janssen and Stoffel 2002
DECR	++ (?)	+/−	Miinalainen et al. 2009

† Embryonic lethal ? Unknown − No phenotype or asymptomatic +/− Mild, + moderate, ++ severe

Such models are of great importance to investigate pathophysiological mechanisms and test new treatment strategies.

FATTY ACID OXIDATION DEFICIENCIES IN THE MOUSE

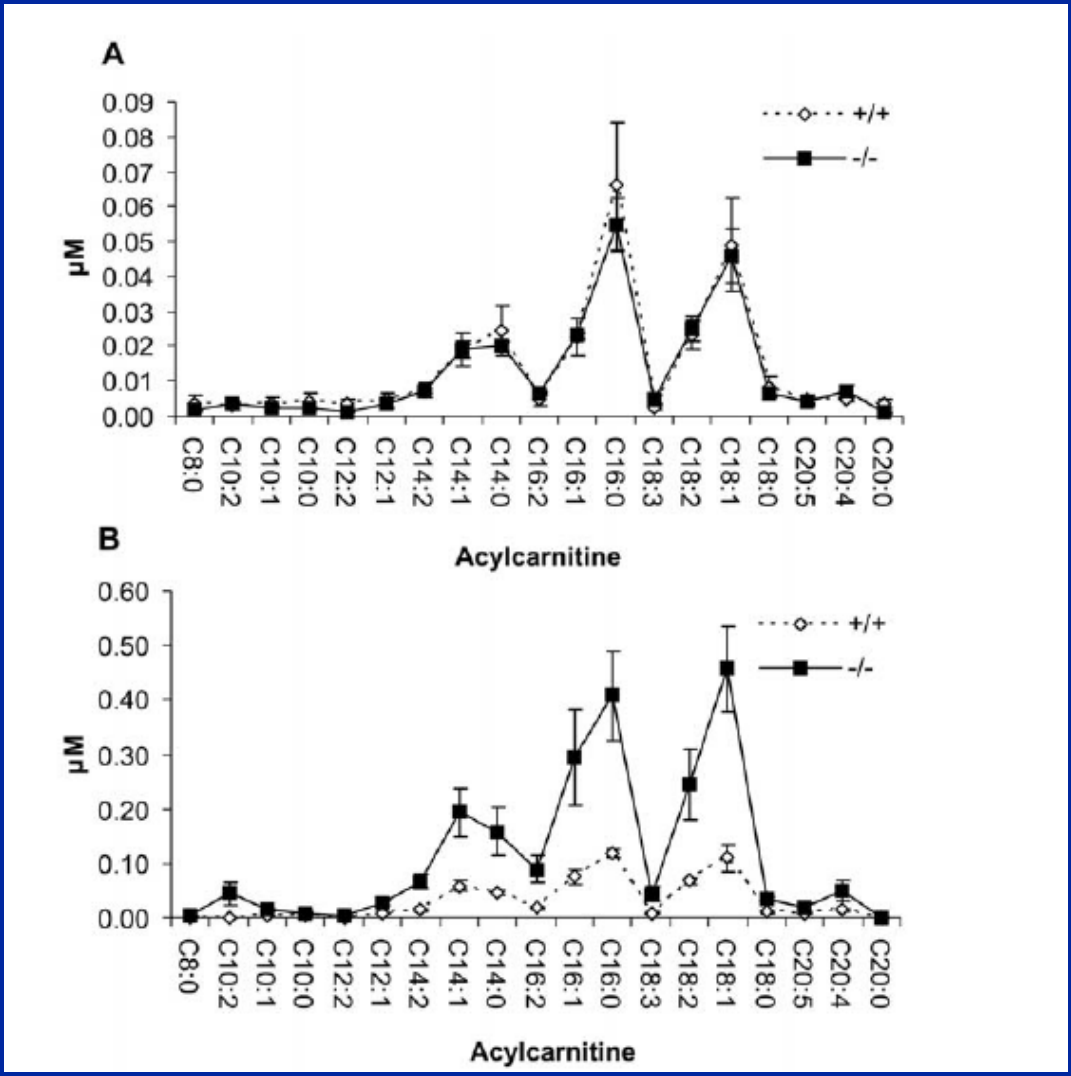
Such single enzyme deficiencies in the mouse may also be very helpful for the identification of still unidentified FAO disorders

Examples :

- 2,4-dienoyl-CoA reductase deficiency
- 3,2-trans-enoyl-CoA isomerase deficiency

FATTY ACID OXIDATION DEFICIENCIES IN THE MOUSE

2,4-DIENOYL-CoA REDUCTASE DEFICIENCY



Most diagnostic metabolite : 2,4-decadienoyl-carnitine

2,4-Dienoyl-Coenzyme A Reductase Deficiency: A Possible New Disorder of Fatty Acid Oxidation

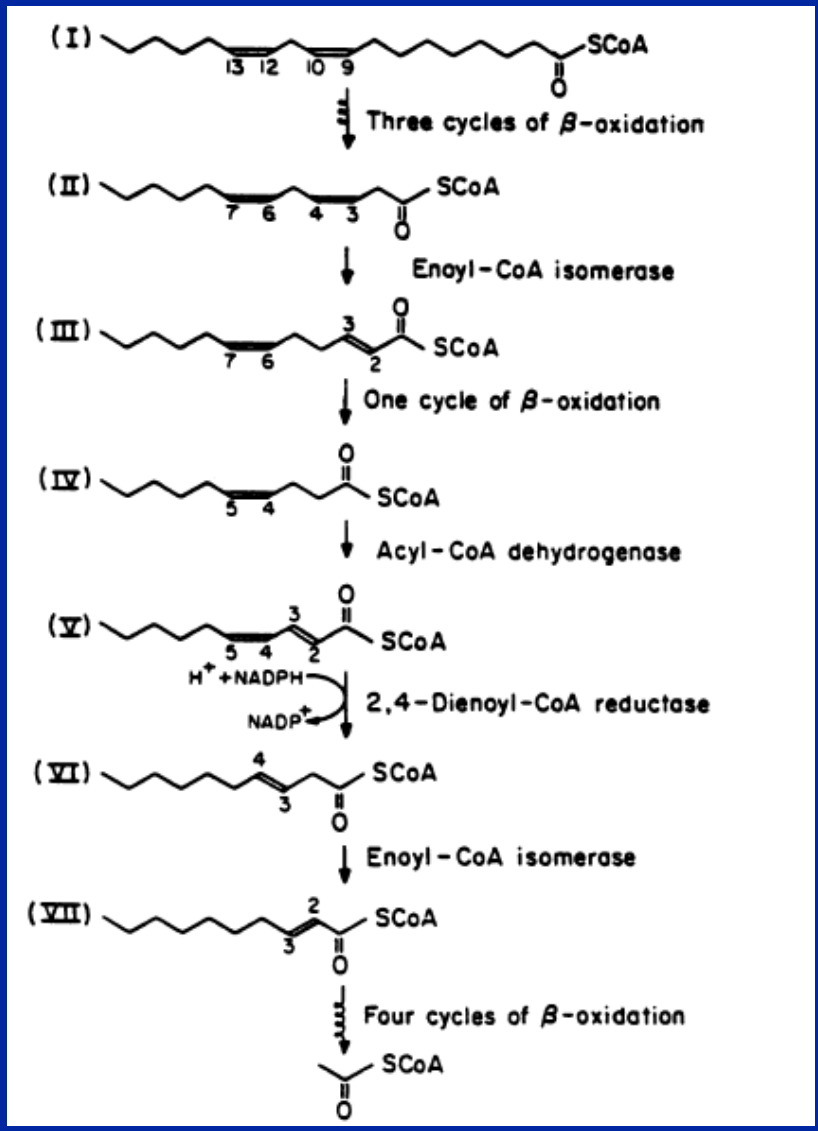
C. R. Roe, D. S. Millington, D. L. Norwood, N. Kodo, H. Sprecher,* B. S. Mohammed,* M. Nada,‡ H. Schulz,‡ and R. McVie§

(J. Clin. Invest. 1990. 85:1703-1707.)

Accumulation of 2-*trans*,4-*cis*-decadienoylcarnitine

Table I. 2,4-Dienoyl-CoA Reductase Activity in Liver and Psoas Muscle of a Patient with a Suspected Deficiency in the Oxidation of Polyunsaturated Fatty Acids

Tissue and source	2,4-Dienoyl-CoA reductase activity	
	2t,4c*	2t,4t‡
	nmol/min/mg of protein	
Liver, control	13±2.6 (100%)	8.4±2.2 (100%)
Liver, patient	5.1 (40%)	5.5 (65%)
Muscle, control	4.6±0.8 (100%)	3±0.28 (100%)
Muscle, patient	0.8 (17%)	1.24 (43%)



FATTY ACID OXIDATION DEFICIENCIES IN THE MOUSE

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 22, Issue of May 31, pp. 19579–19584, 2002

Printed in U.S.A.

Disruption of Mitochondrial β-Oxidation of Unsaturated Fatty Acids in the 3,2-*trans*-Enoyl-CoA Isomerase-deficient Mouse*

Received for publication, November 16, 2001, and in revised form, March 11, 2002
Published, JBC Papers in Press, March 26, 2002, DOI 10.1074/jbc.M110993200

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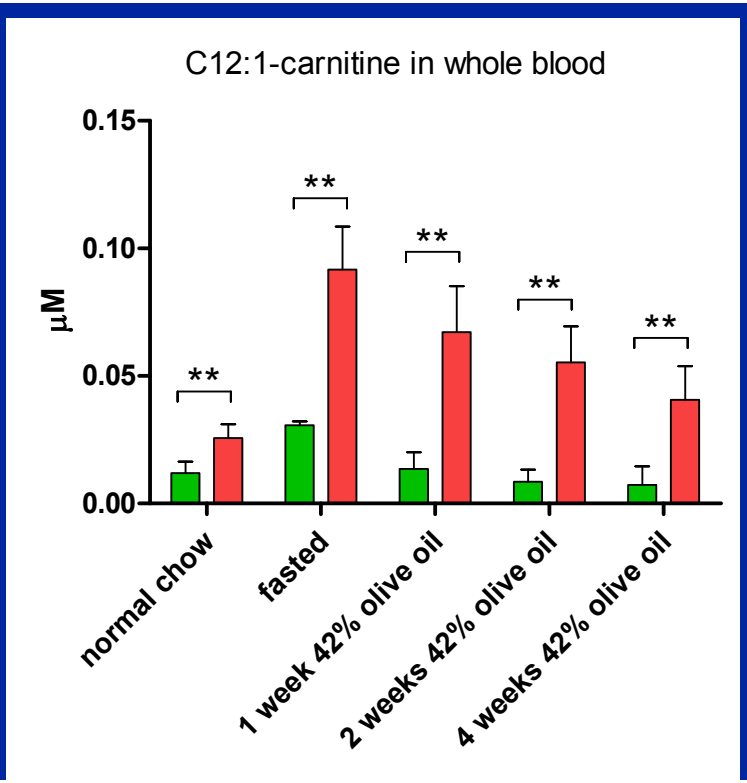
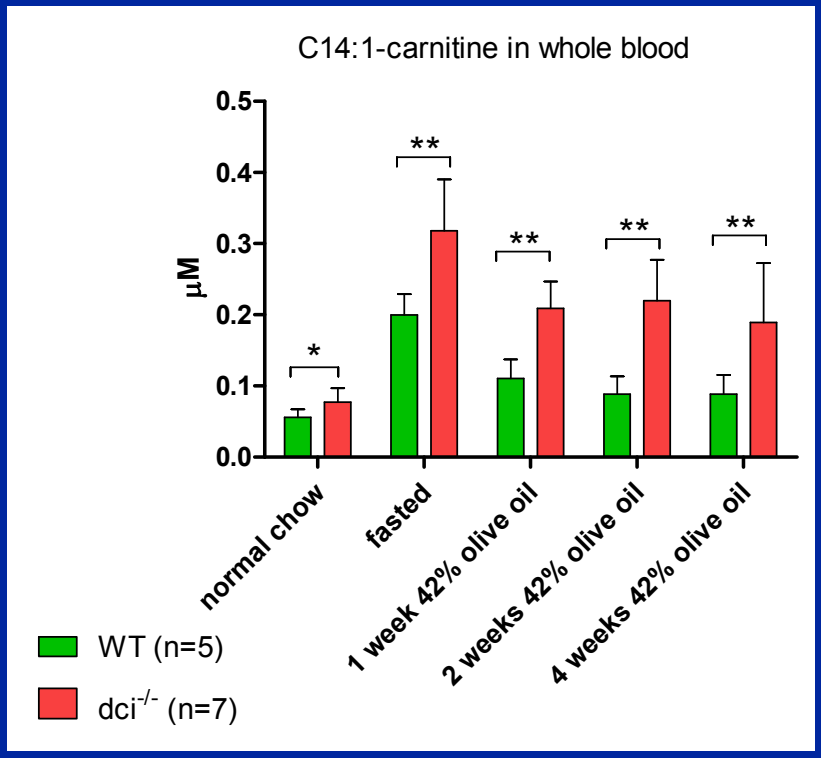
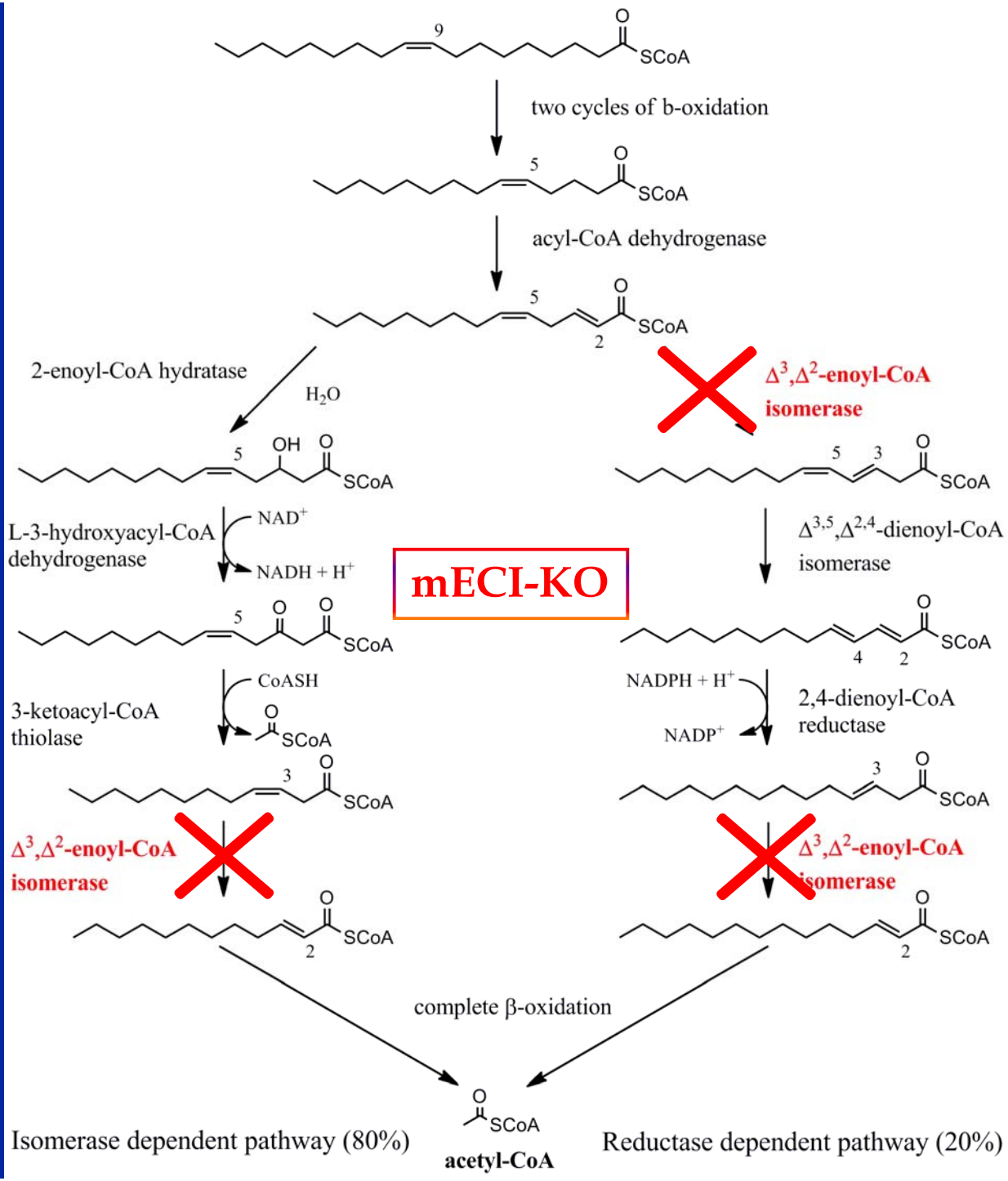




Beta-oxidation of oleoyl-CoA (C18:1)



cis 3-C12:1-CoA

Cis-3-C12:1-CoA

trans 2-C12:1-CoA

CPT2

Cis-3-C12:1-carnitine

ACAD9

- First identified by Zhang etal (2002) BBRC 297, 1033-1044
- Member of the ACAD-family
- Closest homologue : VLCAD ($M_w \approx 70$ kDa ; amino acid sequence: 47% identical, 65% similar to VLCAD)
- Like VLCAD, ACAD9 is a dimer bound to the mitochondrial innermembrane
- Maximal activity with long-chain unsaturated acyl-CoA's
- Tissue distribution : ACAD9 expression especially high in brain (frontal cortex, hippocampus, cerebellum) where VLCAD expression is low to absent.
- PS : ACAD9 expression mimicks that of LCHAD/MTP.

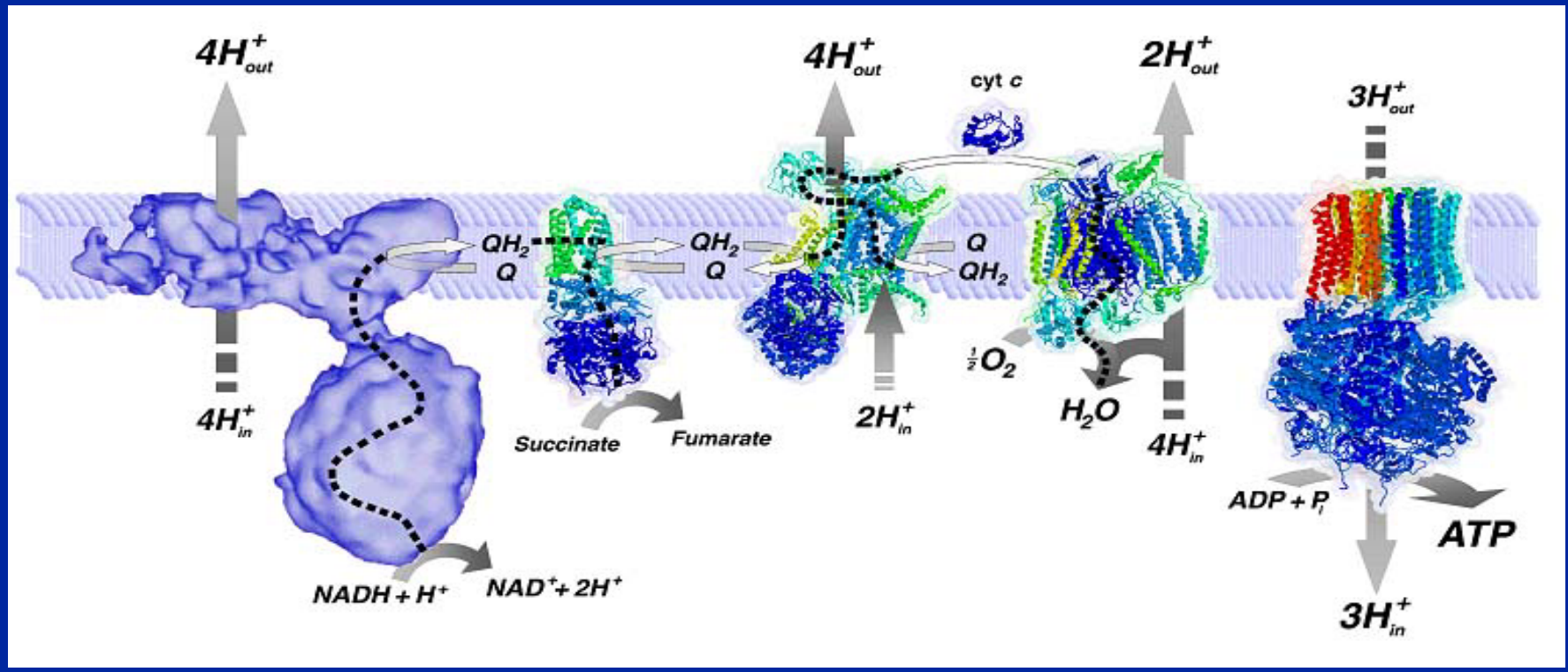
A NEW GENETIC DISORDER IN MITOCHONDRIAL FATTY ACID BETA-OXIDATION: ACAD9 DEFICIENCY

He M, Rutledge SL, Kelly DR, Palmer CA, Murdoch G, Majumder N, Nicholls RD, Pei Z, Watkins PA, Vockley J.

Am J Hum Genet. 2007 Jul;81(1):87-103. Epub 2007 Jun 4.

- Patient 1 was a 14-year-old, previously healthy boy who died of a Reye-like episode and cerebellar stroke triggered by a mild viral illness and ingestion of aspirin.
- Patient 2 was a 10-year-old girl who first presented at age 4 mo with recurrent episodes of acute liver dysfunction and hypoglycemia, with otherwise minor illnesses.
- Patient 3 was a 4.5-year-old girl who died of cardiomyopathy and whose sibling also died of cardiomyopathy at age 21 mo. Mild chronic neurologic dysfunction was reported in all three patients.
- Defects in ACAD9 mRNA were identified in the first two patients, and all patients manifested marked defects in ACAD9 protein.
- Additional information : plasma acyl-carnitine analysis only performed in patient 2 : no abnormalities.
- Fibroblasts ß-oxidation studies performed in patient 1 (normal) and patient 2 (low normal)

THE MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION MACHINERY



Complex	CI	CII	CIII	CIV	CV	total
total subunits	45	4	11	13	16	89
subunits nDNA	38	4	10	10	14	76
subunits mtDNA	7	0	1	3	2	13

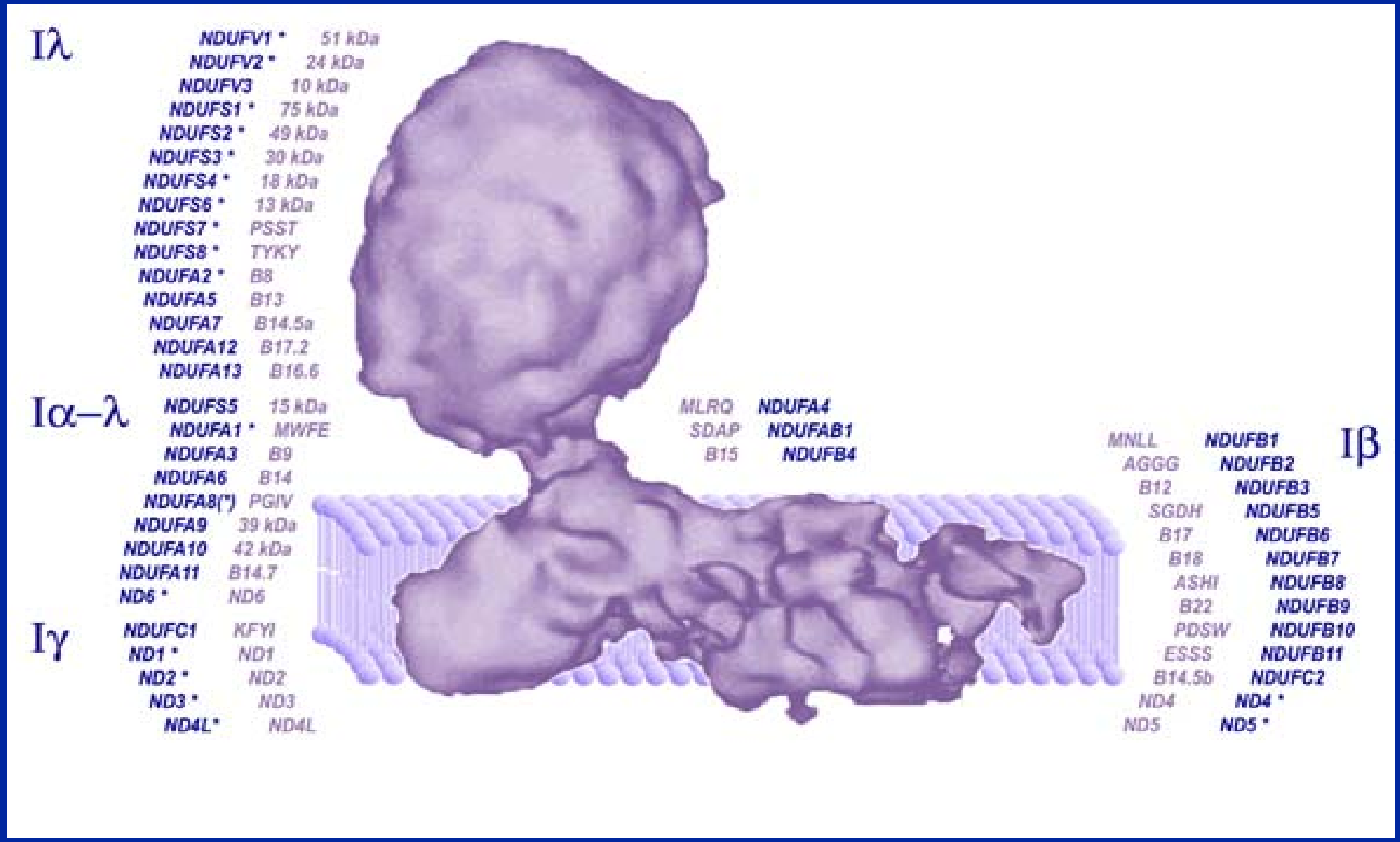
IDENTIFICATION OF ACAD9 AS A KEY PROTEIN INVOLVED IN THE ASSEMBLY OF COMPLEX I

Nouws, Nijtmans etal (2010) Cell Metabolism, in press.

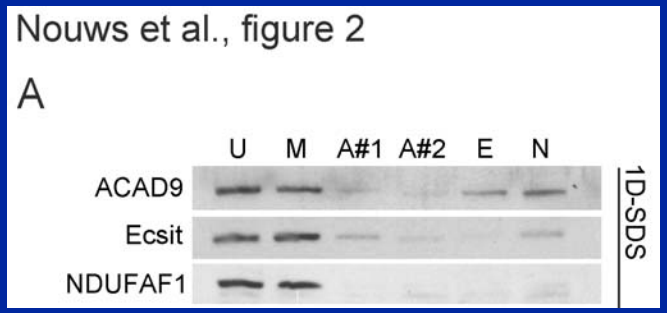
- NDUFAF1 and Ecsit are known assembly factors of complex I
- Immunoprecipitation studies with anti- NDUFAF1 and anti-Ecsit follwed by Western-blotting and nano-LC-MS/MS :

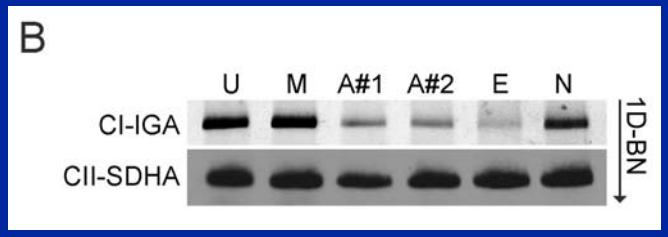
  CONSISTENT COPURIFICATION OF ACAD9 !
- Blue-native SDS-PAGE analysis : ACAD9 co-migrates in a high molecular weight complex of 500-850 kDa (Complex I)

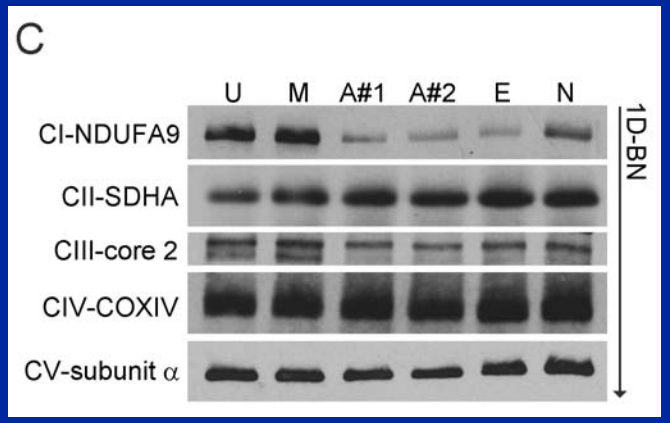
SUB-UNIT STRUCTURE OF COMPLEX 1 OF THE RESPIRATORY CHAIN



RNA INTERFERENCE MEDIATED KNOCKDOWN OF ACAD9 (A#1, A#2), ECSIT (E) AND NDUFAF1 (N) IN HEK293 CELLS.







U: untreated, M: mock transfected.

(A) SDS-PAGE western blot immunodetections of ACAD9, Ecsit, and NDUFAF1.

(B) Blue Native-PAGE analysis of complex I in gel activity (CIIGA) and western blot immunodetection of loading control SDHA.

(C) Blue Native-PAGE western blot immunodetection of oxidative phosphorylation complexes I-V.

GENE SEQUENCING IN COMPLEX I DEFICIENT PATIENTS REVEALED PATHOGENIC MUTATIONS IN ACAD9

- The specific requirement of ACAD9 for complex I assembly prompted sequence analysis of ACAD9 in a large cohort of patients with isolated complex I deficiency (Nijmegen cohort)
- Two unrelated patients with complex I deficiency identified
- Patient 1 : homozygous for c.1553G>A mutation (R518H)
- Patient 2 : compound heterozygous for two mutations including c.187G>T (stopcodon) and c.1237G>A (E413K).
- Transduction of wildtype ACAD9 into the two patients' fibroblasts gave full restoration of complex I activity.

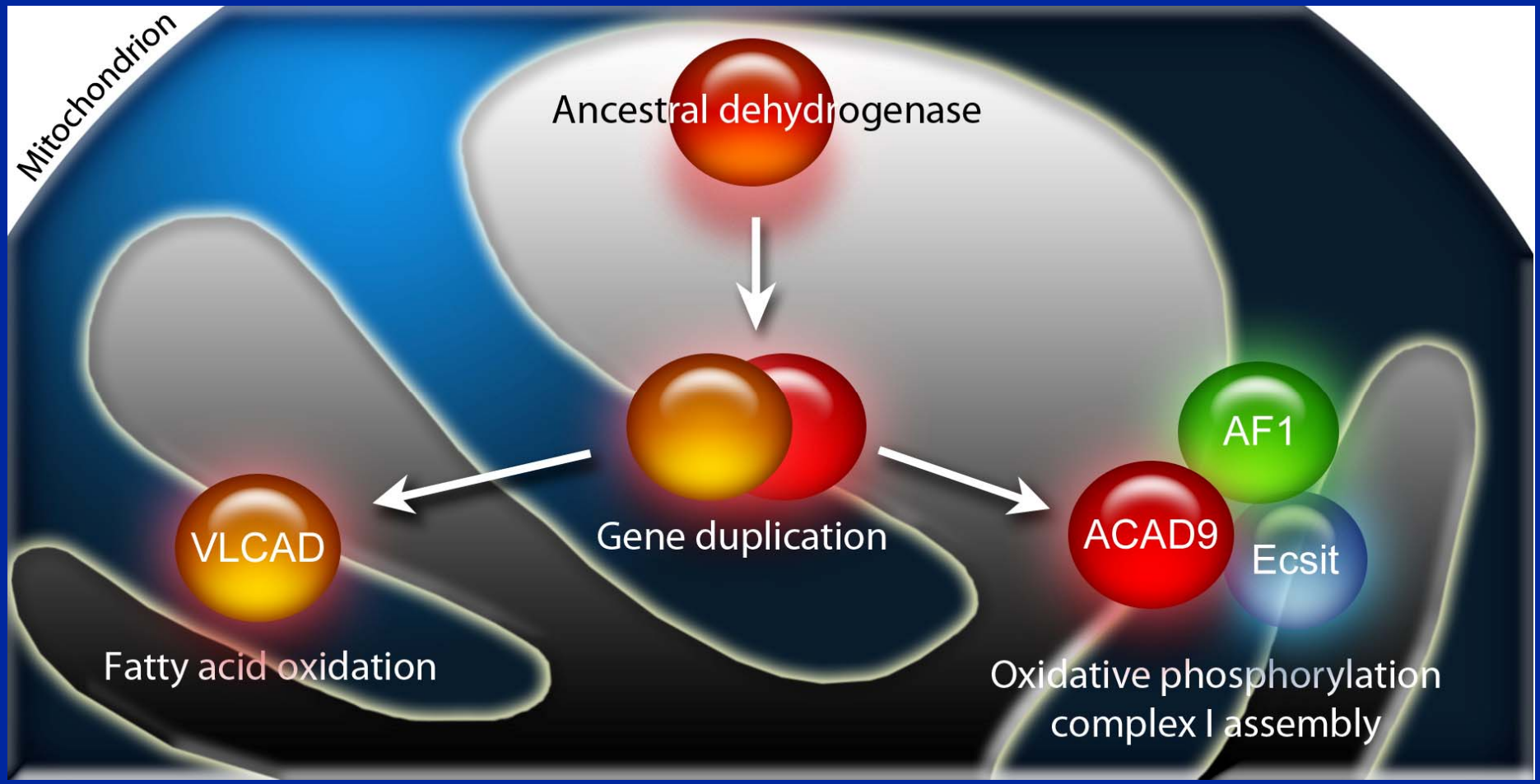
Patients enzyme activities of the mitochondrial respiratory chain complexes measured in cultured skin fibroblasts.

Enzyme activities[a]	Patient I-1	Patient II-1	Controls
NADH:Q_1 oxidoreductase[a]	0.053	0.058	0.10 – 0.26
Succinate: cytochrome *c*			
oxidoreductase[a]	0.24	0.32	0.16 – 0.44
Decylubiquinol: cyt *c*			
oxidoreductase[a]	1.46	2.82	1.25 – 2.62
Cytochrome *c* oxidase[b]	0.95	0.79	0.68 – 1.19
Citrate synthase[c]	165	190	144 – 257

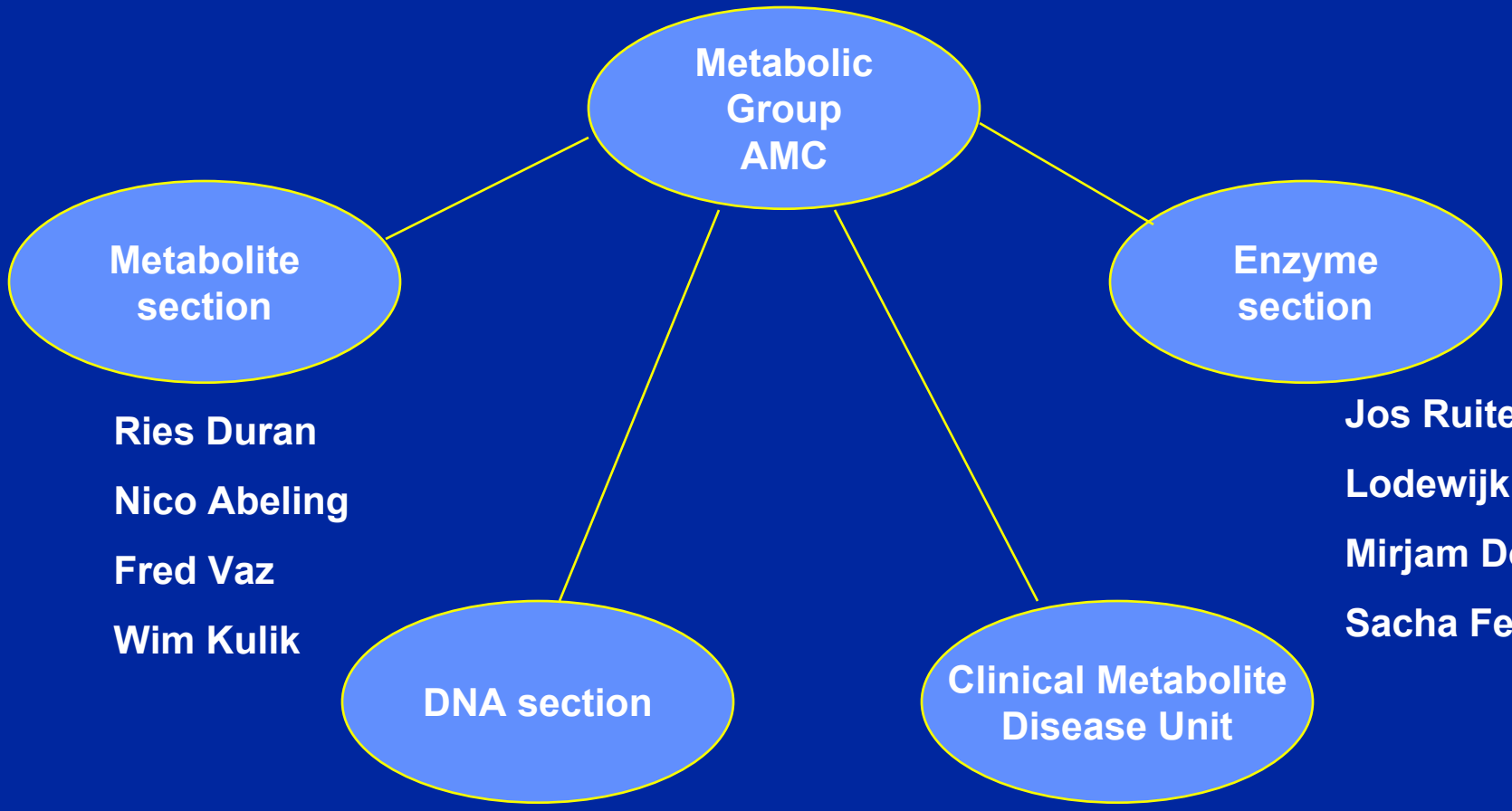
[a] Values are given in mU per mU cytochrome *c* oxidase.

[b] Values are given in mU per mU citrate synthase.

ACAD9 AND ITS INVOLVEMENT IN COMPLEX I BIOGENESIS AND FATTY ACID OXIDATION



ACKNOWLEDGEMENTS



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