UNIVERSIDAD AUTONOMA DE BARCELONA
FACULTAD DE MEDICINA

"Departamento de Pediatría, Obstetricia Ginecología y de Medicina Preventiva"

TESIS DOCTORAL

"MITOCHONDRIAL DISORDERS IN CHILDHOOD: FROM GENERAL CHARACTERISTICS TO NEW ASPECTS"

Director: Guillem Pintos Morell

Àngels Garcia Cazorla.
Barcelona, 2005

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"MITOCHONDRIAL DISORDERS IN CHILDHOOD: FROM GENERAL CHARACTERISTICS TO NEW ASPECTS"

Tesis doctoral presentada por Àngels Garcia Cazorla para optar al título de Doctora en Medicina. Tesis que opta a la mención de "Doctorado Europeo"

Director: Guillem Pintos Morell

Tesis adscrita en el departamento de Pediatría, Obstetricia, Ginecología y Medicina Preventiva. Programa de Pediatria. Plan de estudios 128. 1995-1999

A Edu y Júlia

This study began during an intense period of training in congenital errors of metabolism which I had the chance to undergo in the Professor Saudubray's service at the Necker Hospital. I wish to acknowledge that this was an enormously enriching time for me. Not only did it enable me to discover and immerse myself in a fascinating field of medicine but also, and above all, it enabled me to develop a way of thinking, a method and way of doing things. The great dedication and individual attention shown by all the professionals to each and every patient made a great impact on me. As well as dedicating long hours of meticulous discussion to the pathophysiology and therapeutic possibilities of every single patient, the child and family were also treated with great care, from different perspectives and, once again, with no regard to the time involved.

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ARTICLES AND COMMUNICATIONS DERIVED FROM THIS THESIS

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ABREVIATIONS

AAC: amino acid chromatography
ARTHR: arthrogryposis.
CARD:cardiac.
CM: cardiomiopathy
COMB:combined association of: tachypnea, feeding difficulties and hypotonia.
COX: cytochrome oxidase
CSF: cerebrospinal fluid.
DID: insulin-dependent diabetes.
F: female.
FADH: flavine adenine dinucleotide.
FFA: free fatty acids
FTT:failure to thrive.
HEM: hematologic.
HEP: hepatic
HEPD:hepato-digestive.
HYPERP: hyperpylosity.
HYPOG:hypoglycemia.
KDHC: ketoglutarate dehydrogenase complex
LHON: Leber's hereditary optic neuropathy
M: male.
MELAS: mitochondrial myopathy encephalopathy
MERRF: myoclonic epilepsy and ragged-red fibers
NADH: nicotinamide adenine dinucleotide.
NARP: neuropathy, ataxia, and retinitis pigmentosa
MR: mental retardation.
MRC : mitochondrial respiratory chain
NRL:neurologic
OAC: organic acid chromatography
OPHTH: ophthalmologic.
OXPHOS: oxidative phosphorilation system.
PC: pyruvate carboxylase
PDH: pyruvate dehydrogenase.
PyC: pyruvate carrier
PT: pyruvate transporter
T/ H/F: tachypnea, hypotonia, feeding difficulties.
UNCLASS: non classified hyperlactacidemia

CONTENTS

1-INTRODUCTION

1.1 BRIEF INTRODUCTION COMMENTS

Mitochondrial disorders are the most frequent group of inborn errors of metabolism. Its incidence is estimated in 1 in 10.000 live births (Bourgeron et al 1995). The initial knowledge of these diseases dates back 42 years with the description of a patient presenting a coupling defect between mitochondrial respiratory function and phosphorilation (Luft et al 1962). Since the publication of this report, there has been substantial progress on many different aspects of mitochondrial diseases. Therefore, our understanding of the spectrum with which patients may present as well as biochemical, enzymatic and genetic findings have continuously grown and changed.

Regarding clinical expression, the involvement of high-energy consumption organs is currently well established. Nevertheless, the widespread cellular distribution of mitochondria suggests that all kinds of clinical signs and symptoms may be secondary to its dysfunction. In fact, the spectrum of clinical manifestation is very large ranging from neonatal death to benign myopathic forms with slight exercise intolerance (Munnich et al 1992, Nissenkorn et al 1999, Siacco et al 2001).

Even though hyperlactacidemia is a frequent finding in mitochondrial disorders, this is not a constant result. For instance, normal plasmatic lactate in 15% of the isolated CI deficiency has been demonstrated (Loeffen et al 2000). Normal lactacidemia is still often a barrier that avoids continuing the examinations that are necessary to rule out a mitochondrial disorder. Plasmatic amino acids and urine organic acids are especially useful in mitochondrial enzyme deficiencies that do not concern the respiratory chain.

Lactate in the cerebrospinal fluid (CSF) and some functional tests as glucose overload may help in the diagnostic procedure.

Concerning respiratory chain deficiencies, the study of skeletal muscle activities is considered to be the gold standard diagnostic test. However the defect may not be expressed in skeletal muscle but in other tissues such as liver, heart or muscle. On the other hand, only a few patients have positive genetic results.

Although numerous solid concepts have been established, diagnostic dilemmas as well as many unreliabilities in laboratory investigations still occur. In a recent paper (Smeitink 2003), JAM Smeitink pointed out that the most challenging task in the future would be to develop new criteria of how to diagnose these patients at both the clinical and the laboratory level.

The content of the present thesis reflects the experience with mitochondrial disorders in a paediatric metabolic reference centre, over a very long period of time. It includes general aspects such as diagnostic approach and clinical presentation, poorly documented features such as long-term outcomes of children with neonatal presentation, and new particular findings, not reported before. It has been done thanks to the effort of many professionals dedicated to the study and clinical management of this kind of diseases.

1.2-MAIN CHARACTERISTICS OF MITOCHONDRIAL DISORDERS

1.2.1-THE BIOLOGY OF MITOCHONDRIA AND OXPHOS SYSTEM ENZYMES

Origin and structure

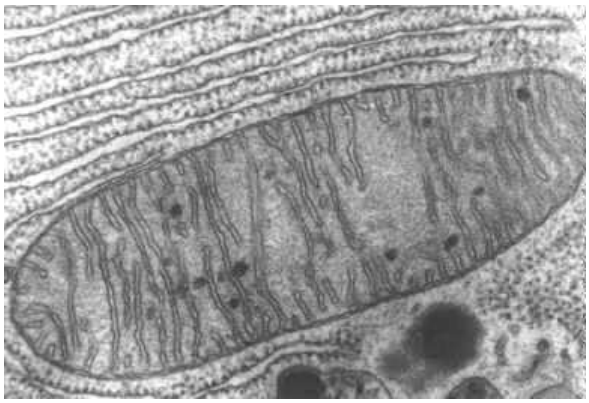
Mitochondria have a bacterial origin. In fact, they are the remnants of protobacteria that populated anaerobic nucleated cells more than one billion years ago. Over time, they established a symbiotic relationship, the bacterium detoxifying the cell of oxygen and the eukariotic cell host providing food substrates. These bacteria evolved towards mitochondria allowing the host cells to have aerobic metabolism and in consequence a more efficient manner of providing energy (Moreira et al 1998, Di Mauro et al 2003, Andersson et al 2003). These organelles are visible by optic microscopy and were first discovered in 1886. The name "mitochondria" comes from the Greek terms: mito (thread), and chondrion (granule).

They are present in all multicellular organisms living in rich oxygen environments. Plants, vertebrates, arthropods and human beings are then included (Gray 92). They can represent about 25% of the total mass cell, and in the hepatic tissue they can be as numerous as 2500 per cell.

Usually they are rod-shaped, but they can also be round, elliptic or cylindrical. Electronic microscopy has revealed their structure. Two separated membranes form them; the outer membrane limits the organelle, the inner membrane is thrown into folds or shelves that project inward and are called "cristae". Inside the space enclosed by the inner membrane is the matrix that contains DNA and ribosomes **Fig 1.**

These diverse substructures carry out different enzymatic functions in every separated space.

Fig 1 Mitochondria structure



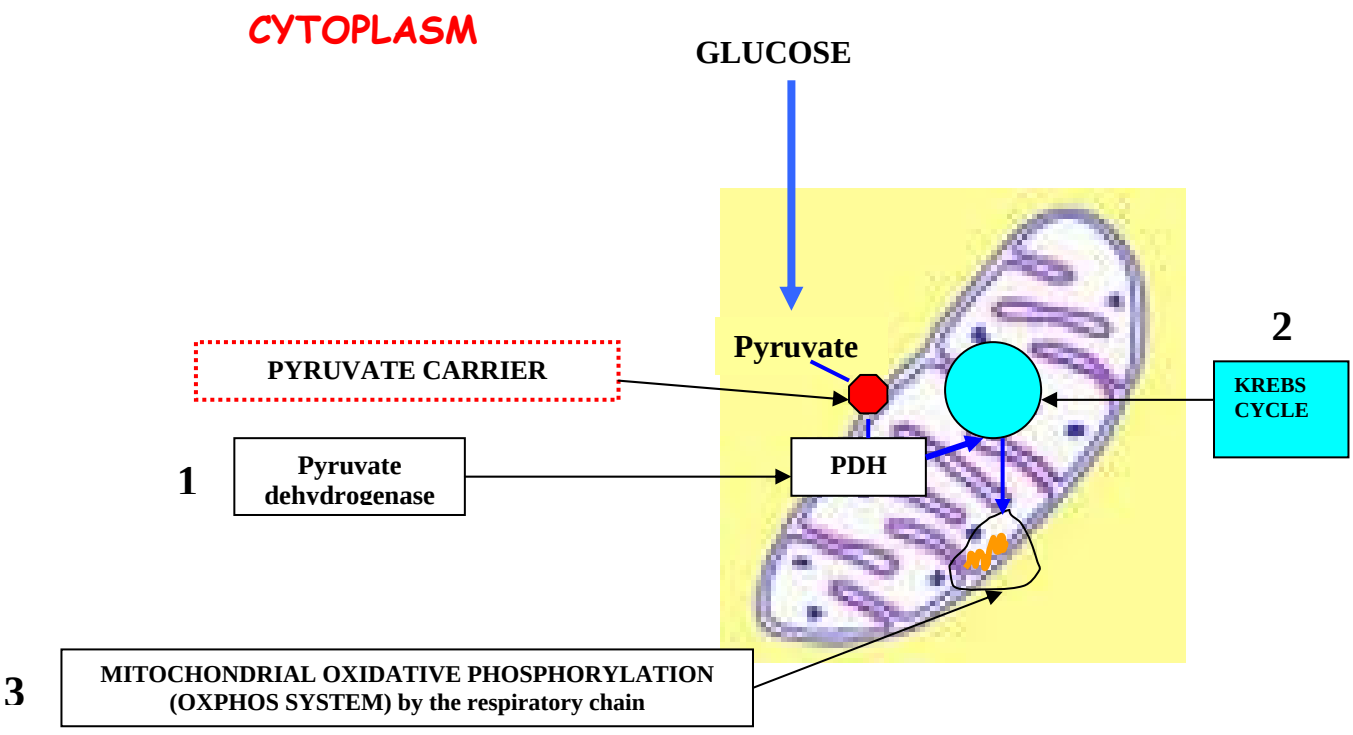
Fawcett, A Textbook of Hystology, Chapman and Hall, 12th edition, 1994

2-Function of mitochondria

The current knowledge about how mitochondria generates energy remains still limited.

The three main stages are represented in the following scheme : (**Fig2**)

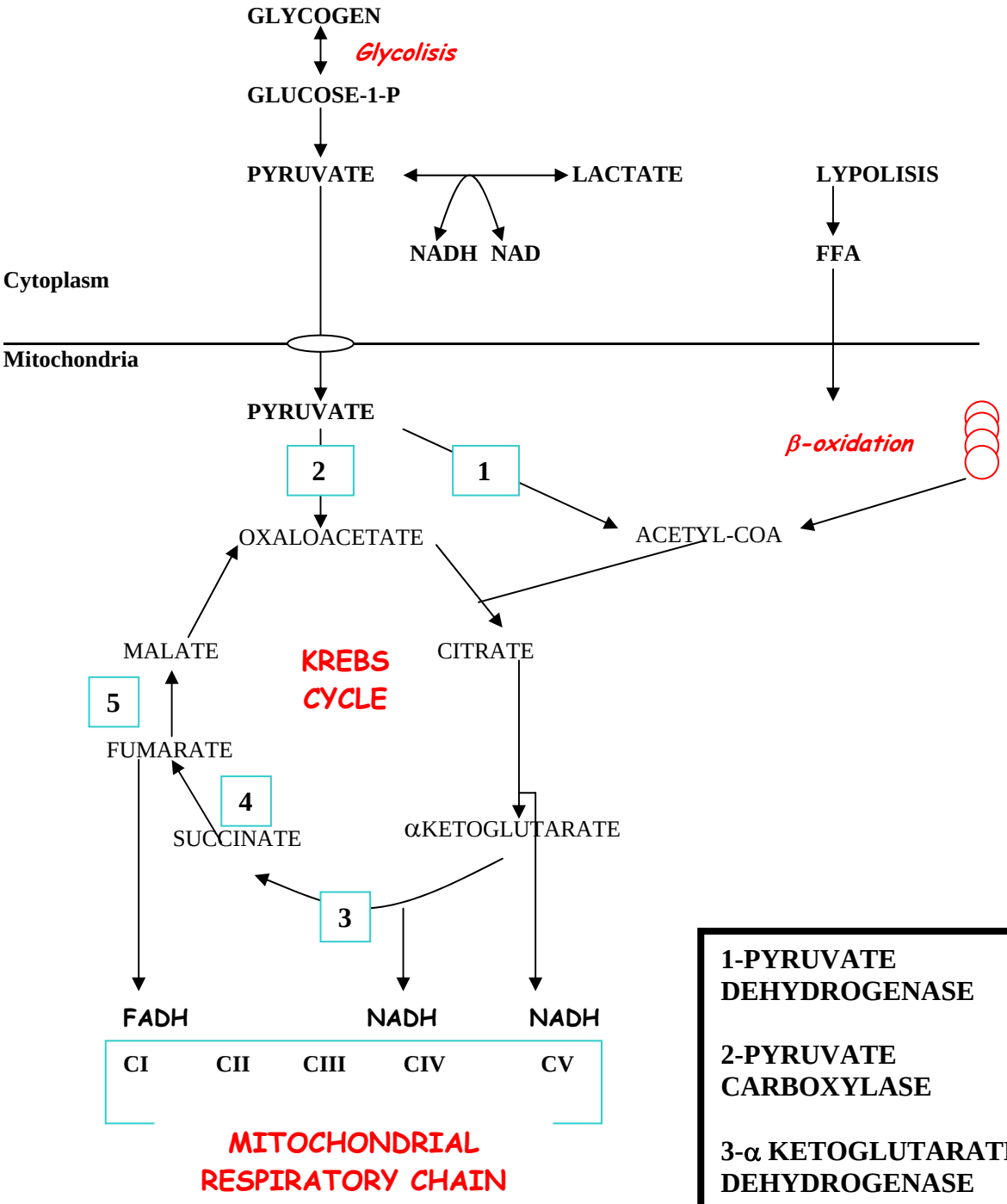
Fig 2



Glucose is broken down by anaerobic glycolysis in the cytoplasm and finally transformed into pyruvate. In anaerobic conditions, lactate dehydrogenase transforms pyruvate into lactate. In aerobic conditions, pyruvate is carried inside the mitochondria and later transformed into acetyl-CoA by the action of the enzymatic complex pyruvate dehydrogenase (PDH. **Nº 1 in figure 2**). Acetyl-CoA is also a product of fatty acid beta-oxidation. This is incorporated into the Krebs cycle (**Nº 2 in figure 2**), where electrons accumulated in form of carbonate compounds are transferred to NAD^+ (nicotinamide adenine dinucleotide) and FAD (flavine adenine dinucleotide). Reduced coenzymes, NADH y $FADH_2$ are the substrates for the next step: the mitochondrial oxidative phosphorilation (OXPHOS) by the respiratory chain (**Nº 3 in figure 2**) (complex I to IV) and complex V.

Figure 3 explains in detail the process described above.

Figure 3



1-PYRUVATE DEHYDROGENASE

2-PYRUVATE CARBOXYLASE

3-α KETOGLUTARATE DEHYDROGENASE

4- SUCCINATE DEHYDROGENASE

5- FUMARASE

1.2.2-PYRUVATE DEHYDROGENASE COMPLEX (PDH) Fig 2 and 3

PDH is a crucial enzymatic complex in the aerobic carbohydrate metabolism. It is located in the mitochondrial matrix, is the link between the glycolytic pathway and the tricarboxylic acid cycle, and is responsible for the irreversible conversion of pyruvate to acetyl-CoA. PDH is a multienzyme complex consisting of three catalytic subunits: pyruvate decarboxylase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3), protein X or E3BP mediates the interaction between E2 and E3, regulatory subunits (pyruvate dehydrogenase kinase, pyruvate dehydrogenase phosphate phosphatase), link proteins and cofactors (thiamine, lipoic acid). (Reed et al 1992, Fouque et al 2003)

Most patients have a deficiency of the E1 subunit, which is X-linked (Brown et al 1994). Many mutations have been reported and occur at an equal frequency in males and females. The spectrum of clinical presentation ranges from severe lactic acidosis and brain dysgenesis within the neonatal period to Leigh's encephalopathy, developmental delay, seizures, episodic weakness, neuropathy, and intermittent episodic ataxia after carbohydrate-rich meals in later onset forms. Neurological manifestation may be chronic and slowly progressive with a long survival.(Reed et al 1992, Lissens et al 2000). Both lactate and pyruvate can be elevated, with a normal lactate/pyruvate ratio. However, in females with a skewed X-inactivation pattern, the clinical and biochemical diagnosis can be difficult (Dahl et al 1995). Glucose over-load can trigger biochemical abnormalities that are not manifested in basal conditions. Pyruvate dehydrogenase activity can be measured in fibroblasts, muscle, lymphoblasts and lymphocytes. A molecular search for a mutation in E1α and 1β, E3 binding protein, or E3 mutation is necessary for confirmation. Some patients with PDH deficiency respond to high doses of thiamine (500 mg to 2000 mg) , possibly in relation to the thiamine

pyrophosphate binding site (Naito et al 1998). The outcome of patients treated with ketogenic diet is variable (Wexler et al 1997).

1.2.3-PYRUVATE CARBOXYLASE (PC). Fig 3

PC is a mitochondrial, biotin-containing enzyme. It transforms pyruvate + CO_2 into oxaloacetate. It is an essential component of the Kreb's cycle and provides the necessary substrate to different metabolic pathways: lypogenesis, glyconeogenesis, glycerogenesis and formation of certain nonessential amino acids.

Although highly variable, there are three main clinical presentation types (Robinson et al 1989, Fernandes et al 2000):

1- Neonatal severe form with lactic acidosis and neurological dysfunction (coma, seizures) and rapid fatal outcome. 2- A later-onset clinical presentation consists in psychomotor delay that can be associated with seizures, pyramidal signs, hepatomegaly, renal dysfunction and periventricular cysts. 3- Another late-onset form manifests as recurrent hyperlactacidemia with slight neurological signs.

In the severe neonatal form, the lactate/pyruvate ratio is elevated, the 3-hydroxybutyric acid/acetoacetate ratio is decreased, and postprandial hyperketonemia may be observed. Hyperamoniemia, and high ornitine, citruline and proline are detected. The diagnosis can be confirmed by measuring the enzyme activity in fibroblasts and other tissues like the liver. There are some identified mutations (Wexler et al 1998).

1.2.4-ENZYMATIC DEFICIENCIES OF THE KREBS CYCLE. Fig 2 and 3

α Ketoglutarate dehydrogenase (KDHC) and Fumarase deficiencies are the most common cause of Kreb's cycle disorders. Both usually present in early childhood with signs of neurological impairment (developmental delay, hypotonia, ataxia) and rarely

survive into adolescence. Fumarase deficiency has a less severe clinical form in which patients develop a static encephalopathy and can survive into adulthood (Bonnefont et al 1992, Rustin et al 1997). The majority of patients have severe hyperlactacidemia. The most useful test for recognizing KDHC and Fumarase deficiencies is urine organic acid analysis, which shows increased excretion of 2-ketoglutaric and fumaric acids respectively. Enzymatic activities in fibroblasts and other tissues confirm the diagnosis.

1.2.5-MITOCHONDRIAL RESPIRATORY CHAIN (MRC)

MRC is located in the inner mitochondrial membrane. Four multienzymatic complex working as electron transporters form this complicated chain: complex l (NADH-CoQ reductase, which has about forty units), complex II (succinate-CoQ reductase, composed by four subunits), complex III (ubiquinone-cytochrome C reductase, eleven subunits), and complex IV (cytochrome C oxidase, thirteen subunits). Complex V or ATPase (fourteen subunits), insures ATP synthesis from ADP and inorganic phosphate in the mitochondrial matrix. (Fig 4).(Hatefi et al 1995).

Mitochondrias have their own DNA (mitochondrial DNA or DNAmt), which is maternally inherited. It codifies 13 subunits belonging to complex I, II, IV and V (Anderson et al 1981). DNAmt regulation is double: nuclear DNA (Mendelian inheritance) on one hand and mitochondrial DNA (maternal inheritance) on the other. This rule has an exception: complex II is entirely codified by the nuclear cell. (Anderson et al 1981). Mutations may also be sporadic.

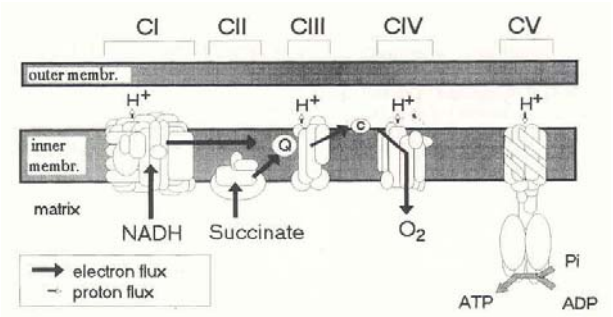


Fig 4. Mitochondrial respiratory chain. Pag 157. "Inborn Metabolic Diseases". J. Fernandes, JM Saudubray, G van den Berghe. Third Editon. 2000

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From a clinical point of view, they are a heterogeneous group of diseases involving different organs. Central nervous system, muscle and heart are frequently affected. The same enzymatic deficit can give place to diverse clinical phenotypes and vice versa; symptoms may be similar even in the presence of different enzymatic deficiencies.

Despite the great clinical heterogeneity, there are some particular manifestations that appear to be associated with some DNAmt mutations such as Leber's disease (LHON: Leber's hereditary optic neuropathy) (Anderson et al 1981), MELAS (mitochondrial myopathy encephalopathy, lactic acidosis and stroke-like episodes) (Sue et al 1999), MERRF (myoclonic epilepsy and ragged-red fibers) (Chinnery et al 2000), and Kearns-Sayre syndrome (Chinnery et al 2000).

The clinical presentation can vary in function of the onset-age (See Table 1)

The important clinical variability is partly due to the double genetic origin (nuclear and mitochondrial) but also to the great number of genes that regulate the respiratory chain (Smeitink et al 2001). In most cases, the results of the enzymatic studies do not permit the definition of the deficient subunit and therefore is not possible to determine the particular genetic origin. Only 5-10% of mitochondrial cytopathies should be related to a DNAmt defect, whereas about one thousand nuclear genes would participate in the correct respiratory chain functioning (MITOMAP).

With respect to mitochondrial DNA abnormalities, each cell contains between hundreds and thousands mitochondria and each owns several DNAmt copies . The same cell may hold normal and mutated molecules. This is known as heteroplasmia. Over different cell divisions, normal and mutated molecules are distributed at random and its proportion could be very variable from one cell to the other, from one organ to the other, and also , over a period of time, within the same organ. (Di Mauro et al 2003, Di Mauro et al 2002). The proportion of mutated molecules decreases through time in rapid turnover cells (blood cells for instance); on the contrary, it tends to increase in cells of organs with a very low or null turnover (muscle, brain). In the same way, the same DNAmt deletion can produce very different clinical pictures (ex: Kearn-Sayre, Pearson). Table 2.

With respect to nuclear genes (Chinnery et al 2000 , Smeitink et al 2001, MITOMAP)**:** the tissue expression of genes is constant and does not depend on the heteroplasmia phenomenon. The greater number of respiratory chain proteins are codified by the nuclear DNA. Furthermore, there are assembling and intergenomic signalling proteins, which are also codified by the nuclear DNA. Table 2.

With respect to laboratory investigations the following examinations should be carried out in face of a patient suspected of mitochondrial disease:

-In blood: lactate, pyruvate and amino acids. It is important to determine lactate and pyruvate at fasting and in the post-prandial state on several occasions and avoiding local hypoxia.

-In urine: amino acids and organic acids.

If the results of these examinations are normal , a glucose overload may be performed (2 grams/kg of glucose; plasmatic glucose and lactate baseline determination and 15, 30, 60, 90, 120, 180 and 240 minutes later).

Additional examinations may include cardiologic study (echocardiography, ECG), brain MRI (spectroscopy should detect abnormal lactate peaks), ophthalmologic study (fundus and electroretinogram) and renal functionalism.

Morphological and biochemical examinations: Skeletal muscle is the tissue of choice because it expresses the defect in the majority of patients (Rustin et al 1994). Light and electronic microscopy, histochemical and enzyme-hystochemical staining are systematically performed. Frequent findings on ultrastructural studies are increased number and/or size of mitochondria, cristae abnormalities and abnormal subsarcolemmal clustering of mitochondria.

Polarography studies (Rustin et al 1994) are useful to detect oxidative phosphorilation defects and also PDH, Kreb's cycle , transporters and oxidation cofactors deficiencies. These examinations need from 50 to 100 milligrams of fresh muscle.

Spectrophotometry analysis (Rustin et al 1994) facilitates the measurement of respiratory chain complexes using specific electron donors or receptors. From 10 to 20 mgs of tissue are needed. This material must be immediately frozen in liquid nitrogen at -80ºC.

A normal activity of the mitochondrial respiratory chain does not exclude its deficiency even if the study has been performed in the tissue in which the disease is expressed. Kinetic disturbances of the enzyme or tissue heterogeneity may explain an abnormal function of the respiratory chain despite of normal enzymatic activity. In this case it is necessary to investigate the defect in other tissues or to repeat the study later, ensuring

the quality of the procedure. Inappropriate freezing conditions may produce a decrease of respiratory chain activities simulating a primary defect.

Molecular studies of mitochondrial DNA are possible. Different deletions, normally sporadic, have been described in the Kearns-Sayre's syndrome (Holt et al 1988), progressive external ophthalmoplegias (Moraes et al 1989) and Pearson's syndrome (Rotig et al 1990). Punctual mutations (MELAS, MERRF and NARP) (MITOMAP) correspond to genes that codify the mitochondrial respiratory chain, or to RNA transfer genes but also to ribosomal RNA (in Leber's optic atrophy). Regarding nuclear DNA genes diverse mutations that codify the synthesis of respiratory chain proteins, assembling and regulating proteins have been reported (Di Mauro et al 2003, Chinnery et al 200, Smeitink et al 2001). It is already known that OXPHOS defects in the pediatric population are probably due in the majority to mutations in the nuclear DNA.

Table 1. Clinical presentation depending on the onset-age

NEONATAL PERIOD	• Severe lactic acidosis with fatal outcome or initial favourable evolution with progressive impairment from 6 to 12 months of life. • Hepatic failure and death during the first days of life • Hypertrophic myocardiopathy +/- Leigh's syndrome • Proximal tubulopathy • Intrauterine growth retardation • Episodic Apnea/tachypnea • Hipotonia, letargy, coma • Feeding difficulties, failure to thrive • Nystagmus, cataracts, ptosis • Dysmorphic traits
1 MONTH-2 YEARS	• Leigh's syndrome • Psicomotor regression, ataxia, myoclonus, white matter alterations, stroke-like episodes • Myopathy, recurrent myoglobinuria • Letargy, coma • Growth arrest of head circumference • Failure to thrive, diarrhea • Proximal tubulopathy • Pancytopenia and external pancreatic insufficiency (Pearson's syndrome) • Insulin-dependent diabetes, Insipidus diabetes, optic atrophy and deafness (Wolfram's syndrome) • External ophtalmoplegia, pigmentary retinitis, palpebral ptosis, myopathy (Kearns-Sayre's syndrome) • Nystagmus, cataracts, ptosis
ADOLESCENCE, ADULTHOOD	• Myoclonus, ataxia, psicomotor delay, leucodystrophy, peripheral neuropathy, stroke-like episodes, migraine • Myopathy, exercice intolerance, myoglobinuria • Hypertrophic or dilated myocardiopathy • Neurosensorial deafness +/- diabetes • Kearns-Sayre's syndrome • Intestinal pseudoobstruction with neuropathy and myopathy (MNGIE's syndrome) • Growth hormone deficiency, hypoparathyroidism, hyperaldosteronism • Ptosis, cataracts, pigmentary retinopathy

Table 2. Relationship between different nuclear and mitochondrial genes and clinical presentation.

MRC Complex	DNAmit	Clinical manifestations of DNAmit	DNA nuclear	Clinical manifestations of DNA nuclear
Subunits CI	ND1,ND2,ND3,ND4,ND4L, ND5,ND6	LHON,MELAS, LHON and dystonia, sporadic myopathy	NDUFS1,NDUFS2, NDUFS4, NDUFS7, NDUFS8, NDUFV1	Leigh, leucodystrophy
Subunits CII			SDHA, SDHB, SDHC, SDHD	Leigh, paraganglyome, pheochromocytome
Subunits CIII	Cytochrome b	Sporadic myopathy encephalomyopathy, septo-optic dysplasia, myocardiopathy	BCSL1	Leigh, GRACILE syndrome
Subunits CIV	*COX1, COXII, COXIII*	Sporadic anaemia, sporadic myopathy, encephalomyopathy, ALS-Like syndrome	*COX10, COX15,SCO1,SCO2,SURF1*	Leigh, hepatopathy, cardioencephalomyopathy, leucodistrophy and tubulopathy
Subunits CV	ATPase 6	NARP, MILS, FBSN		

2-HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

Inherited metabolic disorders are rare causes of disease in childhood. Pediatric metabolic reference units are necessary to insure the appropriate management and study of this highly specialised medicine. The experience of an European reference centre, in a particular subgroup of metabolic diseases, could be of great interest concerning both general guidelines and atypical aspects. In this case, the mitochondrial disorders diagnosed in the “Hôpital Necker-Enfants Malades” from 1977 to 2002 are the subject of the study.

This large background would therefore provide a wide spectrum of information going from general aspects such as clinical presentation and diagnostic approach, poorly documented features such as follow-up studies or characteristics of uncommon enzymatic deficiencies, and new particular findings, not reported before.

2.2 OBJECTIVES

2.2.1-To report the general characteristics of a group of 241 patients with mitochondrial disorders: clinical, biochemical, enzymatic and genetic features.

It is designed to introduce the main characteristics of the whole group of patients from which different particular points will be later developed.

It represents a retrospective review of 241 children affected with hereditary mitochondrial disorders diagnosed in the metabolic unit of the Hôpital Necker-Enfants Malades, from 1977 to 2002. 129 cases of respiratory-chain disorders (MRC), 81 non-classified hyperlactatemia (HL), 17 pyruvate dehydrogenase deficiencies (PDHC), 9 pyruvate carboxylase deficiencies (PC), 4 Krebs cycle defects (KC) and 1 pyruvate transporter deficit are studied.

In summary, this objective could be divided in other three:

a)-To introduce the group of patients that represents the subject of study of different aspects that will be developed in detail in other chapters of the thesis.

b)-To describe the clinical, biochemical, enzymatic and genetic characteristics of this series.

c)-To describe the incidence and characteristics of plasmatic lactate (normal, mild, moderate, severe) in respiratory chain disorders and its relation with onset age, group of symptoms and enzymatic defects.

2.2.2-To develop a diagnostic tree based on the biochemical results.

This diagnostic decision tree is based on the biochemical findings that characterise each subgroup of metabolic disorders described in 2.2.1

2.2.3-To study certain poorly documented aspects of these disorders:

In this part the main objective is to discuss some particular topics that are worthy of consideration due to the lack of information that currently exists about them. This is elaborated through three different studies about aspects that are insufficiently reported in the medical literature. Despite the fact that isolated cases and short series are known, large series have not been described.

a) Long-term outcomes of neonatal mitochondrial cytopathies

This section presents a retrospective study of 57 patients with mitochondrial cytopathies of neonatal onset.

The aims were, on one hand, to determine the long-term clinical and biochemical outcome of patients with neonatal onset mitochondrial cytopathies, and on the other, to identify variables that may modify the natural course of these diseases.

In summary, this objective is divided in other three

1-To describe de clinical and biochemical follow-up of these patients

2-To detect prognosis factors that could modify the course of the disease

3-To describe in detail the outcome of the patients surviving more than 4 years

b) Characteristics of patients with mitochondrial respiratory chain deficiencies, in which the enzymatic defect is found in the liver

This section is a retrospective study of 31 patients with hepatic mitochondrial respiratory chain deficiencies. This particular objective is developed in other two:

1-To describe the clinical and laboratory characteristics of 31 patients with hepatic mitochondrial respiratory chain deficiencies.

2- To develop possible guidelines that facilitate the examination of hepatic biopsy performance in patients suspected of mitochondrial cytopathies.

c) Pyruvate carboxylase deficiency: metabolic and neurological characteristics of nine patients

Two main objectives were defined here:

1- To describe the main clinical and biochemical characteristics of nine patients with the French form of pyruvate carboxylase deficiency in order to draw up some guidelines that allow an early treatment with triheptanoid and citrate.

2-To focus on some poorly documented aspects, such as movement disorders and epilepsy.

2.2.4-To report two clinical cases that represent new findings in the field of mitochondrial disorders:

a) Impaired mitochondrial pyruvate importation in a patient and a fetus at risk

It aims on one hand to describe the main clinical and biochemical characteristics of this rare entity and on the other to develop a method to perform its diagnosis

b) A deletion in the human QP-C gene that originates a complex III deficiency resulting in hypoglycaemia and lactic acidosis.

The main objectives are to report a new presentation form of these disorders (neoglucogenesis-like recurrent hypoglycaemias) and to describe new genetic findings related to the complex III.

3- PATIENTS AND METHODS

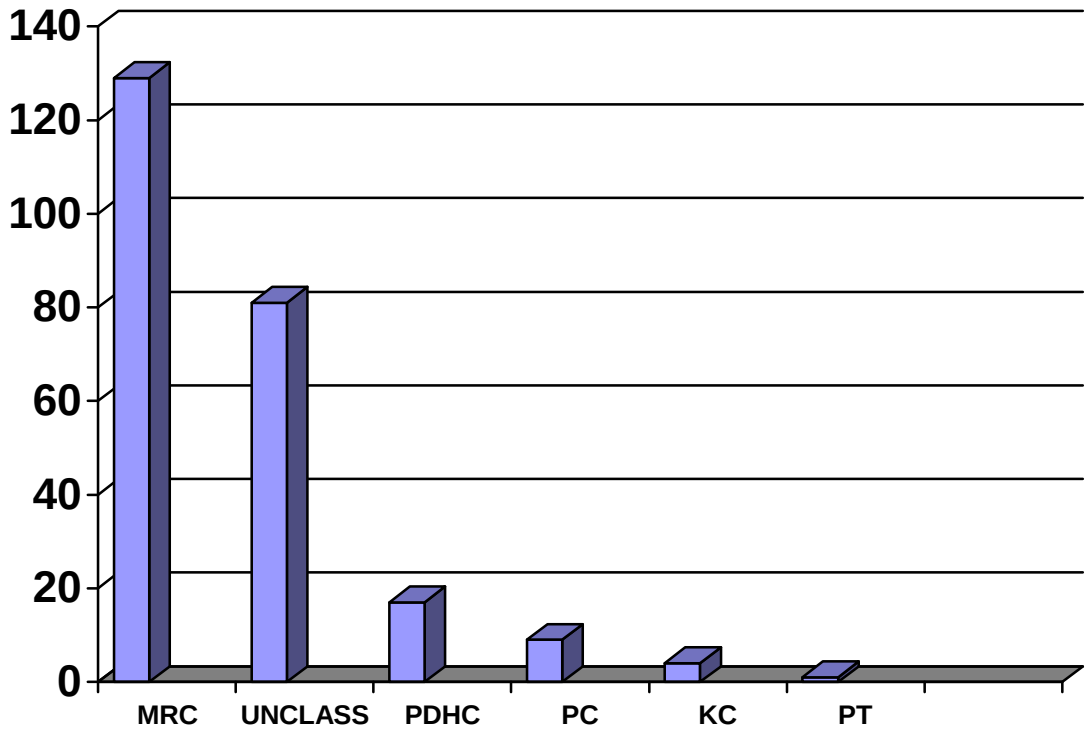
3.1.-PATIENTS

The population that concerns this study is composed by all the children affected with hereditary mitochondrial disorders diagnosed in “Hôpital Necker-Enfants Malades” (Paris) from 1977 to 2002.

This work represents therefore, a retrospective review of 241 children with different disorders that are classified as follows (Fig 1.1):

- 129 cases of respiratory-chain disorders (MRC).
- 81 non-classified hyperlactatemias (UNCLASS)
- 17 pyruvate dehydrogenase deficiencies (PDHC)
- 9 pyruvate carboxylase deficiencies (PC)
- 4 Krebs cycle defects (KC)
- 1 pyruvate transporter deficit. (PT)

Fig 1.1 Distribution of the total population



This total population (241 patients with diverse mitochondrial disorders) has been qualified as **“POPULATION Nº 1”**.

From this global series, different population groups as well as individual cases have been selected and analysed. They are defiened as follows:

- 57 newborns with respiratory chain disorders: **“POPULATION Nº 2”**
- 31 patients expressing the respiratory chain deficit in the hepatic tissue: **“POPULATION Nº 3”**
- 9 patients with pyruvate carboxylase deficiency: **“POPULATION Nº 4”**
- 1 patient with impaired pyruvate transport
- 1 patient with recurrent hypoglycaemias and CIII hepatic deficiency

3.2-COMMON METHODS

3.2.1-) ANALYSE OF METABOLIC INTERMEDIATES

In all the patients , metabolic compounds reflecting the energetic metabolism function have been carried out in plasma and urine.

a) Lactate, pyruvate, acetoacetate and 3-hydroxybutyrate: these metabolites were determined by enzymatic semiautomated methods (Czok R et al 1974, Vassault et al 1990). About 1 to 1.5 ml blood samples were obtained by venopuncture, avoiding hand exercise before the sampling (Braybrooke et al 1975, Kollee et al 1977) and collected in heparinized tubes. From these tubes 0.5 ml whole blood accurately pipetted is immediately deproteinized transferring it into 1 ml of ice-cold perchloric acid (1 mmol/l) tubes. After thorough mixing, samples can be stored at –20ªC and thawed immediately prior to analysis.

b) Plasmatic amino acids: In patients diagnosed before 1980 amino acids were determined by ion-exchange chromatography on a NC 1 Technicon Autoanalyzer whereas in patients detected beyond this date a Compack Prolinea 4 33/s, Ezchrom TM Chromatography Data System was used. About 1 ml blood samples were collected in EDTA tubes. The samples were maintained at low temperature (between 0º to 4º C) before its analyse. They were centrifugated at 2000 x g, 10 minutes at 4º C in no more than 15 minutes. The plasma was separated carefully.

c) Organic acids in urine: From urine collected over 24 hours and equivalent volume of 250 micrograms of creatinine was separated. ClH and undecanodioic acid were added to the sample. After 10 minutes of centrifugation at 2000 x g at 4ºC it was injected in the same chromarographer used for amino acid analyse.

3.2.2-DATA BASE AND STATISTICAL STUDIES

SSPS 9.0 for windows has been used to elaborate a data base af all the patients. 121 different variables for each patient were introduced in it, although not all of them have been later used to perform the definite analyses.

The diverse variables as well as the distinct statistical methods applied in every objective are specified in detail in every population.

3.2.3- METHODS IN THE GLOBAL POPULATION (241 CHILDREN WITH DIVERSE MITOCHONDRIAL DISORDERS: POPULATION Nº 1)

We retrospectively studied 241 pediatric patients (131 females and 110 males) with mitochondrial disorders diagnosed in our metabolic unit from 1977 to 2002. Age of presentation was between 0 days and 11 years.

All these patients followed the same diagnostic procedure:

1-First we ruled out acquired causes of hyperlactacidaemia: infections, hypercatabolic states like malnutrition and hypoxia (tissue hypoxia due to a technically incorrect blood extraction or general hypoxia related to a hypoperfusion).

2-Secondly complementary exams searching for multi-system involvement were performed: cardiac echocardiography, ophthalmologic exam (fundus and electrorretinography), evaluation of renal and hepatic function, and brain imagery.

3- Thirdly we practised first line metabolic exams:

A)-A "redox cycle" or the determination in blood (before and after meals) of lactate (L), pyruvate (P), L/P ratio, ammoniemia (NH3), 3-hydroxybutyrate (3OHB), acetoacetate (AcAc), 3OHB/AcAc ratio, and glucose. These samples were taken from an indwelling venous catheter avoiding hand exercise before the sampling as well as

difficult conditions of extraction. We obtained a minimum of 4 samples for each patient. Definition of hyperlactacidaemia consists in a blood lactate higher than 2.4 mmol/l, pre or post-prandial, found in several samples of the redox cycle.

B)- Blood amino acid chromatography (AAC): after 12h fasting

C)- Organic acid chromatography (OAC) of urine collected over 24 hours.

D)- Morphologic, enzymologic and genetic studies:

In MRC: deficiency of respiratory chain enzymes in muscle, fibroblasts, lymphocytes or liver by spectroscopy and polarography were carried out. These analyses were peformed according to the methodology described by Rustin et al, 1994.

- Polarographic studies were carried out in small muscle biopsies (100-200 mg) or on intact circulating lymphocytes (10 ml of blood) or detergent-permeabilized cultured cells. In all the cases the analysed material was fresh.
- Spectrophotometric studies were achieved with 1-20 mg of muscle (deltoid), liver or endomyocardial biopsies (the last two obtained by needle biopsy). A 25 ml flask of cultured skin fibroblasts and a lymphocyte pellet derived from a 10 ml blood sample were obtained for the studies in these cells. Samples were immediately frozen and kept dry in liquid nitrogen (or at-80ºC)
- Mutations and deletions analyses of the mitochondrial DNA were also performed. About 5 ml of whole blood in newborns and infants younger than 3 months, and 10 ml in older children were required. Southern blot analysis was performed using 5 micrograms of total blood or muscle DNA by standard procedures.

Polarographic, Spectrophotometric and genetic studies were performed in the genetic and research units U375 and 30 (P. Munnich, A. Rotig, P. Rustin. Hôpital Necker, Paris).

- Morphology of the muscle was analysed by light and electronic microscopy, histochemical and enzyme-histochemical staining. (Dr Norma Romero, "Hôpital Salpetrière" Paris).

In PDHC: deficiency of the enzyme was measured in lymphocytes and fibroblasts obtained after culture cells of skin biopsy. Total cell activity was mesured using (1-14C) pyruvate as a substrate , after maximal activation of PDHC by treatment of the cells for 15 minutes with 5 mM of diclhoroacetate before PDHC extraction obtained by three freeze-thaw cycles for fibroblasts and by sonication for lymphocytes (Geoffroy et al, 1997, Denyer et al 1989). Mutations of E1α gene and X protein were also performed in whole peripheral blood using standard methods (Lissens et al 1996) . These studies were determined in the biochemical unit of "Hôpital Bicetre", Paris.(Pr M. Brivet).

In PC, deficiency of the enzyme in fibroblasts and liver according to standard methods (Augereau et al 1985) were determined.

In KC deficiency of the enzyme in muscle were carried out (Biochemical unit, Hôpital Necker, Dr D. Rahier).

4-Statistical analysis was performed as follows:

1-Concerning quantitative variables (lactate: mmol/l): multiple comparisons tests (Tukey HSD and Bonferroni) were used.

We applied the analysis only in MRC disorders and in the following categories:

-Age group: newborns, infants (1 month- 1 year) and children older than 1 year.

-Enzymatic deficiency: CI, CIV, CI+CIV, "other isolated deficiencies" and "other multiple deficiencies"

-Clinical presentation: multi-system or mono-system, the combination of “thachypnea, feeding difficulties and failure to thrive”, neurological, hepatic, failure to thrive, cardiac, and renal .

We compared the mean presentation lactate in each subgroup of the three different categories

2-Concerning qualitative variables we defined 4 categories of HL depending on the lactate concentration:

-Normal lactate < 2,4 mmol/l.

-Mild hyperlactatemia: lactate oscillating between 2.5 to 3 mmol/l.

-Moderate: constant hyperlactatemia between 3 to 6 mmol/l.

-Severe or lactic acidosis: higher than 6 mmol/l.

We used Chi Square test to compare the different lactate category in each subgroup of the following different variables : age, enzymatic deficiency, plasmatic amino acids, urine organic acids and clinical presentation.

3.2.4-METHODS TO DETERMINE THE ROLE OF THE BIOCHEMICAL RESULTS IN THE DIAGNOSTIC TREE.

The following elements were used to design a diagnostic flow-chart :

-Onset age of the disease

-Plasmatic lactate (normal, mild, moderate, severe)

-Lactate/Pyruvate ratio

-Plasmatic ketone bodies

-Plasmatic amino acids

-Urine organic acids

3.3- SPECIFIC METHODS

3.3.1-METHODS IN POPULATION Nº 2: 57 NEWBORNS WITH RESPIRATORY CHAIN DEFICIENCIES

Patients' characteristics

57 patients (30 males and 27 females) with respiratory chain disorders of neonatal onset (age range from 0 to 30 days; mean: 6,35 ,SD 8,5☺ were diagnosed at the metabolic unit, Hôpital Necker Enfants Malades, from 1983 to 2002. A suggestive clinical picture associated with high plasmatic lactate in the majority of cases (50/57: 87,7 % ; mean: 9,7 mmol/l), disturbed plasmatic amino acids (44/57: 77,1%) and urinary organic acids (43/57: 75,4%) led us to suspect the diagnosis.

Concerning the clinical symptoms we mostly found multiorganic presentations. Hypotonia, tachypnea and feeding difficulties formed the most common pattern of association, followed by neurologic and hepatodigestive symptoms.

Depending on these different presentation types, we established the following clinical categories: Multiorganic (53/57: 92,9%), Association of hypotonia, tachypnea and feeding difficulties (25/57: 43,8%), Neurologic (21/57: 36,8%) , Hepatodigestive (15/57: 26,3%), Failure to thrive (8/57: 14%), and Cardiac (3/57: 5,2%). Most of the patients presenting with cardiac disorders are evaluated and followed in the cardiology unit, therefore there is a very low proportion of them in our series.

Initial blood lactate ranged from 1,7 to 25,5 mmol/l (mean: 9,7 SD: 6,65). The following lactate-categories were defined: normal lactate (<2,4 mmol/l): 7 patients (12,2%); mild hyperlactacidemia (2,5-2,9 mmol/l): 1 patient (1,7%); moderate (3-5,9 mmol/l): 11 patients (19,2%); and severe (>6 mmol/l): 38 cases (66,6%)

The diagnosis was confirmed by measurements of respiratory chain enzymes in muscle, fibroblasts, lymphocytes or liver by spectroscopy and polarography. 24 CI, 12 CI+CIV, 7 CIV, 7 generalised (6 of these associated with DNA mitochondrial depletion), 3 CII, 1 CIII, 1 CV, 1 CIII+IV deficiencies, and 1 patient diagnosed on muscle morphology criteria, were observed. We defined five enzymatic categories: CI, CI+CIV, CIV, Generalised and Others (CII, CIII, CV, CIII+IV, muscle morphology).

Other than DNA depletions , genetic studies only found a nuclear mutation in ATP synthetase gene (p8993) in one patient.

Treatment and clinical follow-up

All the patients have been initially treated with different cofactors (thiamin, riboflavin, carnitine), most of them with bicarbonate (40/57: 70,1%), some of them with ketogenic diet (3/57: 5,2%) and some other, more recently, with CoQ10 (3/57: 5,2%). They were visited every 3 months during the first year and every 6 months later on. Clinical exam and general biochemical studies (hemogram, glucose, hepatic and muscle enzymes, and renal function), plasmatic lactate, amino acids and urinary organic acids were performed systematically in every visit. Ophthalmologic and auditive evaluation as well as heart ultrasonography were performed every 6 months during the first year and every 6 to12 months after that. Brain image was carried out initially and on clinical demand.

We established the following outcome categories:

-Clinical outcome categories:

1- Progression towards neurological disease.
2- Progression towards hepatodigestive disease.
3- Progression towards myopathic disease.
4- Progression towards multisystem disease.

-Biochemical outcome categories:

1-Plasmatic lactate normalisation or initially normal lactate remaining unchanged.

2-A decrease but not normalisation in plasmatic lactate.

3- Persistent hyperlactacidemia.

Statistical analysis

We used two different statistical approaches:

1) The probabilities of survival depending on the presentation symptoms, the enzymatic deficiency and the initial lactate category, were estimated by Kaplan-Meier method for univariate analysis with difference calculated using a log-rank test (Kaplan 1958) whereas, the survival probability in function of the initial total amount of lactate (mmol/l) was analysed by Cox regression method.

2) We described four main groups of patients depending on the survival time (survival time categories): 1-Those who died during the first three months of life; 2-those who died between 4 and 12 months of life; 3- those who died between 1 and 3,9 years of life. 4-those who survived beyond four years of life. We compared these groups in function of the initial lactate and clinical presentation, enzymatic defect and clinical and biochemical outcome. This analysis was performed using Chi square test.

Finally, we attempted to relate the clinical and biochemical outcome of these patients with the presentation symptoms, initial lactate, and enzymatic deficiency. Comparison between groups was done using Chi square test.

We did not perform statistical analysis in function of the different treatments due to a great heterogeneity in therapeutic trials, and a short and irregular follow-up in those treated with CoQ_{10}.

3.3.2-METHODS USED IN POPULATION Nº 3 (31 PATIENTS WITH HEPATIC RESPIRATORY CHAIN DEFICIENCIES)

Patients' characteristics

Among our original series of 129 patients with mitochondrial cytopathies, 31 (24 % of the total population) presented an enzymatic defect in the liver. In 24 patients the deficiency was exclusively found in this organ whereas in 7 it was located in different tissues in addition to the hepatic tissue (liver and muscle: 2 cases, liver and fibroblasts: 5 cases). The sex distribution was 15 males and 16 females and the onset age ranged from 1 day to 3 years (mean: 105 days, SD: 232,6 days). In respect of the different age-onset categories we observed that 21/32 (66 %) were newborns and the rest were older than 1 month. Only two patients started the disease beyond 1 year of life.

Hepatic biopsy

With the parents' informed consent liver biopsies were carried out. In 12 patients (12/31: 38%) the choice to examine the hepatic tissue was due to the appearance of liver disease; half of them hitherto underwent a muscle biopsy on account of initial neurological symptoms. In 13 cases (13/31: 41,9%) the hepatic biopsy was performed according to routine established diagnostic procedures in deceased patients; 3 of them did not have signs of liver dysfunction. Finally, in 6 patients (6/31: 19%) without hepatic disease, the examination was indicated by the high clinical suspicion of mitochondrial cytopathy in spite of a previous normal muscle biopsy.

Microscopic studies. Liver biopsies were performed with Menghini needles. The specimens were fixed in 10% buffered formaldehyde, paraffin-embedded, and stained

with hematoxylin and eosin, Masson's trichrome, periodic acid-Schiff (PAS) and Perls'stain.

Respiratory chain studies. The activity of citrate synthase, complex I (NADH: ferricyanide reductase), complex II (succinate dehydrogenase), complex II+III (succinate: cytochrome c reductase), and complex IV (cytochrome c oxidase) in liver homogenates was performed as previously described (Rustin et al 1994).

Southern blot analysis was performed in the liver tissue as previously described (Vu et al 1998).

We have retrospectively analysed several aspects of this subgroup of our total population:

General characteristics:

1 -Clinical and biochemical presentation.

2- Morphologic, enzymatic and molecular findings

3- Clinical and biochemical long-term outcome

4- Characteristics of the patients who never presented with hepatic disease

Statistical studies:

Chi Square test for qualitative variables and Pearson's correlation for quantitative variables were used in order to detect possible relations between them: variables correspond to points nº 1, 2 and 3 described above.

Survival studies

The probabilities of survival depending on the points nº 1,2 and 3 defined above, were estimated by the Kaplan-Meier method for univariate analysis with difference calculated using a log-rank test (Kaplan 1958).

Comparison between patients with hepatic and non-hepatic tissue defects:

From our initial series of 129 patients with mitochondrial cytopathies we selected the patients in whom the defect was found in other tissues (98 patients) and attempted to find differences between both groups in function of onset-age, sex, clinical and biochemical presentation, enzymatic deficiencies and clinical and biochemical outcome. Comparison between groups was done using the Chi square test.

3.3.3- METHODS IN POPULATION Nº 4 (PYRUVATE CARBOXYLASE DEFICIENCY)

We retrospectively studied 9 patients with the French form of PC deficiency (4 females and 5 males) belonging to our initial series of primary mitochondrial disorders (129 patients) diagnosed and studied in our hospital **.**

We have analysed the following aspects:

1- The patients' clinical presentation.
2- Biochemical characteristics. We focused on plasmatic lactate, pyruvate, ketone bodies, ammonium, glucose, sodium, plasmatic amino acids and urine organic acids initially and on several occasions during the follow-up.
3- Enzymology: enzymatic studies were performed in cultured fibroblasts in all the cases. PC activity was determined as previously described (Atkin 1979).
4- Brain imaging: Brain MRI was carried out in 4 patients and CT scan in one case. All MRI were performed on a 1.5-T system, conventional spin echo-T2 weighted images.
5- Detailed description and outcome of abnormal movements.
6- Type of seizures and electroencephalographic findings.
7- Ocular movements and ophthalmologic examinations.
8- Treatment.
9- Global Outcome.

3.3.4- METHODS IN A PATIENT WITH IMPAIRED MITOCHONDRIAL PYRUVATE IMPORTATION

Patient

The patient was the first child of Algerian young consanguineous parents (the child's maternal great-grandfather and paternal grandmother are siblings). The family history was unremarkable except for a spontaneous abortion of a twin pregnancy at 5 months in the mother. The current pregnancy was complicated by gestational insulin-dependent diabetes and hypertension from the 6th month. Routine test to detect risk level of trisomy 21 was positive, but the parents had refused amniocentesis. Delivery was in duced at 37 weeks because of persistent toxaemia. Birth weight was 2710 g, length 46.5 cm and head circumference 31 cm. Clinical exam at birth showed generalized hypotonia, facial dysmorphism (bilateral inner epicanthus, long nasal filtrum, a very thin upper lip, single palmar fold, and small inverted widely spaced nipples, which were located higher than normal on the thorax), hepatomegaly, and respiratory distress. Initial laboratory investigations had ruled out infection but showed metabolic acidosis: pH 7.22, bicarbonate 12 mmol/l with hyperlactacidemia [14.3 mmol/l] (<2.2), transient hypoglycaemia at 2.2 mmol/l (3.5–6), and normal ammonia. The girl was referred to our service at Day 3 of life for further metabolic investigations. Persistent hyperpyru-vicemia [0.8–0.9 mmol/l] (<0.15) and a concomitant hyperlactacidemia [7–8 mmol/l] with a ormal or low lactate/pyruvate (L/P) ratio was confirmed. Ketone bodies and ammonia [57–102 lmol/l] (<50) were slightly elevated. Plasma amino acid chromatography (AAC) [results in micromol/l] showed a moderately elevated proline [286] (149–233) and alanine [383] (233–331). Urine organic acid chromatography (OAC) [results in micromol/ mmol creatinine] showed high excretion of lactate [1190]

(<104), 3-OH-butyric [321] (<74), alpha-ketoglutaric [650] (<63), 4-OH-phenylactic [321] (<3) a d 4-OH-phenyl-pyruvic [78] (<3) acids. These results (persistent hyper-lactacidemia with hyperpyruvicemia, L/P normal or low, a slightly elevated ammonia as well as the AAC and AOC profiles) lead to a suspicion of pyruvate dehydrogenase (PDHC) deficiecy. A treatment with 2-chloropropionate (100 mg/kg/day) and ketogenic diet was started. Thiamine (10 mg/day), carnitine (100 mg/ kg/day), riboflavin (20 mg/day), and bicarbonate (3 meq/ kg/day) were added as a complementary non–specific treatment of hyperlactacidemia. There was no clinical improvement and no decrease in hyperlactacidemia (Table A) with this regime . Complementary exams were performed to further investigate the different aspects of the clinical presentation : Karyotype was normal, cardiac echography revealed an interauricular (ostium secundum type, 4 mm) and interventricular (2–3 mm) communication with no hemodynamic compromise, abdominal ultrasonography was normal and mild renal insufficiency [creatinine 100 micromol/l] (20–50) was found. Ophthalmological examination showed, in the first months of life, a rotatory nystagmus with poor visual contact, normal electroretinogram and visual evoked potential. MRI showed cerebral atrophy with slight ventricular dilatation as well as periventricular leukomalacia and calcifications. Spectroscopy detected a high abnormal lactate peak in the caudate ganglia at 2 months. The evolving clinical course was one of severe multisystem involvement with persistent hyperlactacidemia (Table A). At 5 months, a functional renal test showed abnormal proximal and distal capacity for acidification . At 7 months, plasma creatinine was 113 micromol/l with normal plasma sodium, potassium and urea. At 9 month old the patient presented with persistent tachy-cardia (115 per minute) with normal electrocardiogram. Weight and length were stable at -2 DS, during the first year, but anorexia led to growth failure (-3DS) at 16 months of age. Persistent

hepatomegaly (2–3 cm) with normal liver function was noted. Her neurological status was consistent with very severe developmental delay, severe hypotonia with preservation of tendon reflexes, bilateral Babinsky sign , absence of hand prehension and brisk impulsive movements of arms and legs. The child was not walking at 16 months. Progressive microcephaly was noted at 5 months (-2.5 DS) and the head circumference was at -3DS at 16 months. Transient nystagmus was present and a worsening visual contact was observed. Treatment with bicarbonate (3–10 meq/kg/day), carnitine, ketogenic diet, arginine aspartate, and citrate resulted in no biochemical or clinical improvements (Table A). Enteral nutrition with a 70% lipids regime was started at 17 months. There was progressive neurological deterioration and the child died suddenly at home aged 19 months. A few months after the patient's death, her mother became pregnant again and asked for a prenatal diagnosis. A dichorionic twin pregnancy was discovered at ultrasound examination . Limits of the biochemical diagnosis, the possible occurrence of one normal and one affected fetus requiring a selective pregnancy termination were carefully explained to the parents who maintained their request.

Table A

Blood parameters in the proband

Nutritional state	3 Days			2 Months		5 Months		16 Months		17 Months
	BM[a]	BM[b]	AM[b]	BM[c]	AM[c]	BM[c]	AM[c]	AM[c]	After NGF[c]	BM[c]
Glucose (mM)	2.2	4.3	3.2	4.4	3.3	4.9	4.0	3.6	3.1	4.7
Lactate (mM)	5.39	7.89	7.58	6.12	5.79	7.71	6.91	8.52	5.52	4.90
Pyruvate (mM)	0.56	0.63	0.66	0.69	0.54	0.95	0.65	0.81	0.61	0.83
L/P	9.6	12.5	11.5	8.9	10.7	8.1	10.6	10.5	9.0	5.9
Ammonium (μM)	99	102	57	58	63	66	40	40	87	59
NEFA (mM)	0.15	0.42	0.24	0.48	0.42	0.62	0.29	0.35	0.74	0.18
3OH-butyrate (mM)	0.07	0.18	0.11	0.81	1.39	0.24	1.21	1.08	3.69	0.34
Acetoacetate (mM)	0.45	0.17	0.11	0.50	0.84	0.18	0.56	0.49	1.63	0.41

Abbreviations used: BM, before meal; AM, after meal; NGF, nocturnal continuous nasogastric feeding; NH3, ammonium; NEFA, non-esterified fatty acids.

[a] 2-Chloropropionate treatment.

[b] 2-Chloropropionate treatment and ketogenic diet.

[c] Ketogenic diet.

Methods

Cell lines

EDTA-blood samples for isolation of fresh lymphocytes and/or skin biopsies were obtained from the patient, controls and 4 patients with PDHC deficiency due to a mutation of the PDHA1 gene (two boys and two girls with G298E, dup1159-1162, R304X, a d R119W mutations, respectively). Trophoblasts were derived from chorionic villous biopsies obtained at 12 weeks of pregnancy from each of the twin fetuses at risk and from one age-matched fetus (at risk for a disorder that did not involve the energy metabolism). Fibroblasts and trophoblasts were grown in HAM F10 supplemented with antibiotics, 2.5 mM glutamine and 10–15% fetal calf serum.

PDHC assay

PDHC activity was measured by the release of 14 CO2 from 0.2 mM [1-14 C]pyruvate, using suspensions of lymphocytes or DCA-activated fibroblasts (Sheu et al 1981) disrupted by sonication . Cell suspensions were prepared in an ice-cold homogenizing buffer (100 mM phosphate buffer containing 2 mM EDTA and 1 mM DTT) and adjusted to 1 +/- 0,5 g/l protein . Total PDHC activity was measured after 10 minutes of preincubation at 37ºC with 20 mM $MgCl_2$ and 0.5 mM $CaCl_2$ in order to activate endogenous PDH phosphatase as previously described (Clot et al 1992) with minor modifications: 0.03 mM cytochrome C, 480 U/l cytochrome C reductase, and 5 mM L L-carnitine were added in the incubation medium to prevent inhibition of PDHC activity by its products (Sperl et al 1993).

Functional assays in permeabilized cells (Fig.A)

Incubations were performed in a respiratory medium [225 mM manitol, 25 mM Hepes, 50 lM EDTA, pH 7.4, 5 mM potassium phosphate, and 3 mM ADP] containing 20

microg/ml digitonin and 2 mg/ml bovine serum albumin , as previously described (Pande et al 1993).

Production of [^{14} C]acetylcarnitine and [^{14} C]citric cycle intermediates with [$^{2\text{-}14}$ C]pyruvate

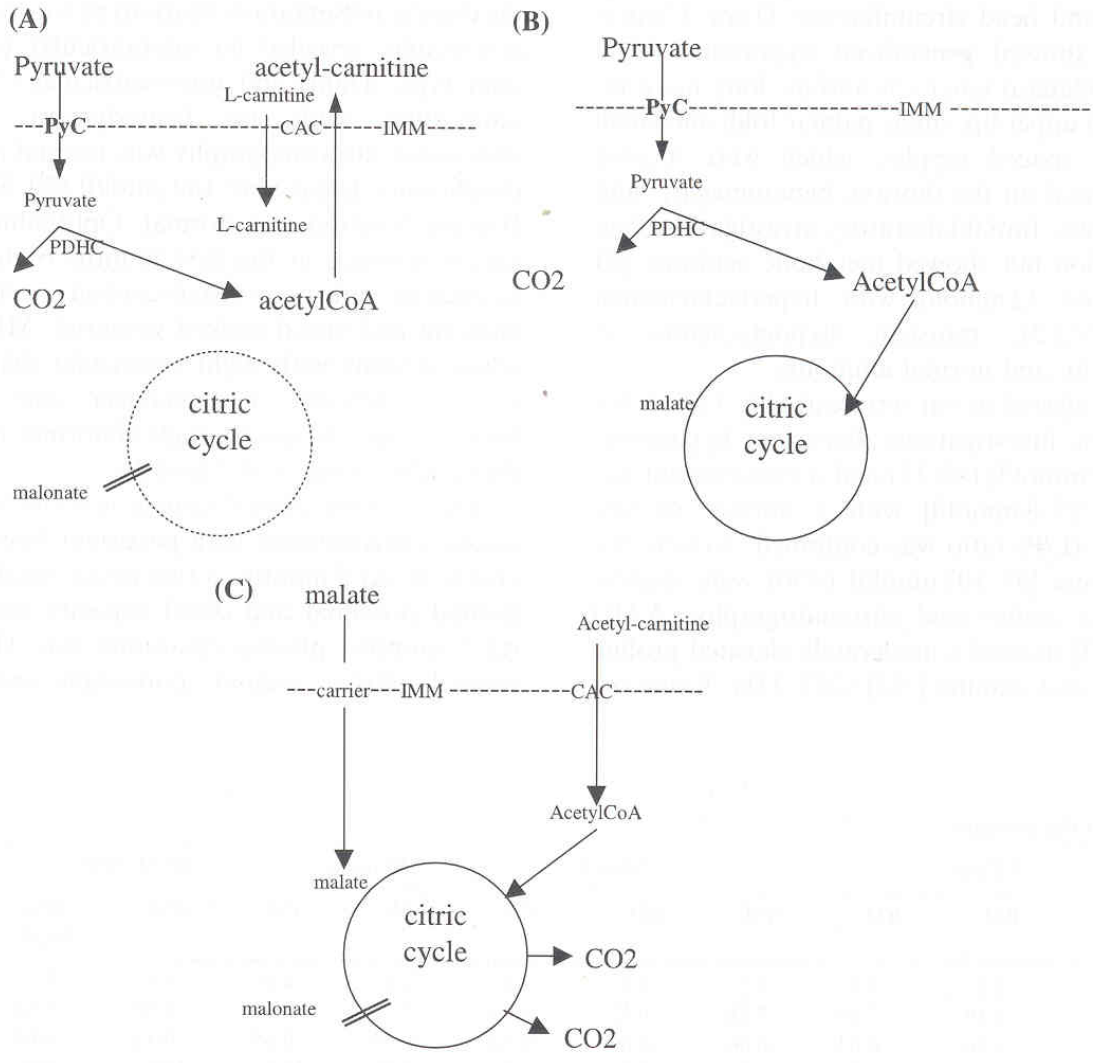
Fibroblasts or trophoblasts were plated in 24-well microtiter plates to assess the oxidation of 0.2 mM [$^{2\text{-}14}$ C]pyruvate to [^{14} C]acetyl-CoA and further conversion of [^{14} C]acetyl-CoA to either [^{14} C]acetylcarnitine or [^{14} C]citric cycle intermediates, in the presence of 2 mM dichloroacetate as previously described (Pande et al 1993), with two modifications : monolayers were directly used for incubation , without scrapping the cells and a excess of L L-carnitine was added in the medium (10 mM). As a control, the oxidation of 0.2 mM [$^{2\text{-}14}$ C]pyruvate into [^{14} C]acetylcarnitine was also measured in disrupted fibroblasts. Homogenates were prepared directly in each well by 3 cycles of freezing and thawing at -196 ºC and 25ºC in the same homogenizing buffer as for the PDHC assay with [$^{1\text{-}14}$ C]pyruvate. Ten minutes of preincubation at 37ºC were performed with 20 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 2 mM DCA to activate PDH-phosphatase and inhibit PDH-kinase. The incubation mixture was essentially the same as in the previous PDHC assay, but 0.2 mM [$^{2\text{-}14}$ C]pyruvate was used instead of 0.2 mM [$^{1\text{-}14}$ C]pyruvate in 250 microlitres of incubation medium instead of 625 microlitres.

Release of ^{14}CO2 with [$^{1\text{-}14}$ C]pyruvate and [$^{U\text{-}14}$ C] malate

Fibroblasts were plated at the bottom of vertical culture flasks (area 7 cm^2). Incubation with 1 mM[$^{1\text{-}14}$ C] pyruvate was carried out either in the presence of 4 mM malate or with 10 mM L L-carnitine plus 5 mM malonate, to avoid PDHC inhibition by trapping the produced acetyl-CoA. Parallel incubation with 10 mM [$^{U\text{-}14}$ C]malate was run in the presence of 10 mM L-acetyl-carnitine as a acetyl-CoA donor and 5 mM malonate to

avoid recycling of malate. The effect of a specific inhibitor of PyC (50 microM alpha-cyano-4-hydroxycinamate) (igma-Aldrich) (Halestrap et al 1974) was checked in control fibroblasts at increasing concentrations of [$^{1\text{-}14}$ C]pyruvate. As a control, effect of the inhibitor on [U-^{14} C]malate oxidation was also monitored.

Fig A



3.3.5 METHODS IN A PATIENT WITH HYPOGLYCAEMIAS AND HEPATIC COMPLEX III DEFICIENCY

Nomenclature

Gene mutation nomenclature follows the recommendations of den Dunnen and Antonarakis (2001). Gene symbols follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al 2001).

Case report

The patient, a girl, was the first child of healthy Turkish consanguineous parents and was born at term after a normal pregnancy and delivery. She developed normally until 8 months of age, when she presented with an episode of acute gastroenteritis. Clinical examination revealed slight dehydration, moderate tachypnea and slight liver enlargement (2 cm). Her weight, length and head circumference were normal. Laboratory investigations revealed hypoglycaemia (1.8 mmol/l; normal: 3.8–5.8 mmol/l), metabolic acidosis (bicarbonates: 6 mmol/l; normal: 23–29 mmol/l; hyperlactataemia (11 mmol/l; normal: <1.7 mmol/l) during metabolic crisis only. Intravenous infusion of glucose and bicarbonate corrected both clinical and biological alterations in a few hours, except for the persistent hepatomegaly and a mild elevation of serum transaminase levels (ASAT: 125 IU/l; normal: <50 IU/l; ALAT: 65 IU/l; normal: <60 IU/l). The amino acid profile was normal in serum but a moderate elevation of alanine (160 micromol/micromol creatinine; normal: <115 micromol/micromol creatinine) was noticed in urine. Ammonia, serum levels of muscular enzymes, serum cortisol and growth hormone levels, and total and free serum carnitine levels were normal. Normal overall fatty acid oxidation and normal activities

of very long and medium chain fatty acylCoA-dehydrogenases were demonstrated in cultured fibroblasts. Fructose 1–6 bisphosphatase and pyruvate carboxylase activities were found to be normal in a liver biopsy. During the course of a fasting test, hypoglycaemia occured 19 h after the last meal and became symptomatic after 21 h (1.1 mmol/l) associated with hyperlactacidaemia (4.16 mmol/L) and low ketogenesis suggesting a functional defect of fatty acid oxidation in these conditions. An abnormality of the respiratory chain was considered as an explanation of the impairment of fatty acid oxidation. Lactate and pyruvate levels monitored in blood before and after meals revealed no abnormality. Nevertheless, enzymatic studies in lymphocytes, liver and fibroblasts demonstrated the respiratory chain defect.

The child is now 4 years of age with normal growth and no signs of psychomotor retardation or neurological impairment. The initial liver enlargement progressively disappeared and liver size was normal at 19 months of age. Cardiac and abdominal ultrasound and ophthalmological work-up was consistently normal. Biological parameters remained unaltered, except for two further episodes of hypoglycaemia (1.3 mmol/l) with metabolic acidosis at 2.0 years and 2.5 years of age.

Methods

Informed consent was obtained from all tested individuals. Fibroblasts were grown in Ham's F10 medium supplemented with 10% fetal calf serum, 2.5 mM pyruvate and 200 uM uridine. Oxygen consumption was measured with a Clark oxygen electrode (Hansa-tech, UK) in a 250-microliter cell, thermostated at 37°C according to Rustin et al., 1994. Results are presented as rates of O2 consumption in digitonin-permeabilized cells. Respiratory chain complex activities were measured spectrophotometrically in lymphocytes, fibroblasts and liver biopsy by standard procedures (Rustin et al 1994). Low temperature difference spectra was performed at liquid nitrogen temperature (–

196°C) according to published procedures (Chance et al 1995, Bourgeron et al 1991). For mutation analysis, direct sequencing of BCS1 and the eleven structural CIII subunits genes (cyt c1, cyt b, core 1, core 2, FeS protein, Rieske 1, QP-C, Hinge, 9.5-kDa protein, 7.2-kDa protein and 6.4-kDa protein) was performed in our patient and five controls. Total RNA and DNA were extracted from liver biopsy, lymphocytes and skin fibroblasts. Reverse transcription/polymerase chain reaction (RT-PCR) for cDNA preparation and PCR amplification of genomic fragments were conducted as described by Valnot et al. 2000 and de Lonlay et al. 2001 with specific oligonucleotides generously given by I. Valnot. PCR amplification was performed in a 50-microliter volume containing 100 ng DNA or cDNA, 1.5 mM MgCl2 , 0.2 mM dNTP, 20 pmol each primer and 0.6 U *Taq* polymerase. Amplification conditions included an initial denaturation at 94°C for 5 min, 30 cycles of 30 s at 95°C, 30 s at 45–55°C (depending on the primers) and 1 min at 72°C and a final extension at 72°C for 10 min. After purification, sequencing was performed on an automated Abi Prism 310 sequencer by using a Big Dye Terminator reaction kit (Applied biosystems).

Table B Biochemical values of respiratorry chain function and enzymes activities in lymphocytes, fibroblasts and liver biopsy were measured according to Rustin et al. (1994). Results are expressed as extreme absolute values or absolute values for controls or patient, respectively (*bold* abnormal values). In controls, activity ratios only were calculated and are given as means ± SD.
a- Values in nanomol O2 /min per milligram protein
b- Values as nanomol sub-strate/min per milligram protein

	Lymphocytes		Fibroblasts		Liver biopsy	
	Controls (n=15)	Patient	Controls (n=25)	Patient	Controls (n=10)	Patient
Substrate oxidations[a]						
10 mM succinate	9–15.2	**6.0**	6.5–14.3	**4.6**		
10 mM Pyruvate + 1 mM malate	5–8.4	4.5	3.3–6.8	3.8		
Decyl Ubiquinol	11–15.7	**7.5**	8.5–23.2	**2.7**		
Respirtory chain complexes[b]						
Complex I					19–26	12
Complex II	14–33	32	10.8–17	10.6	168–277	179
Complex III	75–237	**55**	98–180	**17**	143–192	**10**
Complex IV	85–269	170	72–143	**55**	202–319	210
Complex V					74–167	281
Citrate synthase (matrix)	36–85	**145**	32–72	32	63–131	131
Activity ratio						
Complex IV/III	1.5±0.2	**3.1**	1.0±0.2	**3.2**	1.4±0.2	**21**
Complex IV/II	6.8±0.5	5.3	6.0±0.9	5.2	1.5±0.3	1.2
Complex IV/CS	2.0±0.4	1.2	2.0±0.4	1.7	2.9±0.2	1.6
Succinate/pyruvate	2.1±0.4	1.3	1.9±0.3	1.2		

Screening for the absence of the 4-bp deletion in controls was performed by denaturing high-pressure liquid chromatography (DHPLC) as previously reported (De Lonlay et al 2001). Exon 4 was amplified by PCR from 50 Turkish individuals. The primers used (forward/reverse, 5'–3') for amplification products from genomic DNA were as follows: CAGATAGTTCTTAGGTTGAGA, CAGCTGCATCCACAGACTTCA.

PCR product (10 microliters) from the patient was mixed with PCR product (10 microliters) from a normal reference DNA. Amplification products were then denaturated at 95°C for 5 min and allowed to cool to 25°C for the formation of heteroduplexes. DHPLC was carried out on a Transgenomic WAVE HPLC and DNASep column. Separation was carried out at a flow rate of 0.9 ml/min over a 3.5-min period though a linear acetonitrile gradient (54–63%) at 55.9°C.

4- RESULTS

4.1.- RESULTS IN POPULATION Nº 1 "TOTAL POPULATION REFERRED TO IN THE THESIS: 241 CHILDREN WITH DIVERSE MITOCHONDRIAL DISORDERS"

Among the total population of 241 patients with mitocondrial disorders (131 females and 110 males) we found 129 MRC, 17 PDHC, 9 PC, 4 KC deficiencies, 1 pyruvate transporter deficit, and 81 patients without a definitive diagnosis, even with a constant HL and the absence of an acquired cause. Those 81 have been qualified as "non classified hyperlactatemias".

For each group we have analysed:

1-First symptoms on the basis of presentation age: new-borns (0-30 days), infants from 1 month to 1 year, children older than 1 year.

2-Biological exams at the beginning of the disease: redox cycle, AAC, AOC.

3-Exams leading to a conclusive diagnosis: enzymatic and genetic studies.

5-Role of blood lactate in the diagnostic tree.

Mitochondrial respiratory chain deficiencies: 129 cases (58 males, 71 females)

Clinical presentation

In 57 (30 males and 27 females) of the 129 patients with MRC, clinical symptoms started during the neonatal period, in 48 (16 males and 32 females) during the first year, and in 24 (12 males and 12 females) beyond the first year of life. Clinical presentation depending on the different age-ranges is represented in table 1.1 and figure 1.2.

Newborns had multi-system presentation in the majority of cases (53 out of 57: 92,9%)**.** The most common pattern of symptom combination was generalized hypotonia associated with feeding and respiratory difficulties, with a well-preserved level of consciousness. 25 patients out of 57 (43,8%) had this presentation. Regarding the various system involvements, the nervous system was the most frequently affected and present in 21 cases or 36,8% (generalized hypotonia was the most widespread symptom), the next more frequent were the hepatodigestive presentation (15 cases, 26,3% ; the main sign was hepatomegaly with elevated transaminases) and failure to thrive (8 cases: 14%). 3 infants (5%) had cardiac disorders and 2 patients (4%) had rotation nystagmus.

Patients presenting during the first year of life had predominantly mono-system involvement (34 out of 48: 70,8%). Neurological presentation was also the most frequently found (29 cases; 60%): non-specific developmental delay was the most common manifestation followed by neurological regression and Leigh syndrome. Failure to thrive was observed in 11 patients (23%) . Haematological involvement was discovered in 4 patients followed by cardiac symptoms (4 patients), hypoglycaemia (3 cases), renal proximal tubulopathy (2 cases), 2 patients had hyperpilosity, one patient had pigmentary retinitis and another one hepatomegaly with hepatic cytolysis.

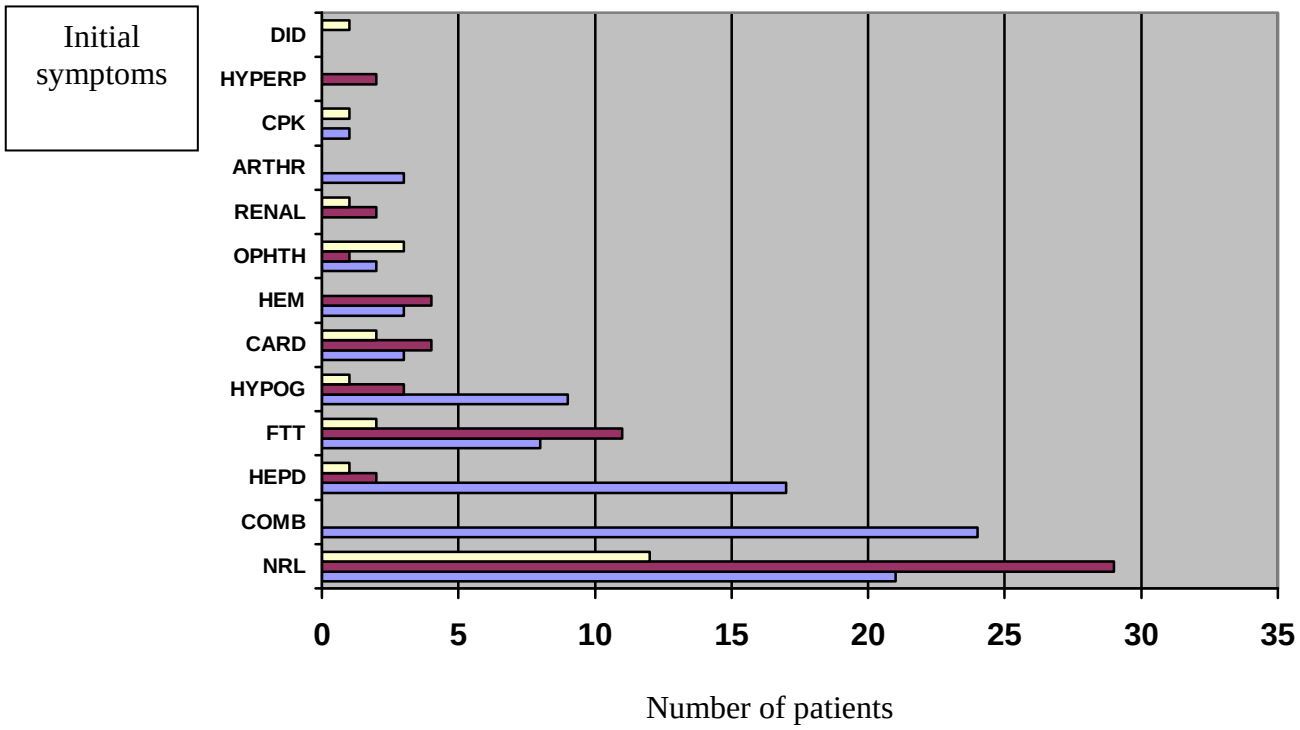
Children older than 1 year presented with mono-system involvement in 23 out of 24 patients (95,8%). Neurological symptoms were predominant (12 patients; 50%). Non-specific psychomotor delay was the most frequent form of presentation followed by Leigh syndrome. The other manifestations observed were: ophthalmologic (3 cases), 2 cases presenting with failure to thrive, 2 cardiomyopathies, 2 patients with renal disorders, 1 with Reye syndrome and 1 with insulin dependent diabetes.

TABLE 1.1- Clinical presentation of CRM depending on the onset age

SYMPTOMS	NEWBORNS	1month-1 year	>1 year	TOTAL
COMBINED	24	0	0	24
NEUROLOGIC	21	29	12	62
HYPOTONIA	15	6	0	21
PSYCHOMOT DELAY	0	13	8	21
REGRESSION	0	9	1	10
LEIGH	1	5	4	10
DYSKINESIAS	1	4	1	6
COMA	2	2	1	5
SPASTICITY	2	0	0	2
SEIZURES	1	1	0	2
TREMOR	2	0	0	2
MICROCEFALY	1	0	0	1
FAILURE TO THRIVE	8	11	2	21
HEPATO-DIGESTIVE	17	2	1	20
VOMITING	2	1	0	3
HEPATIC FAILURE	5	0	1	6
HEPATOMEGALY	10	1	1	12
HIGH TRANSAMINASES	15	1	2	18
COLESTASIS	2	0	0	2
HIPOGLYCEMIA	9	3	1	13
CARDIAC	3	4	2	9
CARDIAC FAILURE	1	2	0	3
DILATED CMP	0	2	2	4
HYPERTROPHIC CM*	2	0	0	2
HEMATOLOGIC	3	4	0	7
ANAEMIA	2	4	0	6
PANCYTOPENIA	1	0	0	1
OCULAR	2	1	3	6
NYSTAGMUS	2	0	0	2
RETINITIS	0	1	2	3
OPTIC ATROPHY	0	0	1	1
RENAL	0	2	1	3
TUBULOPATHY	0	2	0	2
RENAL FAILURE	0	0	1	1
ARTHROGRYPOSIS	3	0	0	3
CPK ↑	1	0	1	2
HYPERPILOSITY	0	2	0	2
DYSMORPHY	1	0	0	1
DID*	0	0	1	1

COMBINED: HYPOTONIA, TACHYPNEA, FEEDING DIFFICULTIES
CM*:CARDIOMIOPATHY.
DID*: INSULIN-DEPENDENT DIABETES

Fig 1. 2 Clinical presentation of respiratory chain disorders.



Blue bars : newborns. Purple bars: infants. White bars: children older than 1 year

DID: insulin-dependent diabetes. HYPERP: hyperpylosity. ARTHR: arthrogryposis. OPHTH: ophthalmologic. HEM: hematologic. CARD:cardiac. HYPOG:hypoglycemia. FTT:failure to thrive. HEPD:hepato-digestive.COMB:combined association of: tachypnea, feeding difficulties and hypotonia. NRL:neurologic

Biochemichal characteristics of MRC (Table 1.2)

Regarding blood lactate, we defined 4 categories of HL depending on the lactate concentration:

1-Normal lactate < 2,4 mmol/l.

2-Mild hyperlactatemia: lactate oscillating between 2.5 to 3 mmol/l.

3-Moderate: constant hyperlactatemia between 3 to 6 mmol/l.

4-Severe or lactic acidosis: higher than 6 mmol/l, permanent or transient.

Lactate ranged from 1.2 to 25.5 mmol/l (SD:5,55) and global mean was 6.4 mmol/l (median: 4,4 mmol/l). Mild HL was observed in 11 patients (8.5%), moderate in 40 (35.6%. Mean:4.34. SD:0.88) and severe in 49 (37,9%. Mean:11.7. SD:5.8). Lactate was normal in 29 patients (22.4%). Lactacidemia was markedly higher in new-borns (Fig S1) (mean:9.7. median: 7,5 mmol/l. SD:6.6. Normal in 7 cases) than in the second group (mean :4.2 mmol/l. median:3,6 mmol/l. SD: 2.3. Normal in 11 patients) and than in children older than 1 year (mean: 2.8 mmol/l. median: 3 mmol/l. SD: 1.2. Normal in 11 cases). L/P ratio was found to be high in all cases (>16). Total post-pandrial ketone bodies and 3OHB/AcAc ratio were increased in 45% and 76% of the cases respectively. AAC was abnormal in 84 cases (64,6%): 44 newborns, 31 infants and 9 children older than 1 year. The most frequent increase was in alanine (81 cases: 62,7%) followed by proline (36 cases: 27,9%) and glutamine (27 cases: 20,9%).

OAC in newborns was abnormal in 83 patients (64,3%): 43 newborns, 34 infants and 6 children older than 1 year. Lactate and fumarate were the predominantly increased acids.

Table 1.2 Biochemical characteristics of MRC disorders

	NEWBORNS	1 MONTH-1 YEAR	> 1 YEAR	TOTAL
LACTATE (mmol/l)				
range/SD	1.2-25.5/6.6	1.2-11.8/2.3	1.2-5.6/1.2	1.2-25.5/5.5
Mean	9.7	4.2	2.8	6.4
Median	7.5	3.6	3	4.4
Nºcases<2.4mmol/l	7	11	11	29
PYRUVATE (mmol/l)	0.1-1.6	0.2-1.4	0.12-1.7	0.1-1.7
L/P	21-74	19-52	16-34	16-74
Total KB increased	32%	92%	12%	45.3%
3OHB/AcAc>1	48%	43%	47%	76.3%
AAC abnormal	77%	64%	37%	64.6%
Increased AA				
Alanine	46/57	30/48	15/24	62.7%
Proline	23/57	9/48	4/24	27.9%
Glutamine	17/57	7/48	3/24	20.9%
OAC abnormal	43/57	34/48	6/24	64.3%
Increased OA				
Lactate	43/57	31/48	5/24	61%
Others	40/57	28/48	5/24	57%

Enzymatic and genetic results

We found 45 patients with complex I (CI) deficiency, 27 with complex IV (CIV), 21 with CI+CIV, 11 with generalized deficiency, 5 with complex III (CIII), 4 with complex II (CII), 4 with different combination deficiencies (2 with CIII+CV, one with CI+II+IV and another one with CII+III+IV), 2 with complex V (CV). In 3 patients we found muscle morphology suggestive of mitochondrial cytopathy without enzymatic deficiencies. These deficiencies were distributed as follows :

Neonatal group. CI= 24; CI+IV= 12; CIV=7; Generalized= 7 (6 of them associated with mitochondrial DNA depletions); CII= 3. CIII= 1; CV= 1; CIII y IV= 1; on muscle morphology criteria =1.

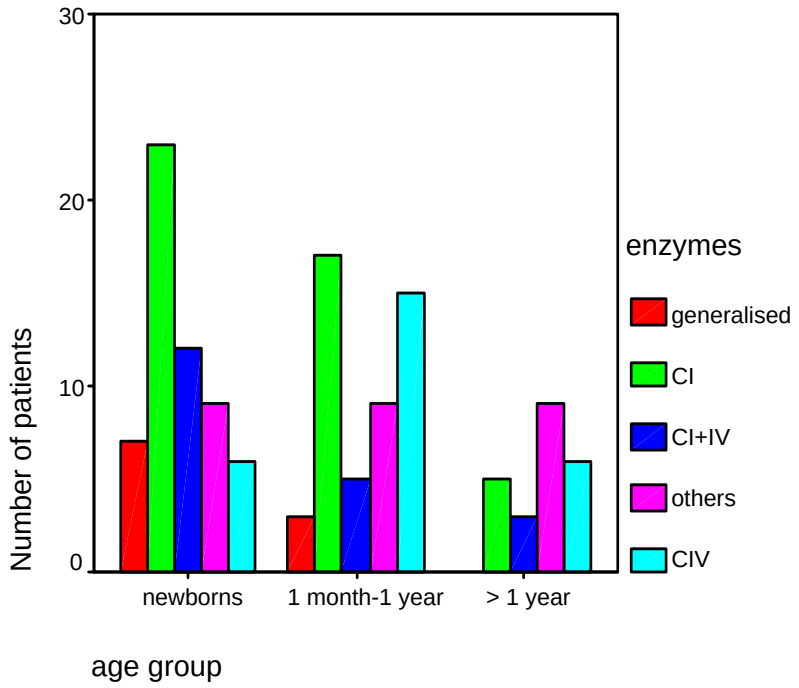
Second group: CI= 16; CIV= 14; CI+IV=6 ; Generalized= 2; CV= 1; CIII= 1; CII= 1; CI+II+IV: 1; CII+III+IV= 1. Mutations: 1 MELAS, 1 Leber, 1 Kearns-Sayre, 2 NARP, 1 Pearson (associated with CI+II+IV deficiencies), and 2 depletions (those with generalized deficiencies)

Children older than 1 year: CIV= 6; CI=5; CI+IV=3; CIII= 3; generalized= 2; Morphology of muscle= 2; CIII+IV= 1. Mutations: 2 MELAS (one of them with associated CI deficit), 3 Kearns-Sayre (two of them with associated CIII and CI+CIV deficiencies).

Figure 1.3 represents the enzymatic deficiencies depending on the onset age.

Muscle was the first tissue where enzymatic deficiencies were localized (80 cases: 61.5%) followed by liver (24 cases: 18.6%), fibroblasts (6 cases: 4.6%), heart (6 cases: 4.6%), combined tissues (liver and fibroblasts: 5 cases; 3.8%, liver and muscle: 2 cases; 1.5%, muscle and fibroblasts:1 case; 0.8%) and lymphocytes (2 cases: 1.5%).

Fig 1.3 Enzymologic studies of MRC disorders



Count

		enzymes					
		generalised	CI	CI+IV	others	CIV	Total
age group	newborns	7	23	12	9	6	57
	1month-1 year	3	17	5	9	15	49
	> 1 year		5	3	9	6	23
Total		10	45	20	27	27	129

Statistics

In patients with a definite diagnosis of MRC we found statistically significant differences (<0.05) concerning mean lactate and the following (analysed by multiple comparisons studies, Bonferroni and Tukey):

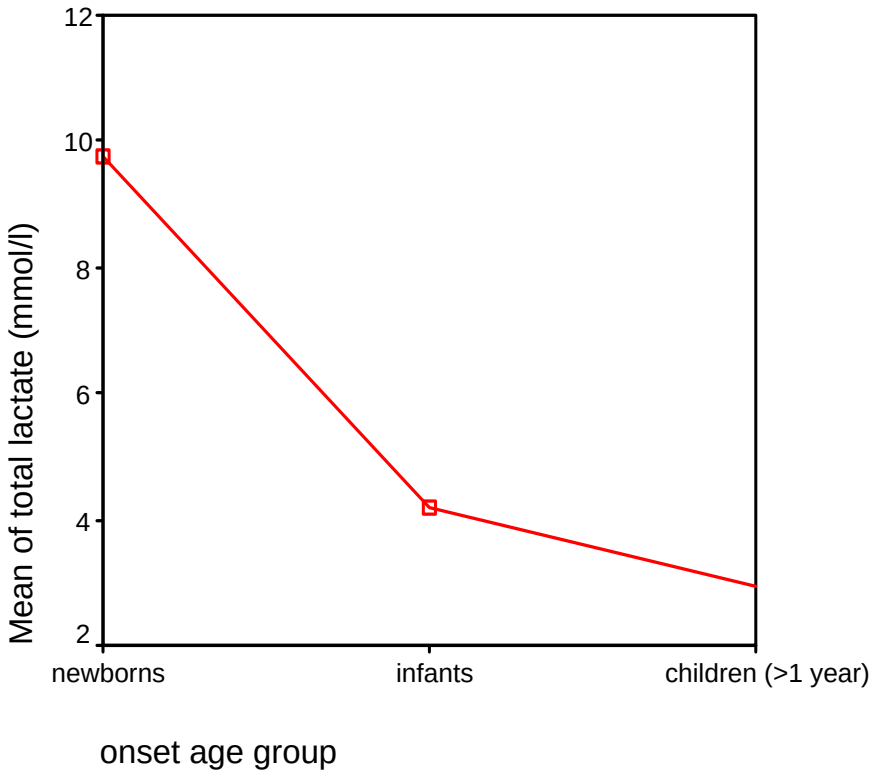
1-Presentation age group (FigS1): Newborns are more likely to present with higher lactates (p<00.5) than older children are. No significant differences were found between infants and children older than 1 year.

2-Enzymatic deficiencies (FigS2): Patients with CI+CIV deficiencies have a significant increase of lactic acid levels (p<00.5) compared with those with CI deficiency. No significant differences were observed within the other groups or between single and combined deficiencies either. Although the highest lactates were seen in CII deficiencies, the few number of cases found in this category as well as in other groups did not allow us to establish statistical comparisons.

3-Clinical presentation (FigS3): no differences were found between monosystem and multisystem presentation. Children with the association of hypotonia, tachypnea and feeding difficulties, or hepatic presentation had higher lactates but no statistically significant.

Statistics: Fig S1,S2 and S3

Fig S1 . Mean lactate depending on the group ages

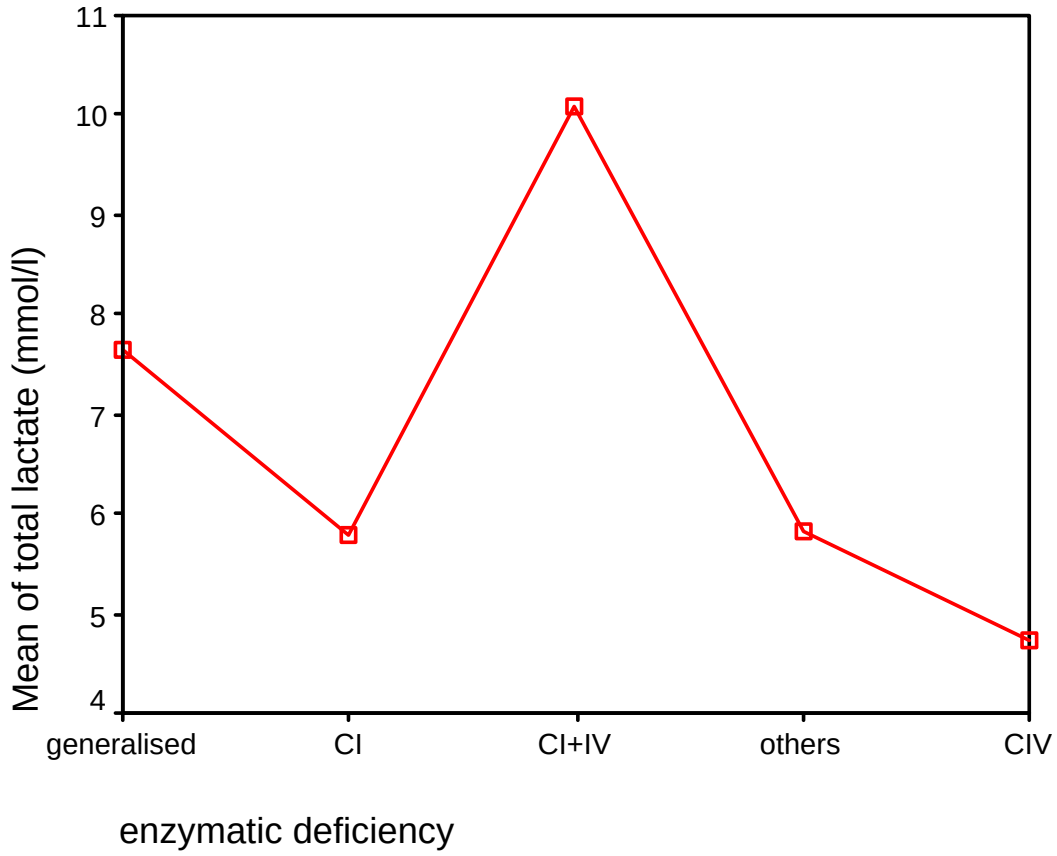


Mean LACTOTAL: mean lactate in mmol/l

1-Newborns
2-Infants (1 month-1 year)
3-Children older than 1 year

p:0,00 between 1 and 2 and 1 and 3.
p:0.492 between 2 and 3

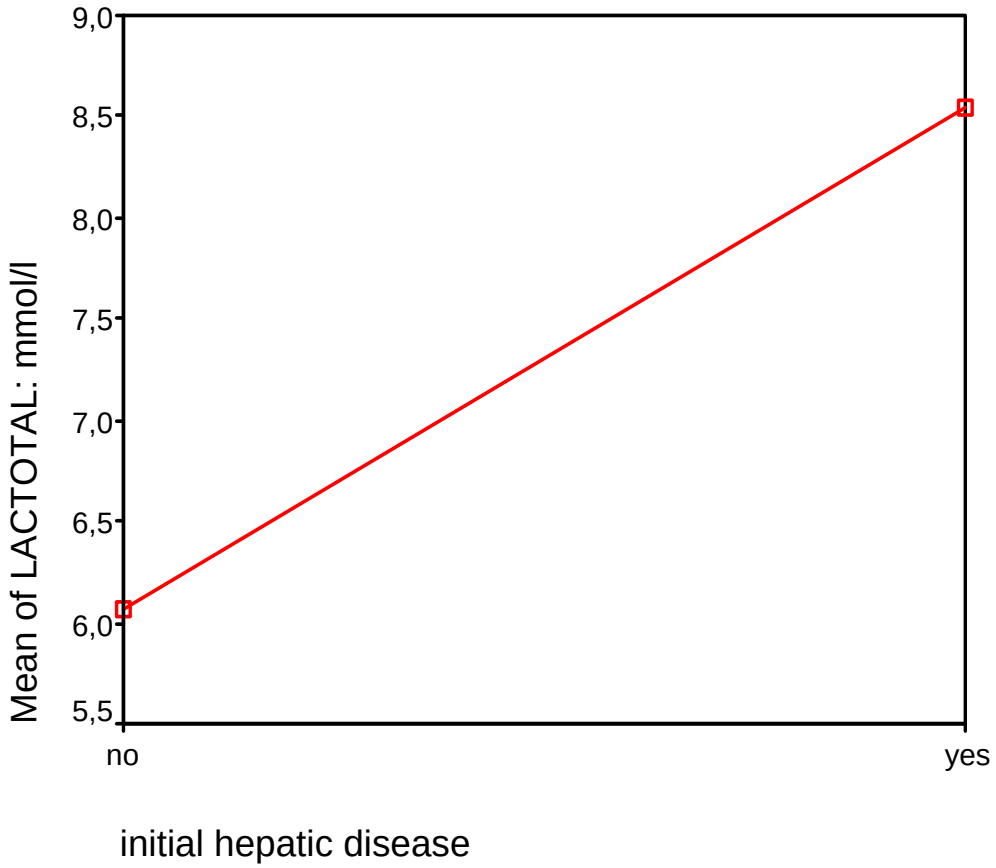
Fig S2 Mean blood lactate and enzymatic deficiency



LACTOTAL

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		398,334	4	99,584	3,484	,010
	Linear Term	Unweighted	54,866	1	54,866	1,920	,168
		Weighted	49,245	1	49,245	1,723	,192
		Deviation	349,090	3	116,363	4,071	,009
Within Groups			3544,181	124	28,582		
Total			3942,515	128			

Fig S3 Mean blood lactate and clinical presentation



LACTOTAL

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		94,537	1	94,537	3,120	,080
	Linear Term	Unweighted	94,537	1	94,537	3,120	,080
		Weighted	94,537	1	94,537	3,120	,080
Within Groups			3847,979	127	30,299		
Total			3942,515	128			

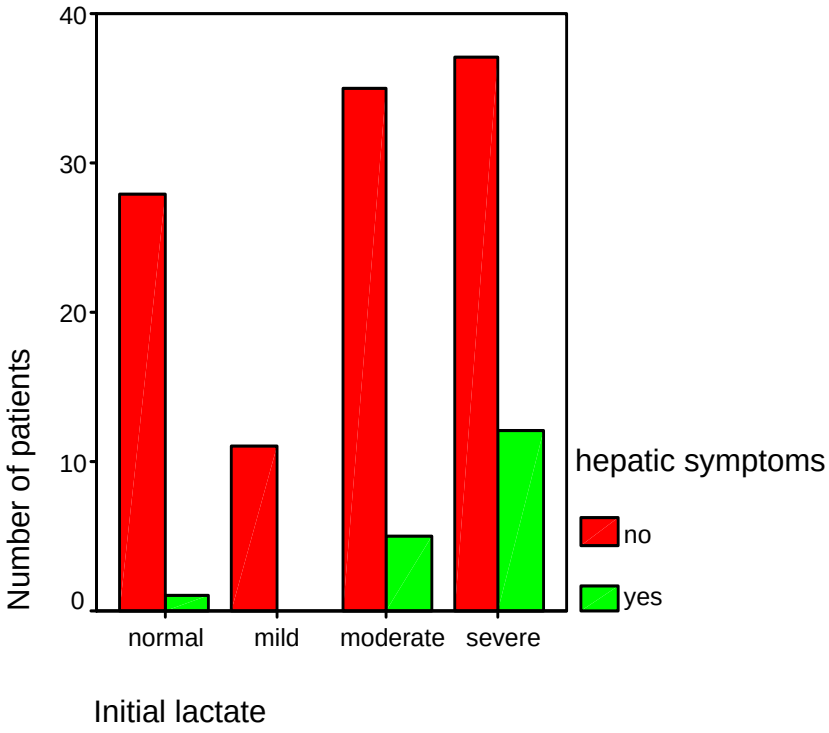
On the other hand, relation between the different lactate categories (normal, mild, moderate or severe lactate) and other different variables (age, enzymatic deficiency, clinical presentation, plasmatic amino acids and urine organic acids) using the Chi Square test, disclosed the following:

1-Presentation age group: newborns had statistically significant higher lactates also using this method. (p: 0,00).

2-Patients presenting with initial hepatic symptoms tended to have higher lactates (p:0,029); fig S4.

3-Abnormal plasmatic amino acids as well as urine organic acids, were mostly found when lactate was very high (p:0,00); fig S5 and S6.

Fig S4. Initial hepatic symptoms and lactate

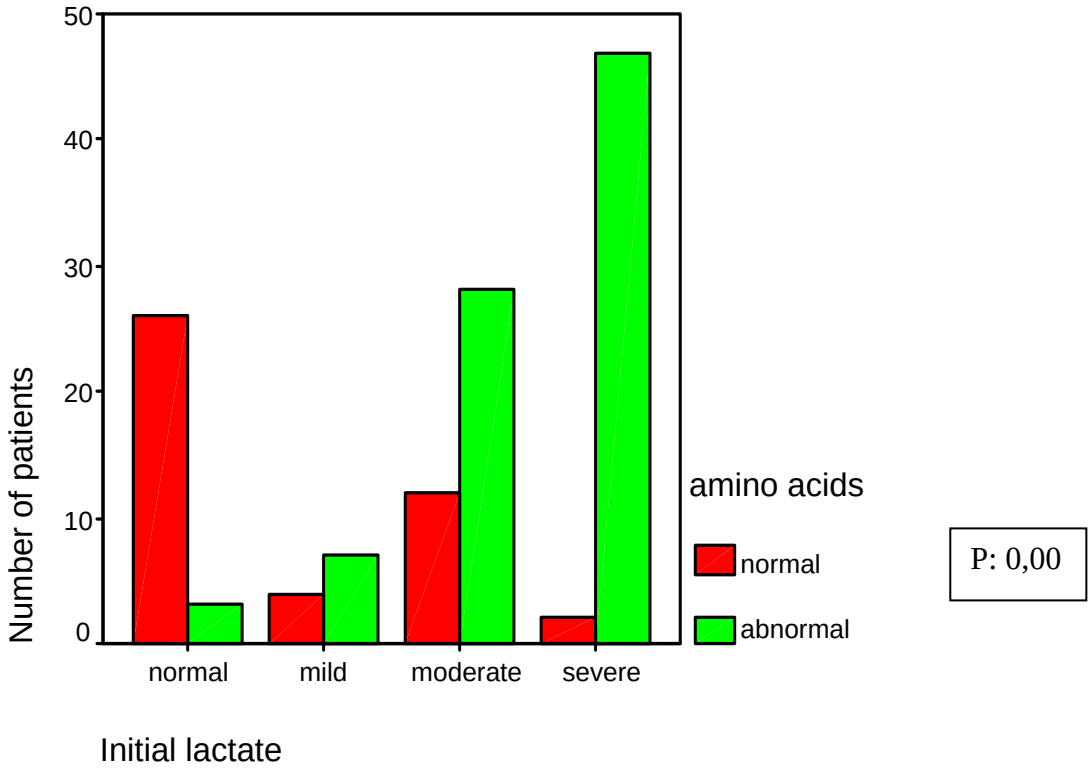


P: 0,029

Count

		Hepatic symptoms		
		no	yes	Total
Lactate	normal	28	1	29
	mild	11		11
	moderate	35	5	40
	severe	37	12	49
Total		111	18	129

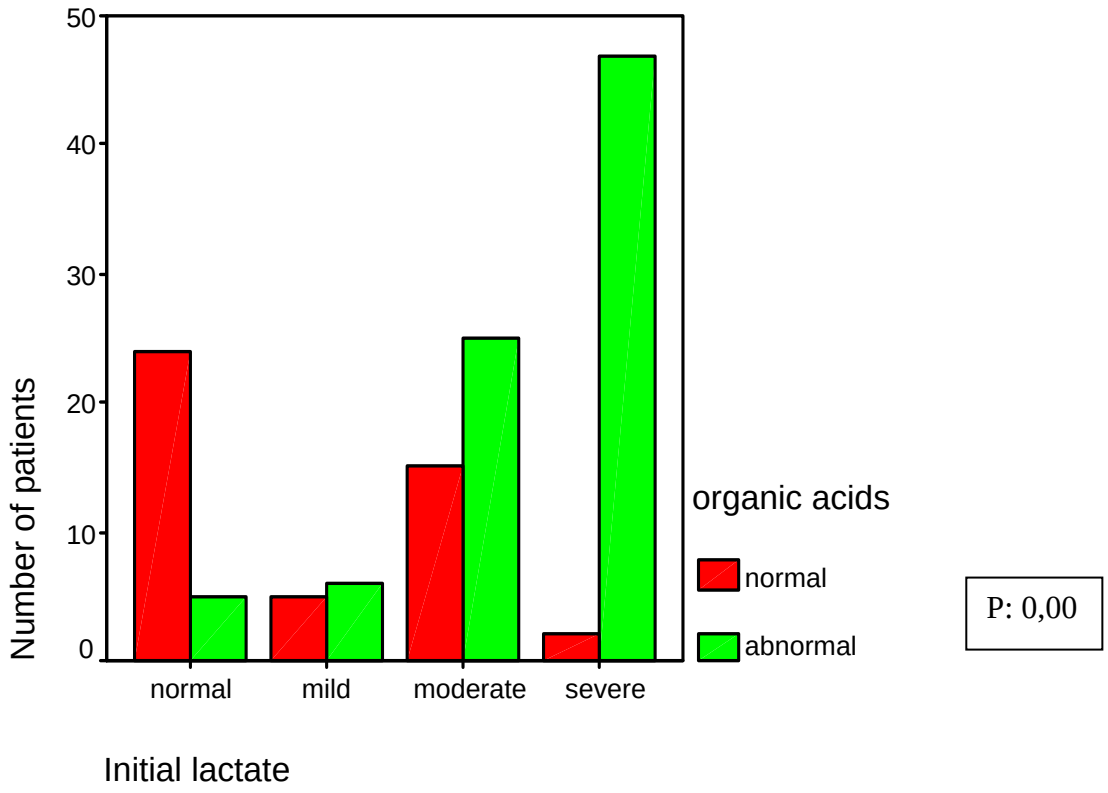
Fig S5. Lactate and plasmatic amino acids



Count

		amino acids		
		normal	abnormal	Total
Lactate	normal	26	3	29
	mild	4	7	11
	moderate	12	28	40
	severe	2	47	49
Total		44	85	129

Fig S6. Lactate and urine organic acids.



Count

		organic acids		
		normal	abnormal	Total
lactate	normal	24	5	29
	mild	5	6	11
	moderate	15	25	40
	severe	2	47	49
Total		46	83	129

PDHC deficiencies: 17 cases, (Table 1.2)

This group is composed of 13 males and 4 females: 4 newborns (all the females), 9 infants and 4 children older than 1 year (age range: 1- 6 years). Initial symptoms were hypotonia and tachypnea in the neonatal period, Leigh Syndrome and strabismus in infants, and acute ataxia, peripheral neuropathy and Leigh Syndrome in the oldest children. Biochemical exams showed: metabolic acidosis in 11 cases (constant in new-borns), hyperlactacidaemia (range: 3,1 to 16,3 mmol/l; mean:8,9; SD:3,4). Post-prandial lactate increase was present in all the cases as well as hyperpyruvicaemia (range: 0.2 - 1.3 mmol/l), L/P ratio always below 10, and normal ketone bodies with 30HB/AcAc <1. AAC showed increased alanine and proline and the AOC Kreb's cycle derivates. Diagnosis was confirmed by measuring PDH activity in lymphocytes or fibroblasts (residual activities were between 15 and 40%). Gene E1alpha was abnormal in 5 cases and X protein in 1 case.

Pyruvate carboxylase deficiency: 9 cases. (Table 1.2)

The results of this group will be exposed in detail later as an individual objective (4.5- Pyruvate carboxylase deficiency: metabolic and neurological characteristics of nine patients).

Krebs cycle deficiencies: 4 cases (Table 2)

The 4 patients are males; among them 3 are brothers starting in the neonatal period with tachypnea and hypotonia. 1 case presented with coma at 3 months of life. Biological examinations showed severe metabolic acidosis and hyperlactacidaemia (range:2,8-23,5, mean:11,6;SD:9,5), L/P ratios and ketone bodies were elevated (normal 3OHB/AcAc ratio), glutamine was markedly increased and urinary derivates from the

Kreb's cycle were present (αketoglutarate, succinic, fumaric, and malonic acid). Diagnosis was confirmed by dosage of αketoglutarate dehydrogenase in muscle.

Pyruvate transporter deficiency: 1 case

This case is developed in detail as a different objective: 4.6-Impaired mitochondrial pyruvate importation in a patient and fetus at risk.

The patient was the first child of healthy consanguineous parents. She presented at birth with hypotonia, mild facial dysmorphism, hepatomegaly, periventricular cysts, marked metabolic acidosis and hyperlactacidemia (5-14 mmol/l) with normal L/P ratio. PDHC activity was normal in lymphocytes and fibroblasts. The results of functional assays to measure oxidation rates from radiolabeled pyruvate and malate were consistent with a defect of mitochondrial pyruvate transport in the patient. Mutation analyses has not been identified as yet in any organism. The evolving clinical course was one of severe multisystem involvement with persistent hyperlactacidemia (8,5-4,9 mmol/l) and progressive neurological deterioration. The child died suddenly at home aged 19 months.

Non classified hyperlactacidaemias: 81 cases

This group is composed of 39 males and 42 females. 42 of them starting in the neonatal period, 30 between 1 month and 1 year and 9 from 1 year on. It is characterised by the absence of a conclusive diagnosis, persistent hyperlactacidaemia (range:2,5-18,3, mean:5,2;SD:4,3) and a progressive neurological impairment. Nevertheless most of the cases (48/81) have been observed before 1990 when enzymatic investigation was not complete . They have not been evaluated later because some of them have died and the rest of them have not had regular clinical monitoring.

Those who have been followed up developed neurologic degradation (Leigh Syndrome as the most frequent outcome) followed by ophthalmological, cardiac and hepatic involvement.

Table 3. Comparative clinical and biological results in PDHC, PC and KC

	Onset age	Main symptoms	Associated symptoms	Basic biology (mmol/l)	AAC AOC	Enzymes and genes
PDHC	7 days to 6 years	**New-borns** tachypnea **1-12 months** Leigh Syndrome **1-6 years** ataxia neuropathy Leigh Syndrome	Strabismus Recurrent respiratory infections. **IRM:**corpus callosum agenesys. White matter alterations	**BIC:**8-12 **LAC:**4-16 **PYR:**0.2-1.3 **L/P**:<10 **KB** normal **B/A:**normal	**AAC** aa elevated: alanine proline **OAC** Lactate pyruvate	5 E1 αgene 1 X protein
PC	New-borns Between 1 and 3 hours of lifle	Tachypnea Preserved level of consciousnes	**MRI:** periventri-cular Fronto-parietal. cysts HyperNH3 Hypoglycaemia. HyperNa	**BIC:**4-17 **LAC:**7-22 **PYR:**0.5-12 **L/P:**20-60 **KB** increased **B/A:**normal	**AAC** aa elevated: proline citrulline lysine aa decreased: glutamine aspartate **OCA** Lactate 3OHbut 2OHbut	0-10% activity PC liver, fibroblasts.
KREBS CYCLE	1-3months	Hypotonia Tachypnea	Cardio-myopathy.	**BIC:**10-18 **LAC:**2.8-23	**AAC** aa elevated:	αKetoglu-tarate DH

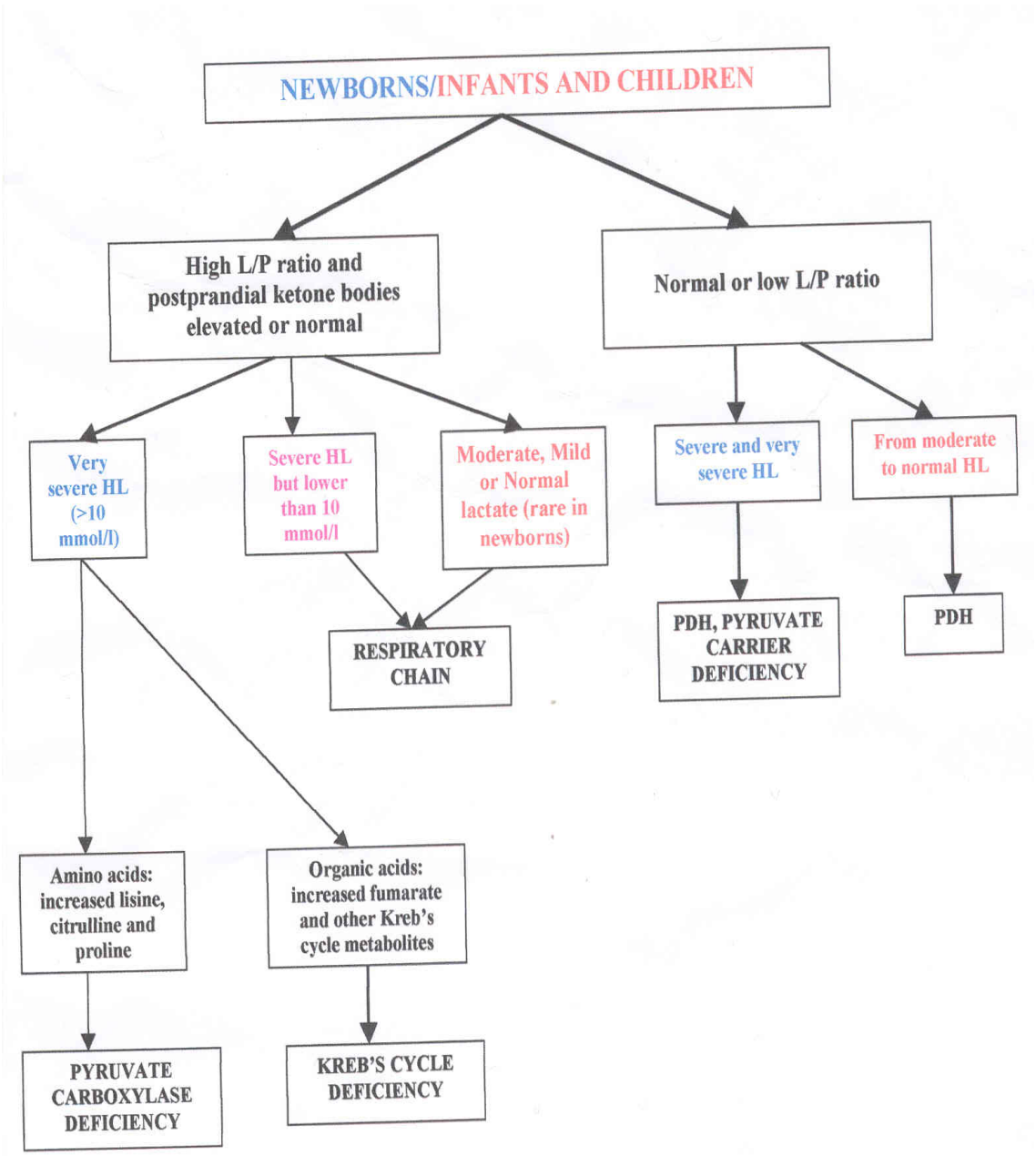
		Coma	Ophtalmo-plegia. Elevated liver enzimes	**PYR:**0.1-0.2 **L/P:**22-46 **KB** normal Increased in. postprandial **B/A:**normal	glutamine **OCA:** αKetogluta-rate Lactate Succinic Fumaric	deficiency muscle
	Onset age	**Main symptoms**	**Associated symptoms**	**Basic biology (mmol/l)**	**AAC AOC**	**Enzymes and genes**

4.2- DIAGNOSTIC TREE BASED ON THE BIOCHEMICAL RESULTS.

If we try to design a diagnostic flow-chart based on lactate levels and other first line biochemical data, we would suggest the following:

1) With high L/P ratio and postprandial ketone bodies elevated or normal; the most probable deficiencies are:
    a) If very severe HL (>10 mmol/l): -French phenotype of PC deficiency (AAC could help with a particular profile) -CRM of neonatal onset. –KC deficiencies.
    b) Severe but lower than 10 mmol/l: infants with CRM disorders.
    c) Moderate, Mild and Normal: infants and older children with CRM disorders.
2) With low or normal L/P ratio:
    a) If severe and very severe HL: neonatal PDHC and pyruvate carrier deficiencies.
    b) From moderate to normal lactate: infants and children with PDH deficiencies.

With this orientation we can design an algorithm depending mainly on the onset age, that is represented as follows:



Letters in BLUE: more likely to happen in NEWBORNS
Letters in RED: more likely to happen in INFANTS AND CHILDREN
Letters in PURPLE :may happen at any age

4.3-RESULTS IN POPULATION Nº 2 "LONG TERM FOLLOW-UP OF NEONATAL MITOCHONDRIAL CYTOPATHIES: A STUDY OF 57 PATIENTS"

At the time of data collection 33 patients had died (18 males and 15 females), 12 remained alive (6 males and 6 females) and 12 had been lost in the follow-up. Concerning the 12 patients who were lost, the minimum follow-up from the beginning of symptoms was 30 days (maximum 3 years) with a median of 180 days . Regarding the 33 patients who died, the age of death ranged from 2 days to 3 years with a median of 90 days. Most of the patients died during the first three months (16/33), 12 patients from 3 to 12 months, and 7 patients beyond 1 year of life. The 12 patients who are currently alive have a follow-up ranging from 30 days to 18 years (median: 3,2 years; mean: 1986,6 days: 5,4 years, SD: 1976 days: 5,4 years;); 2 patients younger than 2 months; 4 patients from 1 to 3 years; 6 patients older than 4 years whose ages are: 4,5,6; two patients who are 12 and one who is 18 years. (fig 3.1).

The clinical, biochemical, enzymatic and outcome characteristics of the patients are shown in Table 3.1.

Clinical outcome mostly tended towards a multisystem disorder (39/57:68,4%) followed by neurological (9/57: 15,79%), hepatodigestive (6/57:10,53%) and myopathic (3/57: 5,26%) disease. (For details see table 3.2)

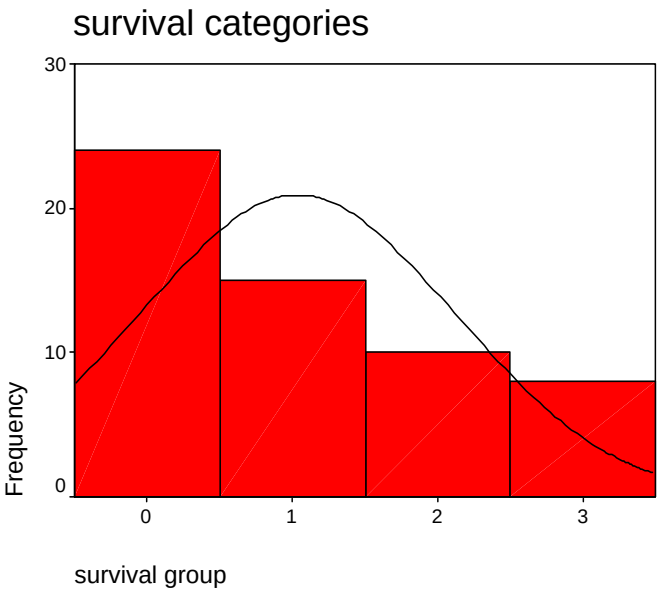
Biochemical progression regarding blood lactate showed persistent hyperlactacidemia in the majority of the cases (31/57: 54,39%). Plasmatic lactate normalisation and consistently normal lactate were found in 13/57 (22,81%) cases. The same number of patients had a lactate decrease but it was not normalised.

Table 3.1- Clinical, biochemical, enzymatic and outcome characteristics of the patients.

		Initial lactate				Enzymatic defect					Clinical outcome				Lactate outcome		
INITIAL SYMPTOMS	**nº**	**N**	**M**	**Mo**	**S**	**CI**	**CI+CIV**	**CIV**	**G**	**O**	**N**	**H**	**M**	**Mu**	**NL**	**DL**	**HL**
"H+T+F"	**25**	3		4	**18**	**8**	**8**	2	2	5	2	2	2	**19**	7	4	**14**
NEUROLOGIC	**21**	3	1	3	**14**	**8**	3	4	2	4	7		1	**13**	5	4	**12**
hypotonia	19	3	1	2	**13**	**8**	2	3	2	4	7		1	**11**	5	4	**10**
leigh	1				**1**	**1**					**1**						**1**
dyskinesias	1				**1**	**1**					**1**						**1**
coma	2	**1**		**1**		**1**		**1**					**1**	**1**	**1**	**1**	
hypertonia	2			**1**	**1**		**1**	**1**						**2**			**2**
seizures	1				**1**			**1**			**1**						**1**
tremor	2	**1**			**1**	**1**			**1**					**2**	**1**		**1**
microcefaly	1				**1**					**1**	**1**					**1**	
HEPATO-DIGESTIVE	**15**			3	**12**	**7**	3		4	1	2	4		**9**		3	**12**
vomiting	2				**2**	**1**			**1**					**2**		**1**	**1**
liver failure	5			2	**3**	**3**	1		1		1	1		**3**		1	**4**
hepatomegaly with high transaminases	10			2	**8**	**4**	2		3	1		2		**8**		2	**8**
cholestasis	2			**1**	**1**	**2**					**1**	**1**					**2**
HIPOGLYCEMIA	**9**	1		1	**7**	**4**	**4**		1		1	2	1	**5**	3	1	**5**
FAILURE TO THRIVE	**8**			**5**	3	**5**			1	2	1	2		**5**	1	2	**5**
CARDIAC	**3**	1		**2**	1	**1**	**1**		**1**		1			**2**	1		**2**
cardiac failure	1	**1**							**1**		**1**				**1**		
hypertrophic cardiomyopathy	2			**2**	1	**1**	**1**							**2**			**2**
HEMATOLOGIC	**3**			1	**2**	**3**						1		**2**		1	**2**
anaemia	2			**1**	**1**	**2**						**1**		**1**		**1**	**1**
pancytopenia	1				**1**	**1**								**1**			**1**
ARTHROGRYPOSIS	**3**	**2**		1				**2**		1			1	**2**	**2**		1
OCULAR (NYSTAGMUS)	**2**		**1**		**1**	**1**	**1**				**1**			**1**	**1**		**1**
CPK ↑	**1**	**1**						**1**					**1**		**1**		
DYSMORPHY	**1**	**1**								**1**	**1**				**1**		

Numbers in red indicate the greatest values for every category (initial lactate, enzymatic deficiency, clinical and lactate outcome)
Nº: number of patients who presents the correspondant symptoms
H+T+F: hypotonia, tachypnea and failure to thrive
Initial Lactate: N (normal), M (mild), Mo (moderate), S (severe)
Enzymatic deficiency: G: generalised; O: others
Clinical outcome: N: neurological; H: hepatodigestive; M: myopathic; Mu: multysistem
Lactate outcome: NL: normalised lactate or initially normal remaining unchanged; DL: decreased lactate; HL: persistent high lactate

Fig 3.1 Distribution of patients according to the survival age



Survival groups
0: 0-3 months: 24 patients (41,4%)
1: 4-12 months: 15 patients (25,9%)
2: 1-3,9 years: 10 patients (17,2%)
3: beyond 4 years : 8 patients (13,8%)

Table 3.2 Details of clinical outcome

Outcome type	Clinical expression	Nº patients	Most prevalent disorders
MULTIORGANIC	NEUROLOGICAL	38/39: 97,4%	Mental retardation, Leigh Syndrome, Regression, seizures, abnormal movements
	HEPATIC	26/39: 66,6%	Hepatomegaly with elevated transaminases, liver failure
	FAILURE TO THRIVE	21/39: 53,8%	Growth below –3 SD, anorexia
	CARDIAC	12/39: 30,7%	Hypertrophic and dilated cardiomyopathy, heart failure
	MYOPATHIC	6/39: 15,1%	Hypotrophia, elevated CPK, weakness
	NEUROSENSORIAL	5/39: 12,8%	Nystagmus, poor vision, cataracts, pigmentary retinitis, deafness
	RENAL	4/39: 10,2%	Proximal tubulopathy
NEUROLOGICAL	SEVERE ENCEPHALOPATHY	4/9: 44,4%	Severe mental retardation, spasticity, seizures
	PSYCHOMOTOR DELAY AND ABNORMAL MOVEMENTS	2/9: 22,2%	Moderate to mild psychomotor delay and dyskinesias (dystonias, myoclonias)
	PSYCHOMOTOR DELAY	2/9: 22,2%	Moderate to mild isolated psychomotor delay
	LEIGH SYNDROME	1/9: 11,1%	
HEPATIC	CYRRHOSIS	3/6: 50%	
	LIVER FAILURE	2/6: 33,3%	
	HEPATOMEGALY	1/6: 16,6%	
MYOPATHIC	ELEVATED CPK	1/2 : 50%	
	WEAKNESS	1/2: 50%	

1)Survival studies using Kaplan-Meier method.

We found no statistical differences in survival probability depending on the diverse clinical presentation forms (combined, neurologic, hepatic, failure to thrive, cardiac or multiorganic), nor the different initial lactate categories (normal, mild, moderate, severe, normal), nor the diverse enzymatic deficiencies . Neither did we find differences in survival probability depending on the blood lactate presentation values (significance of 0,6241) following the Cox regression test . However, we observed a common tendency in all the survival figures (Figs 3.2): patients who survived beyond 2,5 –3 years were more likely to remain alive for a long period of time whatever the initial symptoms, lactate or enzymatic deficiency.

Fig 3.2 Survival figures. (Y axis: survival probability. X axis: time in days)

Fig 3.2.1 Hepatic presentation (p: 0.5901)

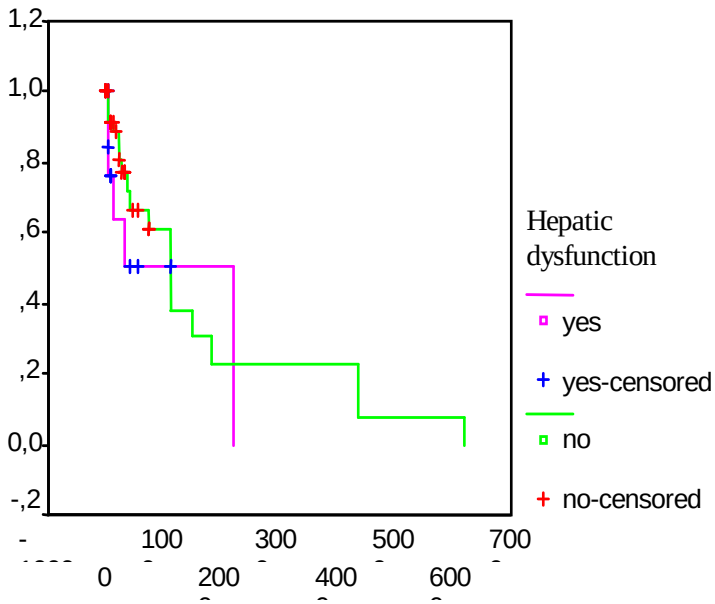
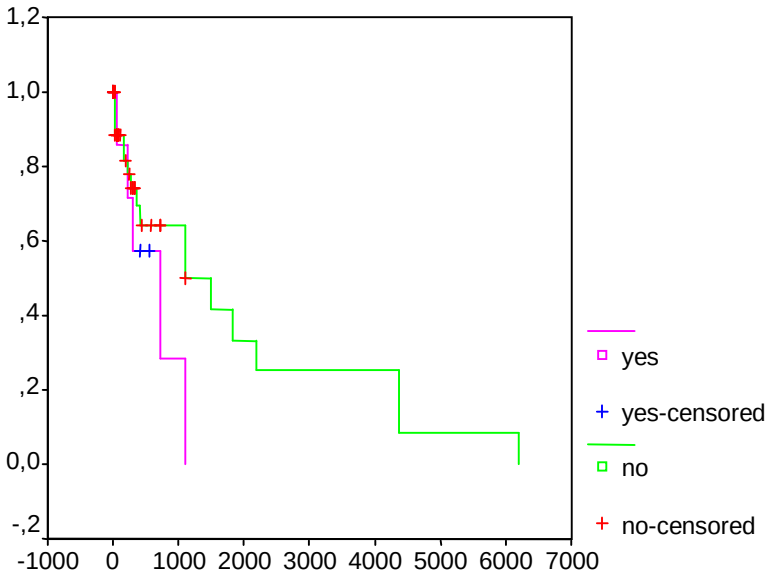


Fig 3.2.2 Failure to thrive presentation (p: 0.3923)



Comments for figs 3.2: Although no statistical significance is found in any survival study, children overcoming the approximate age of 2,5 years are more likely to survive for a long period of time

2)Relation of clinical and biochemical outcome with the initial clinical and biochemical characteristics using Chi Square test (table 3.4 and figure 3.3)

Survival time category

We found statistical significance comparing survival time categories with lactate outcome (p:0,000; 2-sided) (fig 3.3). In fact, 21 out of 24 patients (87,5%) with persistent hyperlactacidemia died during the first 3 months of life. On the other hand we also found a positive correlation (p:0,019; 2-sided) with enzymatic deficiency: those patients with CI+CIV deficit and those with generalised deficiency had a clear shorter survival time (most of them, 11 out of 12 patients and 4 out of 7 respectively, died during the first 3 months of life). Table 3.4 summarises the statistical analysis

Table 3.4. Summary of statistical analysis (Chi square test)

	Survival time	Clinical outcome	Lactate outcome	Initial lactate	Initial symptoms	Enzymatic defect
Survival time		0.256	0.000	0.059	0.384*	0.019
Clinical outcome	0.256		0.015	0.048	0.017 (NRL)	0.006
Lactate outcome	0.000	0.015		0.000	0.028 (HEP)	0.290
Initial lactate	0.059	0.048	0.000		0.333*	0.224
Initial symptoms	0.384	0.017 (NRL)	0.028 (HEP)	0.333		0.384
Enzymatic defect	0.019	0.006	0.290	0.224	0.384*	

NRL: neurological symptoms
HEP: hepatic symptoms
* Significance average of the different initial symptoms (none of them was significant)

Initial presentation

-Initial symptoms and clinical outcome:

Although most of the patients developed a multisystem disorder as they evolved, we could find statistical significance (p: 0,017; 2-sided) due to patients later progressing towards a neurological disease: most of these patients presented initially with symptoms of nervous system involvement (7/9).

-Initial symptoms and biochemical outcome:

In general, a notable proportion of the patients had persistent hyperlactacidemia but we only found clear statistical significance in those who presented with hepatic symptoms (p:0.028;2-sided). These newborns always had hyperlactacidemia that was severe and persistent in the majority of the cases (80%). (fig 3.3b)

-Initial lactate and clinical outcome:

We found a high proportion of patients (73,6%) presenting with severe hyperlactacidemia and later developing a multiorganic disorder. This finding has a positive statistical significance (p: 0,048 ; 2-sided). (fig 3.3c)

-Initial lactate and lactate outcome:

An important statistical significance (p:0,000 ; 2-sided) was found. In fact, we observed that the lower the initial lactate, the more the chances of normalising or decreasing it. On the contrary, severe hyperlactacidemias were more likely to remain at these very high levels.

-Enzymatic deficiency and clinical outcome:

Patients affected with CI deficiency (82,61%) and CI+IV (83,33%) were inclined to develop multisystem involvement. These results have statistical significance (p:0,006 ; 2-sided). (fig 3.3d)

-Enzymatic deficiency and lactate outcome: we did not find statistical significance between these two variables (p: 0,290; 2-sided).

Fig 3.3 Relation of clinical and biochemical outcome with initial clinical and biochemical characteristics. Y axis: number of patients.

Fig 3.3a. Lactate outcome and survival groups (p: 0.000)

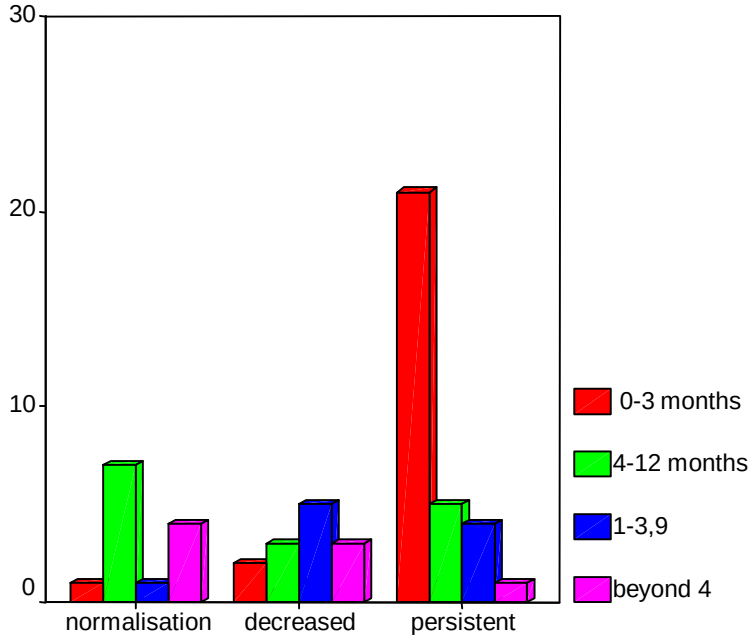


Fig 3.3b. Lactate outcome and hepatic symptoms (p: 0.028)

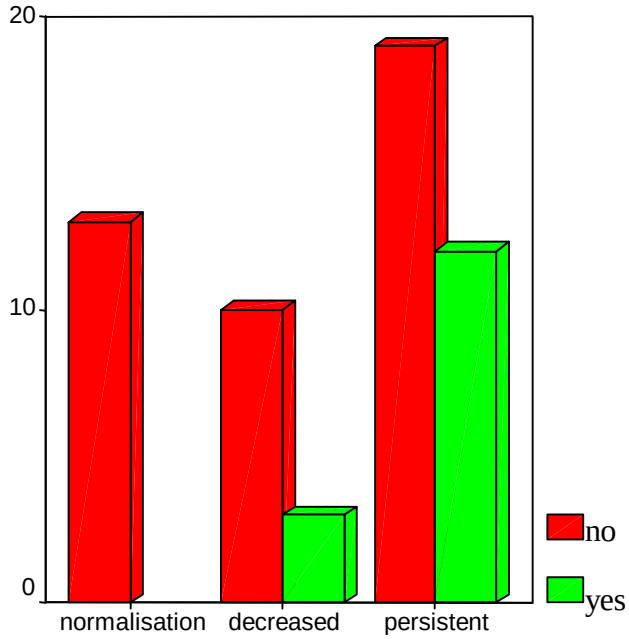


Fig 3.3c- Initial lactate and clinical outcome (p: 0.048)

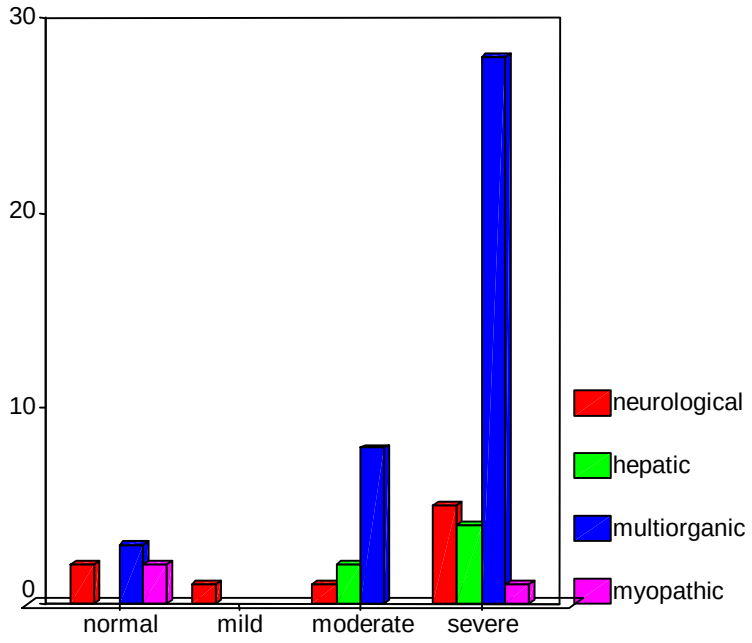
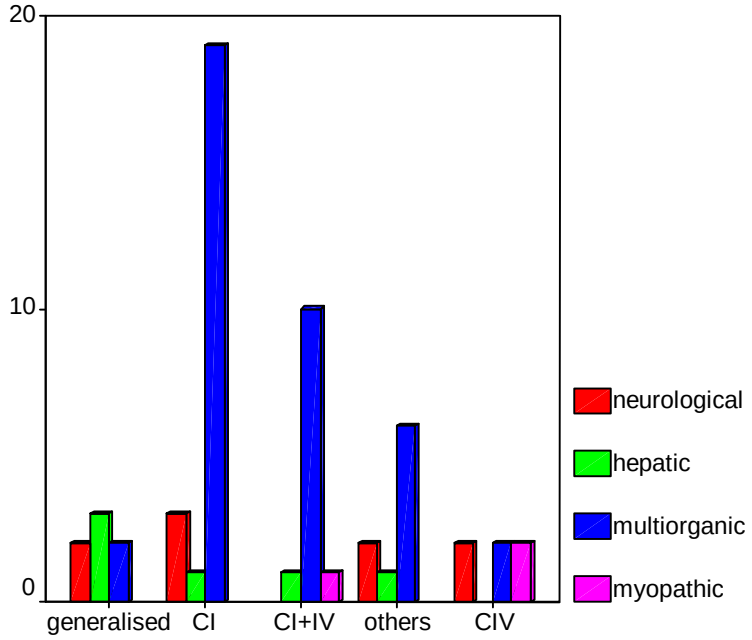


Fig 3.3d- Enzymatic deficiency and clinical outcome (p: 0.006)



Description of the patients surviving more than 4 years (table 3.3)

Among patients with evolution to neurological disease (4, 6 and 18 years of age currently), two patients (nº 3 and 6 of table 1) presented with severe liver failure but hepatic function normalised over time (at 9 months and 2 years respectively). In patient nº 6 the diagnosis was possible at 17 years of age after a liver biopsy in spite of the absence of hepatic involvement since 15 years ago. In this case a muscle biopsy performed at 2 years of life was normal.

Regarding patients with evolution to myopathic disease (12 years of age each one, patients nº 4 and 5), the presentation was severe and life-threatening in patient nº 4 (CI (14 mU/UCS)+CIV (200 mU/UCS)); furthermore multiorganic involvement was present during the first two years of life but it gradually normalised giving rise to myopathic dysfunction only at 3 years of age. At 9 years electromyography and nerve conduction studies disclosed signs of anterior horn involvement. This patient has now mild proximal limb weakness (3/5) with autonomous motor function and exercise intolerance, muscle enzymes are slightly elevated (CPK: 245 and LDH: 1272) and growth retardation (height and weight are at –2,5 SD), but brain MRI and cognitive function are normal. Patient nº 5 presented with feeding difficulties, tachypnea and hypotonia. Marked limb hypotonia with weak deep reflexes were present from the first months of life. From the first years of life limb weakness as well as mild bilateral ptosis were present. At 5 years muscular balance was +3/5 in proximal limb muscles. Now, the boy is 12 years old, he has normal intelligence, myopathic face with persistent muscle weakness (+3 to 4/5) but autonomous gait, Gower's sign, and generalised slowness in physical activities. Delayed bone age and growth hormone deficiency has also been found.

Table 3.3 Characteristics of patients surviving more than 4 years

Nº patient	Current age/sex	Enzymatic defect	Initial symptom	Initial Lactate mmol/l	Clinical outcome	Current Lactate mmol/l
1	4 years/F	CIII+CIV	Hypotonia	1.8	Moderate MR, Hypoacusia, Basal ganglia calcifications Dystonia	Normal
2	5 years/F	Generalised	T/H/F	15.2	Slight MR, tubulopathy, cardiomiopathy	8-10
3	6 years/F	CI	Liver disease	9.3	Slight MR	Normal
4	12 years/F	CI+CIV	T/H/F	6.1	Muscle weakness. Spinal atrophy-like findings. Normal IQ	Normal
5	12 years/F	CIV	T/H/F	1.7	Muscle weakness. Normal IQ	Normal
6	18 years/M	CIV liver	Liver disease	7.6	Severe encephalopathy Dystonia	Normal

T/ H/F: tachypnea, hypotonia, feeding difficulties. MR: mental retardation.
F: female. M: male.

4.4-RESULTS IN POPULATION Nº 3 "CHARACTERISTICS OF PATIENTS WITH MITOCHONDRIAL RESPIRATORY CHAIN DEFICIENCIES EXPRESSING THE ENZYMATIC DEFICIENCY IN THE HEPATIC TISSUE: A STUDY OF 31 PATIENTS" .

Table 4.1 summarises the main characteristics of our series.

1-Clinical and biochemical presentation

Clinical presentation. The majority of our patients (18/31: 58,1 %) manifested multiple organ involvement. 14 patients (43,8%) presented with neurological symptoms, hypotonia, psychomotor delay and myoclonus being the most prevalent. The remaining 10 first appeared with hepatic disease (28,1%) ; half of them had biochemical evidence of liver synthetic failure (hypoglycemia, hypoalbuminemia, coagulopathy); hepatomegaly and high transaminases were observed in 7 cases and cholestasis in one case. 20 patients (65%) did not initially reveal any sign of hepatic dysfunction (12 newborns, 7 infants and 1 child older than 1 year) .

9 patients (28,1%) presented with failure to thrive and 6 newborns (18,8 %) with non specific combination of hypotonia, tachypnea and feeding difficulties. We did not find cardiac or muscular disorders as initial symptoms in this series.

We could not demonstrate a positive statistical relation between clinical presentation and onset age, biochemical presentation or enzymatic deficiency.

Biochemical presentation. Initial fasting lactate ranged between 1,4 mmol/l and 22,5 mmol/l (mean: 7,7 mmol/l; SD: 5,7). Distribution depending on lactate categories showed: 4 patients (12,5%) with normal lactate, mild hyperlactacidemia in 2 cases (6%), moderate in 9 (28,1 %) and severe in 16 (50%). Plasmatic amino acids chromatography was abnormal in 23 patients (72%): alanine was increased in 23 cases

(71,9%) and ranged from 409 to 1760 mmol/l (mean: 786,6, SD: 345); proline was high in 10 patients (31,3%) and ranged from 460 to 598 mmol/l (mean: 509; SD: 49,2); glutamine was elevated in 8 cases (25%) and ranged from 430 to 1045 mmol/l (mean: 797,5 mmol/l; SD: 269,8). Urine organic acids were altered in 24 patients (75%); lactate was found in 23 patients (71,9%) and other Kreb's cycle metabolites in 22 cases (68,8%). No statistical differences were found when comparing initial biochemical features with onset age, clinical presentation, enzymatic deficiency or outcome clinical and biochemical characteristics.

2-Morphologic, enzymatic and molecular studies

Ultrastructural changes in liver disclosed microvesicular steatosis, focal cytoplasmic biliary necrosis and iron overload in all the patients. Portal fibrosis was additionally found in one newborn presenting with failure to thrive and cholestasis (patient nº 18 in table 1).

We came across 24 isolated (75%) and 7 combined (21,9%) deficiencies. Complex I deficiency was the most frequently found (15 patients; 46,9%) followed by Complex IV (6: 18,8%) and combined CI+CIV defect (5: 15,6%). Three patients (9,4%) were affected with Complex III deficiency and one with a combination of CI, CIII and CIV.

Molecular analysis revealed mitochondrial DNA depletion (20%) in a patient with generalised complex deficiencies, Pearson's deletion in a patient with combined CI+CIII+CIV deficiency, and a new nuclear mutation in a patient with a CIII deficiency.

We discovered that onset age and enzymatic deficiency had a clear statistical relation (p: 0,017); see fig 4.1. Newborns disclosed CI and CI+CIV deficiencies whereas older children tended to suffer CIV deficiency.

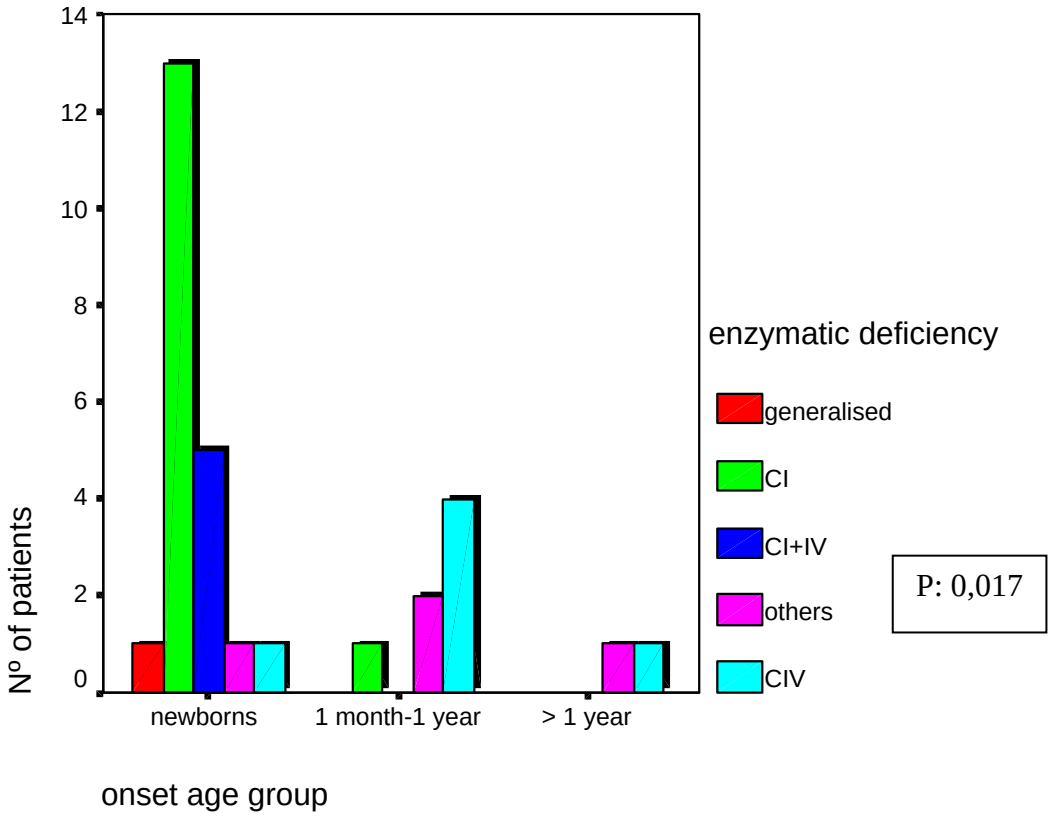
TABLE 4.1

	Onset age/Sex	Presentation symptoms	Initial lactate mmol/l	Survival	Follow-up time	Clinical outcome	Normalisation of hepatic symptoms/age	Lactate outcome	Enzymatic deficiency/Molecular results	Tissue/Activity
1	1d/F	Hypotonia, tremor,myoclonus	2,2	Unknown	9m	Refractory epilepsy and hypertransaminemia (Alpers)	No	Normalised	CI	H
2	5d/M	Hypotonia, myoclonus, nystagmus	2,9	Alive	1y	Myoclonic epilepsy, psychomotor delay		Normalised	CI	H
3	1d/F	H+T+F	5,5	Deceased	21d	Hepatomegaly and high CPK at 7 days. Tremor, hypertonia.	No	Remains high	CI	H
4	1d/M	Hypotonia	22,5	Deceased	1,5y	Leigh syndrome at 9 months. Renal tubulopathy at 19 months		Decreased	CIII	H
5	1d/M	Failure to thrive, vomiting	7,2	Unknown	8m	Failure to thrive, hepatomegaly at 4 months, DCM, psychomotor delay	No	Decreased	CI	H+F
6	1d/M	Hypotonia	16,8	Unknown	15m	Hepatomegaly at 8 months. DCM at 14 months. Severe epileptic encephalopathy,	No	Decreased	CI	H+F
7	2d/M	H+T+F	17,7	Unknown	8m	Hepatomegaly at 7 months. Slight psychomotor delay.	No	Normalised	CI	H+F
8	7d/M	Failure to thrive, Liver failure	4,4	Deceased	1,5y	Hepatomegaly at 1 month. Hypotonia, psychomotor delay	No	Remains high	CI	H
9	7d/F	Failure to thrive, Liver failure	4	Deceased	15m	Hepatomegaly at 1 month. Psychomotor delay	No	Remains high	CI	H
10	1d/M	Hypotonia, Liver failure	10,2	Deceased	3y	Hepatomegaly and high transaminases at 6 months. Epileptic encephalopathy (Alpers)	No	Decreased	CI	H
11	1d/F	Hypotonia, hepatomegaly, high transaminases	16,2	Deceased	3d	Hypotonia, hepatomegaly, high transaminases	No	Remains high	CI	H
12	1d/M	H+T+F	19,2	Deceased	3d	H+T+F, hypoglycemia		Remains high	CI+CIV	H
13	1d/M	H+T+F	12,3	Deceased	11m	Hypotonia, psychomotor delay. Hepatomegaly, high transaminases at 11 months	No	Normalised	CI	H
14	1d/F	H+T+F	2,1	Deceased	3m	Hypotonia. Liver failure and cholestasis at 1.5 months. Muscle hypotrophy	No	Normalised	CI+CIV	H
15	30d/F	Hypotonia, pyramidal signs	4,2	Deceased	4m	Non epileptic myoclonus, pyramidal signs. Liver failure at 4 months	No	Remains high	CIV	H

16	1d/M	Tachypnea	6,1	Deceased	3m	Generalised hypertonia. HCM.		Remains high	CI	H
17	3d/F	H+T+F, Hepatomegaly, high transaminases	15,2	Deceased	12d	Liver failure, generalised hypertonia. Renal failure.	No	Remains high	Generalised	H+M
18	15d/F	Failure to thrive, cholestasis, anemia	3,4	Unknown	10m	Heptaomegaly, high transaminases, villous atrophy, failure to thrive	No	Remains high	CI	H
19	1d/M	Hypotonia, liver failure	7,6	Alive	18y	Normalisation of liver dysfunction. Severe myoclonic encephalopathy. HCM	Yes/2 year	Normalised	CI+CIV	H
20	26d/M	Hypotonia, hepatomegaly, high transaminases	9,2	Deceased	1m	HCM. Hepatomegaly, high transaminases	No	Remains high	CI+CIV	H+M
21	15d/F	Hypotonia, hepatomegaly, high transaminases	10,3	Deceased	2m	HCM. Hepatomegaly, high transaminases	No	Remains high	CI+CIV	H
22	10m/F	Coma	4,1	Alive	14y	Mental retardation		Normalised	CIV	H
23	5m/M	Renal tubulopathy, failure to thrive, anemia	6,3	Alive	2y	Hepatomegaly, high transaminases. Renal tubulopathy	No	Remains high	CI+CIII+CIV/ Pearson	H
24	8m/F	Hypoglycemia	3	Alive	1,5y	Hypoglycemia		Decreased	CIII	H+F
25	8m/F	Neurological regression	3	Deceased	2y	Leigh, muscle hypotrophy, strabismus		Remains high	CI	H
26	3m/F	Neurological regression	6,4	Alive	2,5y	Myoclonus, hepatomegaly at 7 months, esteatosis, cataracts	No	Normalised	CIV	H
27	3m/M	Failure to thrive	4,1	Unknown	4m	Hepatomegaly, renal tubulopathy	No	Remains high	CIV	H
28	6m/M	Psychomotor delay	6,1	Alive	10m	Leigh, hypertransaminemia at 1 year	No	Normalised	CIV	H
29	4m/M	Hypotonia, psychomotor delay	1,6	Unknown	22m	Leigh, failure to thrive		Normalised	CI	H
30	2y/F	Failure to thrive, liver failure, cholestasis	5,6	Deceased	2y	Liver failure	No	Remains high	CIII	H+F
31	3y/F	Dystonia, coma	1,4	Unknown	10y	Cerebellar signs, neurological regression		Normalised	CIV	H

M:male, F: female, d:days, m:months, y: years. H+T+F: hypotonia, tachypnea and failure to thrive. N: normal. A: abnormal. MM: muscle morphology. H: hepatic. H+F: hepatic+fibroblasts. H+M: hepatic+ muscle. HCM: hypertrophic cardiomiopathy. DCM: dilated cardiomyopathy. Words in red mark liver involvement.

Fig 4.1. Onset-age and enzymatic deficiency



Count

		enzymatic deficiency					Total
		depletion	CI	CI+IV	others	CIV	
onset age	newborns	1	13	5	1	1	21
	1 month-1 year		1		2	4	7
	> 1 year				1	1	2
Total		1	14	5	4	6	30

3-Clinical and biochemical long-term outcome

Clinical outcome. Most of the patients developed multisystem involvement (24/31: 77%). We describe three main clinical outcome groups:

1- Patients with "neurohepatic" disease: 16/31 or 52% (among them 14 newborns). In these children neurological manifestations preceded the hepatic dysfunction (see figures 2 and 3). In general, nervous system involvement presented very early (from 1 day to 9 months; mean: 16,5 days) whereas hepatic disease appeared later (from 1 day to 1 year; mean: 143 days). CI was the most prevalent defect (10/16 cases) followed by CIV (3 cases), CIV+CI (2 cases) and 1 case of generalised deficiency. We distinguished 2 different clinical outcome subtypes within this "neurohepatic" group. On one hand there is a group of 4 patients (nº 1, 6, 10 and 19 in table 1) was characterised by severe refractory myoclonic epilepsy of late-onset (from 8 months to 9 years; 8 months in patient nº 6, 9 months in patient nº 1, 3 years in patient nº 10 and 9 years in patient nº 19) and progressive brain cortical atrophy. All of them previously had hypotonia and different degrees of psychomotor delay. Hepatic abnormalities (hepatomegaly, high transaminases), started between 6 and 9 months except in one patient (nº 19) who presented with liver failure at one year of age. He is the only patient of our series who gradually normalised his liver function (no evidence of hepatic disease at 2 years of life). This subgroup can be diagnosed as Alpers syndrome although patient nº 19 presents some atypical features. The other subtype corresponds to patients who did not develop epilepsy but other neurological disorders such as hypotonia, psychomotor delay, spastic tetraparesis and different movement disorders (tremor, non epileptic myoclonus); hepatic alterations did not differ from those of patients with Alpers syndrome.

2- Patients with predominant neurological disease although in some cases other organs were also affected: 8/31 or 26%. Leigh disease is the most prevalent in this subtype.

3- Patients with predominant hepatic disease although in some cases other organs were also affected: 6/31 or 19%. One case of Pearson's disease and another of villous atrophy are included in this subgroup.

Fig 4.2-Hepatic signs and onset-age

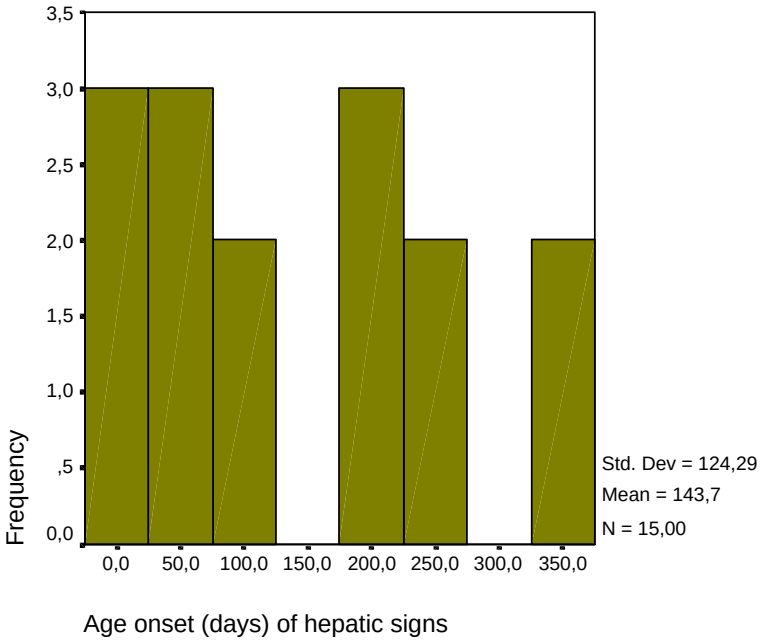
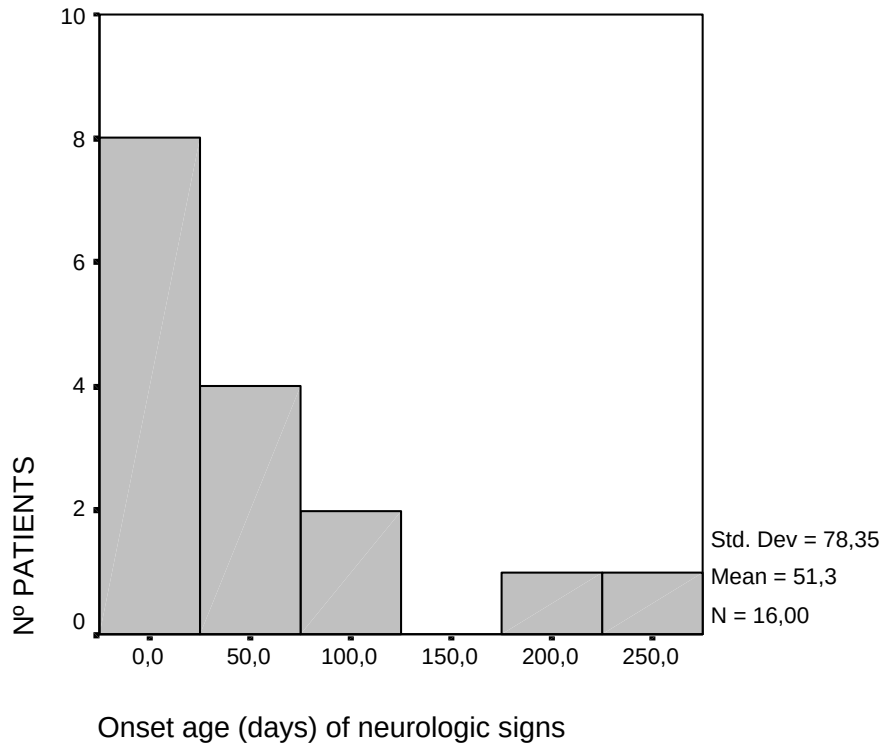


Fig 4.3-Neurological signs and onset-age



Tables 4.2,4.3,4.4 show the different neurological, hepatic and other systemic outcome signs as well as onset age in function of these different three categories.

Table 4.2. Nº of patients with different neurological disorders, and onset age in function of the clinical outcome subtypes

	Neurological	Hepatic	Neurohepatic
Psychomotor delay			5
Leigh syndrome	3		1
Cerebellar syndrome	1		
Epilepsy	1		4
Severe encephalopathy			1
Generalised hypertonia	1		1
Hypotonia	1		1
Non epileptic myoclonus			2
Mental retardation	1		
Myoclonus			1
Onset age (days) **Minimum** **Maximum** **Mean**	 1 1190 227,7		 1 1095 110

Table 4.3. Nº of patients with different hepatic disorders, and onset age in function of the clinical outcome subtypes

	Neurological	Hepatic	Neurohepatic
Hepatomegaly		3	7
High transaminases		1	4
Liver failure		2	4
Cholestasis			1
Onset age (days) **Minimum** **Maximum** **Mean**		 15 730 181	 1 365 136

Table 4.4 Nº of patients with different systemic disorders in function of the clinical outcome subtypes

	Neurological	Hepatic	Neurohepatic
Dilated myocardiopathy			2
Hipertrophyc myocardiopathy		3	1
High CPK			1
Muscle hypotrophy	1		1
Cataracts			1
Renal failure			1
Renal tubulopathy	1	2	

One of the patients of this series is out of this classification due to an atypical course in the form of repetitive hypoglycemias as the main sign of the disease (this patient is described in detail in ” A deletion in the human QP-C gene causes a complex III deficiency resulting in hypoglycaemia and lactic acidosis”).

Therefore, considering all the collected data of clinical outcome, we discovered 9 patients (28%) without hepatic disease over time in spite of a long-term follow-up in the majority of them (longer than 1 year in 6 patients, 9 and 12 years of age being the longest pursuit).

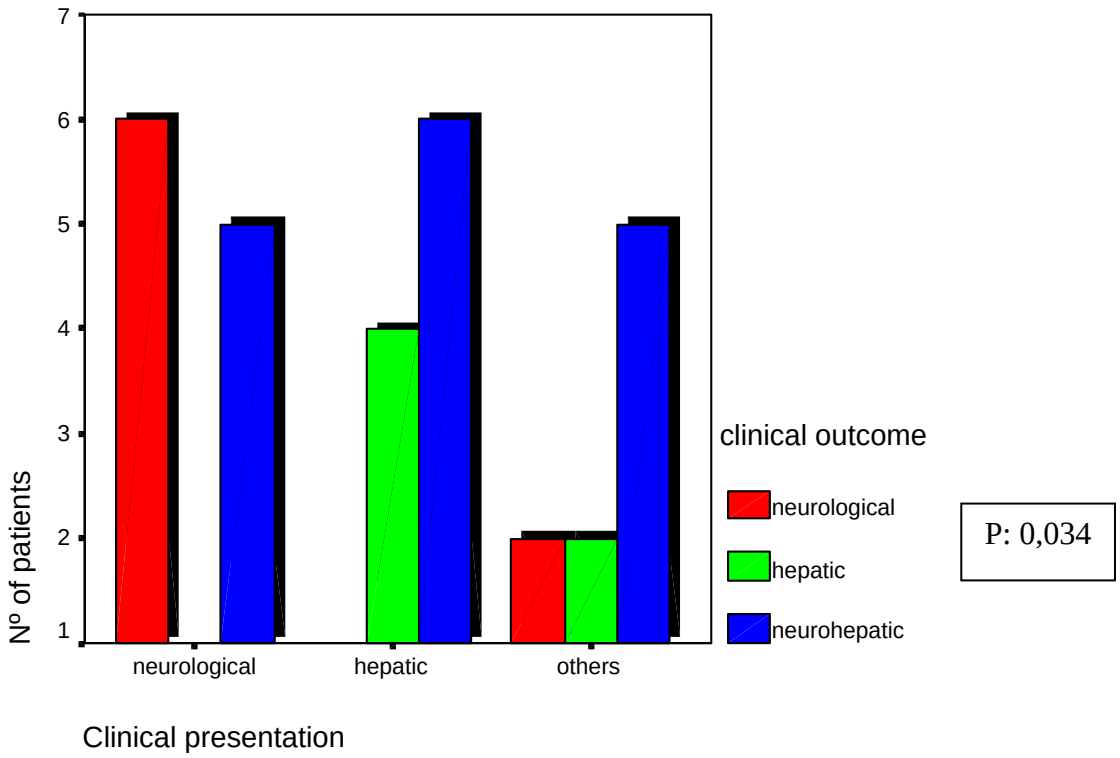
Among the patients without hepatic involvement at the beginning of the disease (20 patients), 11 developed liver disease over time (from 60 to 730 days; mean time: 179 days).

Statistical studies revealed a positive significance (p: 0.034) between clinical presentation type and clinical outcome. In this respect we observed that “neurohepatic” outcome was very frequent whatever the initial clinical type. The proportion of patients with a predominant neurological outcome was higher amongst those with initial

neurological symptoms. The prevalence of hepatic outcome forms was higher in those cases with initial liver disease. See fig 4.5

Biochemical outcome. Most of the patients manifested constant hyperlactacidemia over time (15/31: 48,4%); in 11 patients lactate normalised or was always normal (35,5%) and in 5 (16,1%) it decreased but remained high. Constant hyperlactacidemia was more common in patients who did not survive beyond three months of life (p 0,033); fig 4.6

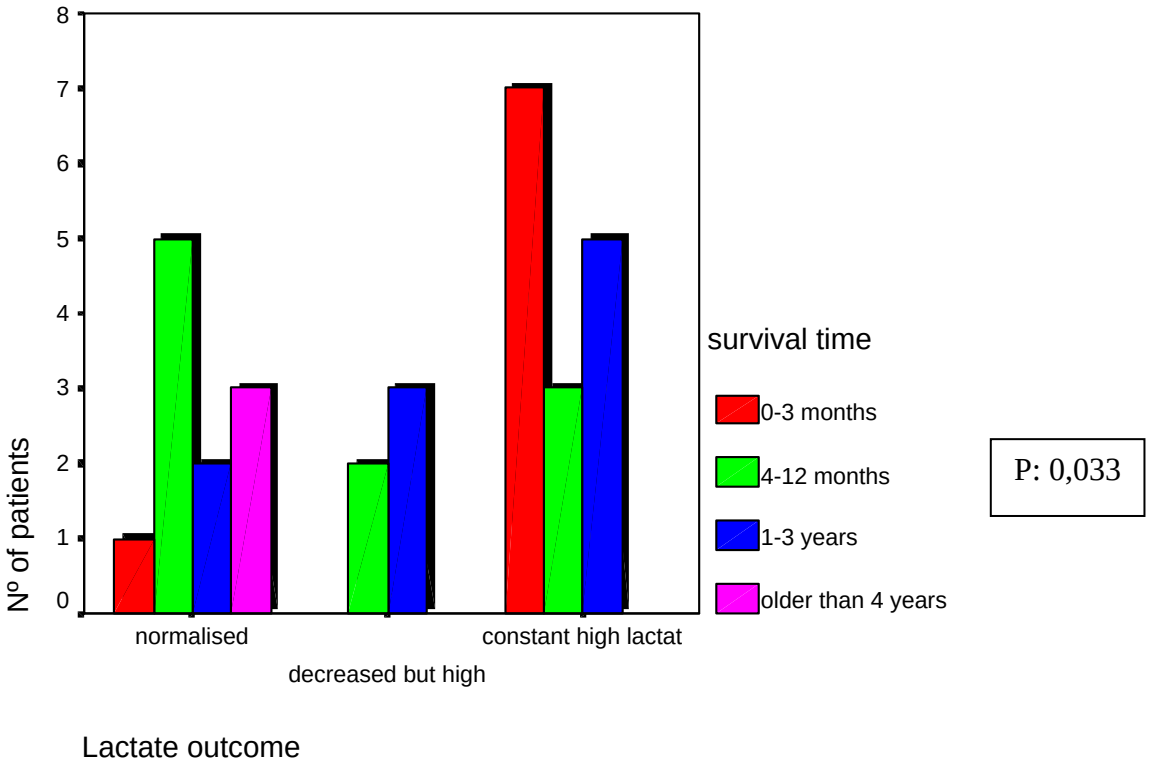
Fig 4.5 Relation between initial symptoms and clinical outcome



Count

		CLINICAL OUTCOME TYPES			
		NRL	HEP	N-H	Total
Initial clinical groups	NRL	6		5	11
	HEP		4	6	10
	Others	2	2	5	9
Total		8	6	16	30

Fig 4.6-Relation between lactate outcome and survival time



Count

		survival time				Total
		0-3 m	4-12 m	1-3 y	> 4 y	
Lactate outcome	normalised	1	5	2	3	11
	decreased but high		2	3		5
	constant high lactate	7	3	5		15
Total		8	10	10	3	31

4- Characteristics of the patients who did not present with hepatic disease

These are patients number 2,4,12,16,22,24,25,29 and 31 of table 1. This group is composed of 5 males and 4 females who presented symptoms between 1 day and 3 years (mean: 225,5 days; 7 patients were younger than 1 year and among them 4 were newborns). Patient nº 24 displayed a very uncommon clinical picture (recurrent hypoglycemias) and is discussed in detail in "" A deletion in the human QP-C gene causes a complex III deficiency resulting in hypoglycaemia and lactic acidosis". Two newborns, patients nº 12 and 16, (CI+IV and CI respectively) had a rapid fatal course and therefore a very short follow-up (3 days and 3 months). Four other patients (nº 2: CI and 4: CIII, newborns; and nº 25: CI and 29: CI , both infants) were monitored for a longer period of time: from 1 to 2 years. They developed neurological disorders such as myoclonic epilepsy and Leigh syndrome. Finally there were two patients (nº 22 and 31, both CIV deficiencies) whose symptoms arose at 10 months and 3 years respectively, and who have been followed for an extensive period of time: 14 and 10 years. Patient nº 22 presented with coma and moderate hyperlactacidemia (4 mmol/l) and progressed towards moderate mental retardation only. Patient nº 31 presented with coma, dystonia and normal plasmatic lactate, disclosing later cognitive regression and signs of cerebellar dysfunction.

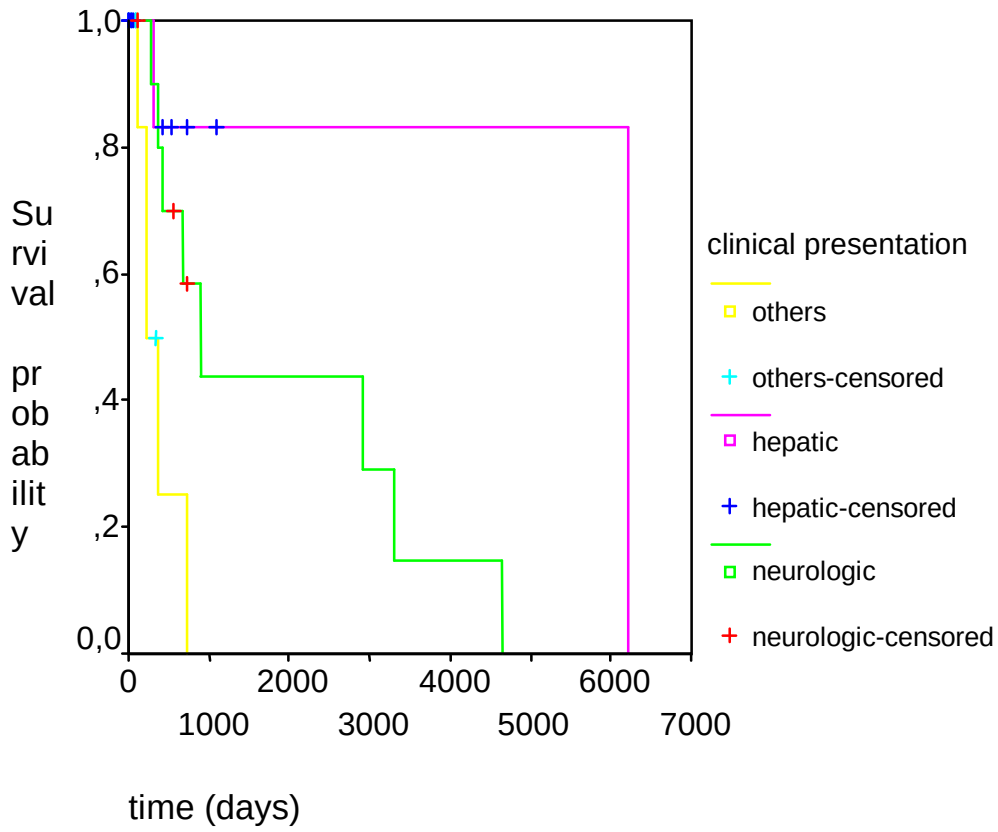
5-Survival

At the time of the data collection, medical histories ranged from 3 days to 18 years (mean: 853,6 days ; SD: 1444,4 days). 16 patients (51,6%) had died, 7 had survived (22,6 %) and 8 (25,8%) were lost in the follow-up. 18 patients (68,1%) did not survive one year of life and among them 8 (25,8%) had a very early mortality (younger than 4

months). 13 patients (41,9%) survived beyond year of age and 3 out of 13 were older than 4 years (10, 14 and 18 years of age).

Survival studies using the Kaplan-Meier method, attempted to find statistical significance (log rank 11,5; df 2; sig 0,0032) only when the data was analysed according to the presentation type (hepatic, neurological or others). Patients who initially presented with a form of hepatic disease were more likely to survive for a longer period of time (see fig 4.7).

Figure 4.7. Survival function depending on the clinical presentation (hepatic, neurologic, others); p: 0.0032



Statistical data of figure 5.7

Clinical presentation	Nª Cases	Nº Censored cases	Survival time (mean in days)	Standard deviation
NEUROLOGIC	11	3	1898,23	599,76
HEPATIC	10	8	5220,8	1270,5
OTHERS	10	5	362,5	111,69

6-Comparison between patients with hepatic and non-hepatic tissue defects:

We detected statistical significance analysing several variables:

-The survival probability (fig 4.8) was markedly lower in patients with liver enzymatic deficiencies compared with those expressing the defect in other tissues (p 0,05).

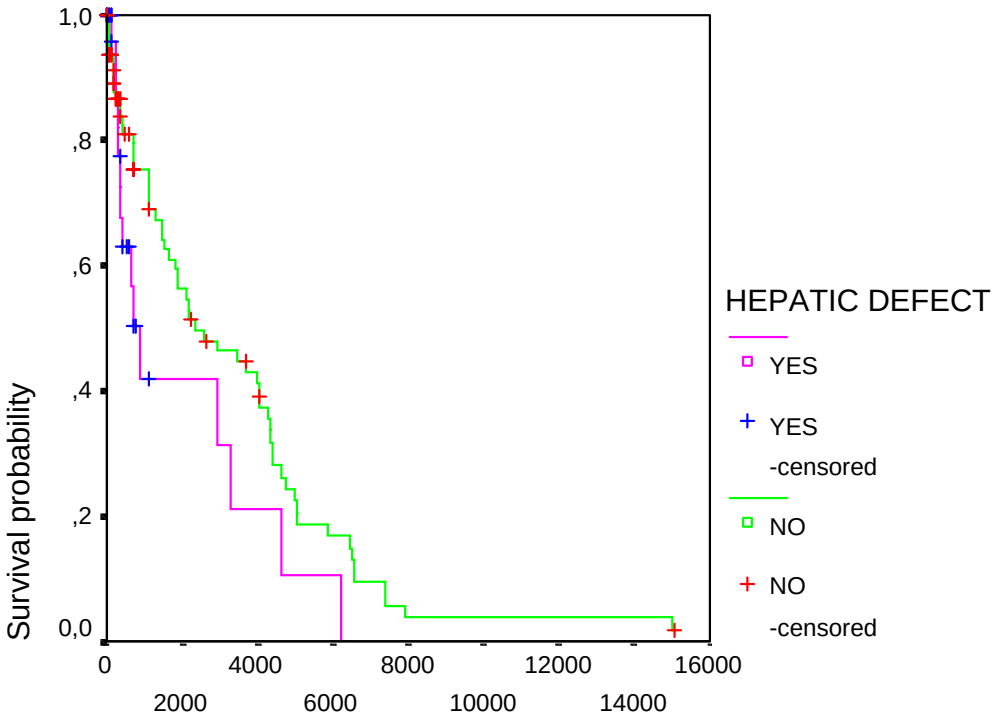
-The onset age of the disease was also clearly different in both groups: patients expressing enzymatic defect in the liver did not tend to present beyond the neonatal period and above all beyond 1 year of life (p 0,008). See fig. 4.9

-Patients with initial signs of hepatic dysfunction were more likely to have the enzymatic defect in the liver (p 0,009). See fig 4.10

- Patients with an enzymatic hepatic defect had a higher tendency to develop hepatopahy over time (p 0,00).

No other variables (enzymatic deficiencies, biochemical presentation/outcome) showed relevant significance between the groups.

Figure 4.8. Survival depending on the defect expressed in hepatic tissue



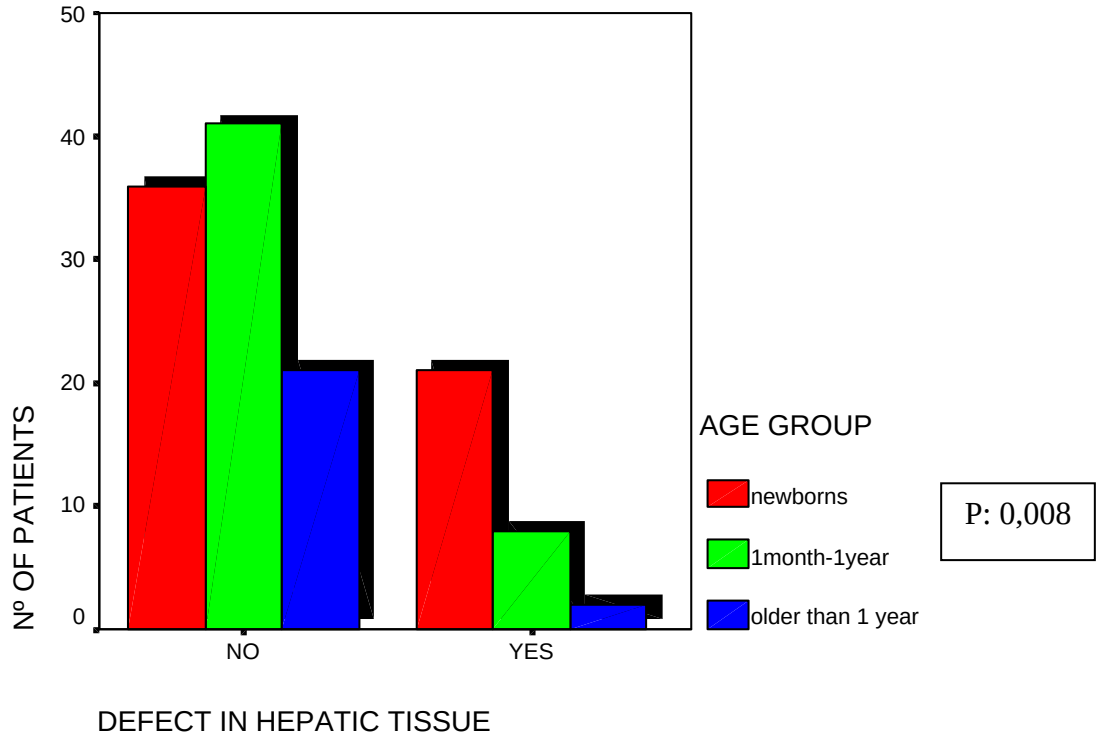
Hepatic enzymatic defect	Nª Cases	Nº Censored cases	Survival time (mean in days)	Standard deviation
NO	98	36	3377,74	408,24
YES	31	16	2058,58	201,2

Table 4.5 . Enzymatic defect expressed in the liver and other tissues

Count

		ENZYMATIC DEFECT					
		Generalised	CI	CI+IV	otros	CIV	Total
HEPATIC DEFECT	NO	9	29	16	23	21	98
	YES	1	15	5	4	6	31
Total		10	44	21	27	27	129

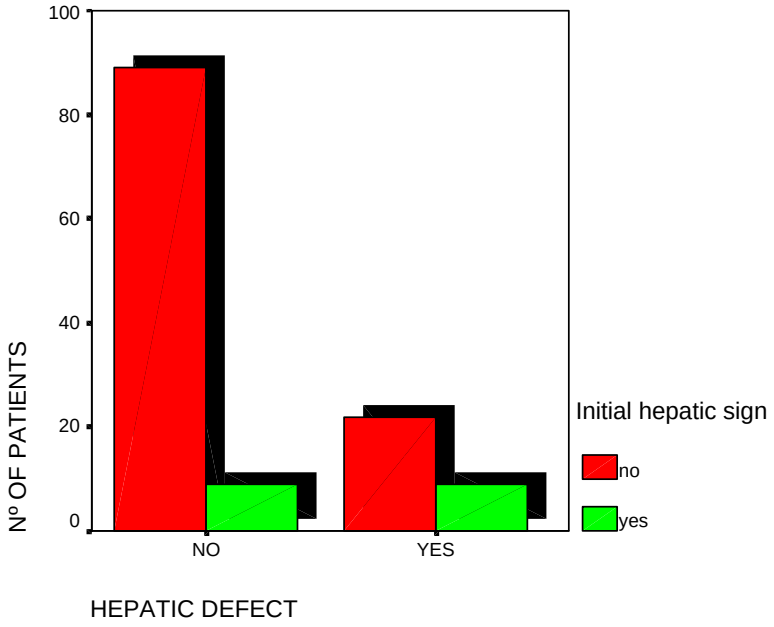
Figure 4.9. Age-onset comparison between patients expressing the defect in the liver and in other tissues



Count

		AGE GROUP			
		newborns	1m-1year	older than 1 year	Total
Hepatic defect	NO	36	41	21	98
	YES	21	8	2	31
Total		57	49	23	129

Figure 4.10. Initial hepatic signs in patients expressing the defect in the liver and in other tissues



Count

		Initial hepatic signs		
		NO	YES	Total
HEPATIC DEFECT	NO	89	9	98
	YES	22	9	31
Total		111	18	129

4.5- RESULTS IN POPULATION Nº 4 "PYRUVATE CARBOXYLASE DEFICIENCY: METABOLIC AND NEUROLOGICAL CHARACTERISTICS OF NINE PATIENTS".

Patients' clinical presentation

The general clinical characteristics of this group are represented in table 5.1.

All the patients had a very early neonatal onset (from 1 to 72 hours of life; median: 10 hours). The birth weight was within the normal limits (range: 3150 to 3500 g) in all the cases except in patient number 1 (2130 g at term).

Without exception they presented severe axial hypotonia and tachypnea due to lactic acidosis as first symptoms. In opposition to that, an excellent level of consciousness was initially present in the majority of the cases (7/9).

Other associated manifestations were as follows:

-Hepatic involvement was detected in 4 patients over the first two weeks . High hepatic enzymes (4/4), enlarged liver (4/4) and liver failure with coagulopathy (2/4) were the most frequent findings. Pathological studies were carried out in one patient disclosing portal fibrosis and steatosis.

-Abnormal movements were registered in 6 patients, seizures in 3 and bizarre ocular behaviour in 5. These aspects will be developed in detail later.

-Facial dysmorphy was seen in 2 patients with epicantus, long filtrum and thin upper lip.

Abnormal movements

We observed abnormal movements in 6 patients. They were detected during the first 72 hours of life in 4 cases whereas they appeared later (1 week, 3 weeks) in the other 2 .

We detected a similar pattern of movement disorder in every patient. The constant finding was high amplitude, rhythmic rapid movements of the limbs that could be simultaneous in the four extremities or in some occasions limited to the upper or lower ones. Due to their rhythmic character they should be classified as high amplitude tremor. These could appear spontaneously but were predominantly triggered by manipulation of the child and had in general a short duration (from half a minute to 4-5 minutes), although occurring very frequently. Abnormal movements could not be stopped by external stimuli such as holding the moving limbs of the patient. They contrasted with major axial hypotonia which was associated and the comparative good consciousness level. Electroencephalography studies did not reveal synchronous changes. Facial hypomimia, global hypokinesia and severe slowness of physiological movements were also observed.

These tremulations were detected throughout the follow-up in the patients with fatal outcome (5/6) and displayed unvarying characteristics over time. By contrast, in one of the patients with a long survival (patient number 6 of the table 5.1), these movements normalised at 15 days of life.

Furthemore, non epileptic erratic myoclonus and pedal limb movements were observed in two patients.

Ocular behaviour

Abnormal ocular behavior was detected in 5 cases (first symptoms from 1 to 15 days of life; median: 2 days). Different types of simultaneous abnormal movements were present in all of the cases. The combination of fixed gaze and nystagmus was constant. Fixed gaze with neither ocular pursuit nor associated EEG discharges was observed for long periods of time. This finding alternated with frequent nystagmus-like movements,

that were pendular in 3 cases but rapid multidirectional in 2 cases. Frequently abnormal rapid movements were triggered by external stimuli such as noise or manipulation.

Epileptic activity

Seizures were clinically observed in 3 patients and appeared between 15 to 45 days of life. Generalised tonic-clonic type was common. EEG also disclosed diffuse abnormalities (multiple poly-spikes) and in one case hypsarrithmic-like pattern without flexion or extension spasms. Due to the infrequent nature diazepam was considered the most suitable on-the-spot treatment. Therefore a baseline antiepileptic treatment was not administered.

Biochemical characteristics

The general biochemical characteristics of this group are represented in table 5.2.

Severe hyperlactacidemia was a common finding. Plasmatic lactate ranged from 6,5 to 27 mmol/l (mean: 14,2 mmol/l; median: 10,3 mmol/l; SD:8,1) with a high lactate/pyruvate (L/P) ratio (range: 24,6 to 68; mean: 41,2; median: 35). Total ketone bodies were elevated in the majority of the cases (7/9) ; the sum of Acetoacetate and 3-OH-butyrate disclosed values between 0,4 and 4,2 mmol/l (mean: 2,2; median:2,5) with low Acetoacetate/3-OH-butyrate ratio in most of the cases (6/9) (range: 0,5-1,8; mean: 1; median: 1). Hyperammoniemia was a frequent finding (5/9) (range: 48-143 umol/l; mean: 99,7 umol/l; median: 123 umol/l). Hypoglycemia was constant and ranged from 1 to 2,2 mmol/l (mean: 1,6 mmol/l; median: 1,7 mmol/l). Hypernatremia was found in 4 cases (range: 135-158 mmol/l; mean: 144,4; median: 147). There was a constant presence of abnormal plasmatic amino acids (Fig 5.1). The most common profile of plasmatic amino acids was high proline, citrulline and lysine (5/9) . Citrulline was invariably high (range: 46-400 mmol/l; median: 87; mean 136) contrasting with low to

very low glutamine (in the first 5 patients, diagnosed before 1980, reliable glutamine levels were not available given the used technique for measuring amino acid, Technicon TSM1 amino acid analyser, in wich there was an interference between threonine and glutamine. Therefore the given values are abnormally high). Alanine was high in 3 patients and low in 2. Urine organic acids analysis always showed abnormalities: lactate , 2-OH-butyrate and 3-OH-butyrate were the most frequent detected metabolites with low alpha-ketoglutarate and other Krebs cycle intermediates.

Enzymatic studies

Enzymatic activities were performed in all the patients and ranged from 1 to 10% of these of the controls. Most of patients had very low values: mean 4,4 %, median 3,1%

Brain imaging

In one patient, prenatal cranial ultrasound revealed choroidal plexus cysts at 33 weeks of pregnancy. These alterations were confirmed in a MRI study at 7 days of postnatal life. MRI was carried out in 4 patients. These examinations were performed between 1 and 2 weeks of life. The most frequent finding revealed by MRI studies were high signals in periventricular white matter with frontal predominance on T_2-weighted images, associated with multiple cysts of diverse localization . Brain CT was performed in 1 patient. Extensive subcortical and periventricular white matter hypodensities were observed (Fig 5.2)

Basal ganglia cysts and cerebellar hemorrhage was observed in one patient . Non-progressive intraventricular hemorrhage was detected also in one case.

Treatment

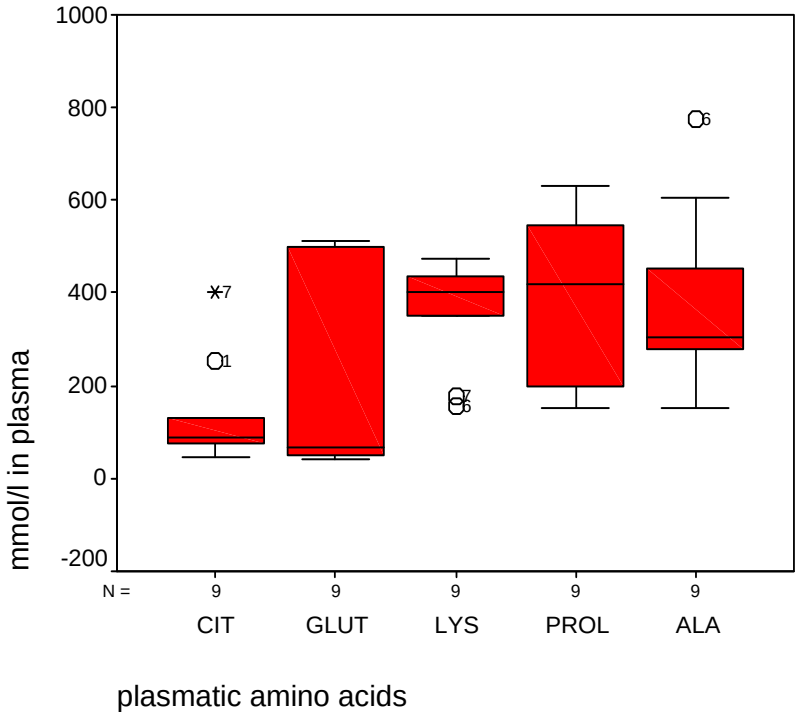
Aspartate, alphaketoglutarate, arginine, bicarbonate, biotin and thiamin were used in different combinations in all of the cases. Breast feeding was recommended for as long as possible.

Outcome

7 patients died (age of death was from 21 days to 4 months; median 60 days) and 2 were lost in the follow-up at the age of 3 months and 2 years. Regarding the patients who died, all of them had a severe persistent hyperlactacidemia and deteriorated neurologically (generalised trunkal hypotonia, progressive spasticity, persistent abnormal movements) except in one case where the only neurological abnormality was hypotonia (patient nº1). Liver disease remained constant in those who initially manifested hepatic involvement.

Concerning the patients lost in the follow-up, one of them disclosed psychomotor delay and pyramidal signs at 3 months of life (date of the last control) and the other was followed up until the age of 2 years old. In this last case lactate was normal from 15 months of life and clinical examination revealed moderate psychomotor delay and spastic tetraparesis.

Figure 5.1. Plasmatic amino acids



CIT: citrulline. (VN: 26 +/-7). GLUT: glutamine. (VN: 578 +/- 92). LYS: lysine. (VN: 183 +/-39). PROL: proline. (VN: 180 +/-47). ALA: alanine. (VN: 312 +/- 78).

Figure 5.2. Brain CT showing extensive subcortical and periventricular white matter hypodensities.

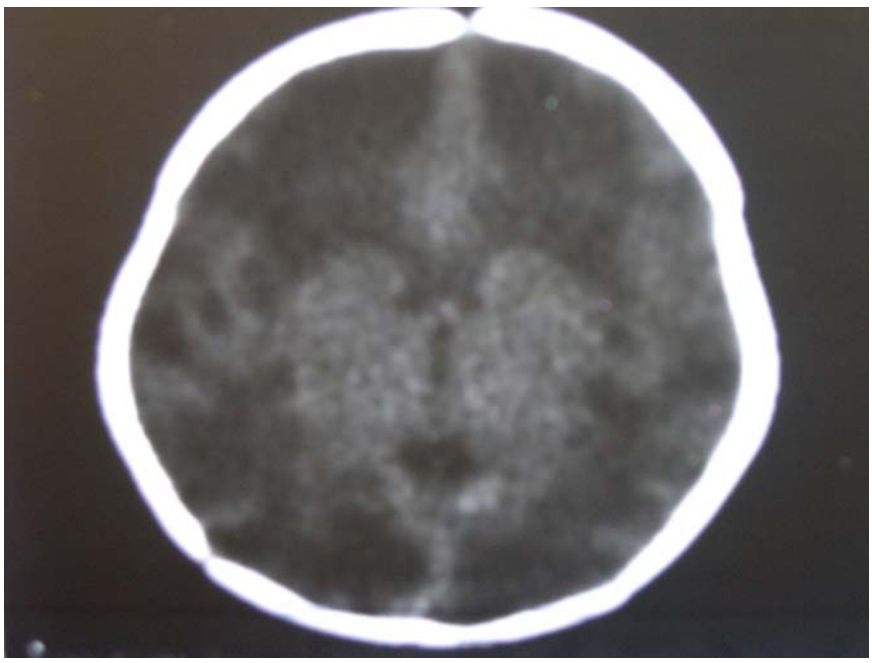


Table 5.1. General characteristics

Nº Sex	Onset age	Survival	Initial symptoms	Abnormal movements/ age	Ocular movements/ age	Neuroimaging /age	Seizures/ age	EEG/ age	Hepatic symptoms/age	Outcome
1/F	24 hours Plexus cyst 33 weeks	Deceased at 3 weeks	Hypotonia, tachypnea, good level of consciousness Facial dysmorphy	No	Normal	MRI (1 week): white matter periventricular high intensity and cysts	No	Normal/15 days	High hepatic enzymes from the first day Enlarged liver	Persistent tachypnea, high lactate and failure to thrive. No neurological degradation. Spontaneous sudden bradycardia at 3 weeks.
2/F	3 hours	Deceased at 2 months	Hypotonia, tachypnea. Depressed level of consciousness. Facial dysmorphy	High amplitude tremulations of the limbs at 48 hours. Hypokinesia , hypomimia	Abnormal. Conjugated episodic upgaze and downgaze. Fixed gaze 2 days	MRI (2 weeks): white matter periventricular high intensity and cysts. Basal ganglia cysts. Cerebellar hemorrhage	Generalised clonic movements at 45 days	Subintrant crises	High hepatic enzymes. Enlarged liver and liver failure at 2 weeks	Severe hyperlactacidemia and neurological degradation at 15 days: abnormal movements, macrocephaly, seizures
3/F	10 hours	Deceased at 4 months	Hypotonia, tachypnea, dehydration. Good level of consciousness	High amplitude tremulations Limb hypertonic crises, hypomimia, at 3 weeks	Abnormal. Nystagmus,oculomotor apraxia and fixed gaze at 15 days	Not done	Generalised clonic movements at 29 days	Hypsarrithmia-like at 29 days	Enlarged liver and high enzimes at 12 days. Portal fibrosis and steatosis	Neurologic degradation: seizures, severe hypotonia, lack of visual contact, limb hypertonia, persistent high lactate
4/ M	2 hours	Deceased at 2 months	Hypotonia, tachypnea, limb tremulations, coma	High amplitude tremulations , hypomimia at 4 hours	Abnormal. Pendular nystagmus, fixed gaze at 1 day	CT scan (1 week): white matter hypodensity	Generalised clonic movements at 15 days	Not done	No	Persistent high lactate. Neurological degradation, seizures, limb hypertonia

Nº Sex	Onset age	Survival	Initial symptoms	Abnormal movements/ age	Ocular movements/age	Neuroimaging /age	Seizures/ age	EEG/ age	Hepatic symptoms/age	Outcome
5/ M	24 hours	Lost in the follow-up	Hypotonia, tachypnea, good level of consciousness	No	Normal	Not done	No	Not done	High transaminases, enlarged liver, hepatic failure from the first day	Psychomotor delay, pyramidal signs at 3 months
6/ M	72 hours	Lost in the follow-up (last control at 2 years)	Hypotonia, tachypnea. Good level of consciousness At 15 days: normal examination	High amplitude tremulations of the limbs at 3 days	Normal	MRI (2 weeks): white matter periventricular and subcortical high intensity and cysts	No	Multifocal spikes at 12 months	No	Normal lactate since 15 monts of age. Moderate psychomotor delay and pyramidal signs
7/ M	1 hour	Deceased at 2 months	Hypotonia, tachypnea, Good level of consciousness	No	Normal	MRI (3 weeks): Extensive white matter periventricular high intensity and cysts	No	Not done	No	Neurologic degradation at 1 month: generalized hypotonia, coma
8/F	2 hours	Deceased at 1 month	Hypotonia, tachyp nea, good level consciousness Tremulations	Tremulation pendular movements of the limbs, hypomimia at1 day	Pendular nystagmus, fixed gaze at 1 day	Not done	No	Not done	No	Persistent hyperlactacidemia, neurological degradation
9/ M	24 hours	Deceased at 1.5 months	Hypotonia, tachypnea, good level consciousness.	High amplitude limb tremulations at 1 week. Hypokinesia	Pendular nystagmus, fixed gaze at 1 week	No	No	Not done	No	

Table 5.2 . Biochemical results

Nº patient	Cit mmol/l	Glut mmol/l	Lys mmol/l	Prol mmol/l	Ala mmol/l	NH_4 umol/l	Lact mmol/l	L/P ratio	Gluc mmol/l	KB mmol/l	KB ratio	Na mmol/l	Enzyme activity
1	253↑	46↓	453↑	538↑	202↓	66	15,8↑	24,6↑	1,7↓	2,19↑	1,54↑	158↑	2,8%
2	46↑	43↓	353↑	362↑	280	58	27↑	30↑	2↓	3,4↑	0,7	147↑	10%
3	87↑	50↓	476↑	630↑	421	142↑	10,3↑	32↑	1,42↓	2,6↑	1	140	1%
4	92↑	397	425↑	547↑	451↑	143↑	22↑	40↑	1↓	1,2	0,5	147↑	5%
5	77↑	66↓	372↑	150	300	123↑	24↑	35↑	1,8↓	2,5↑	1,2↑	139↑	3,5%
6	79↑	513	157	421↑	774↑	131↑	7,5↑	28↑	2,2↓	4,2↑	1,8↑	136↑	10%
7	400↑	54↓	178	197	150↓	54	6,9↑	68↑	1,42↓	0,43	0,6	150↑	2,5%
8	130↑	498	435↑	612↑	306	48	8,6↑	61↑	1,5↓	0,42	0,5	148↑	2,3%
9	60↑	512↑	402↑	167	604↑	133↑	6,5↑	53↑	2,2↓	3,1↑	1,4↑	135	3,1%

Cit: citrulline. Normal values: 26 +/-7. Glut: glutamine. Normal values: 578 +/- 92. Lys: lisine. Normal values: 183 +/-39. Prol: proline. Normal values: 180 +/-47. Ala: Alanine. Normal values: 312 +/- 78. Lact: lactate. L/P: lactate/pyruvate. Gluc: glucose. KB: ketone bodies KB ratio: 3-OH-Butyrate/Acetoacetate. Enzyme activity was measured in fibroblasts.

4.6- RESULTS OF THE PATIENT WITH IMPAIRED MITOCHONDRIAL PYRUVATE IMPORTATION

PDHC assays in disrupted lymphocytes and fibroblasts.

PDHC activity was shown normal in homogenates of fresh lymphocytes and cultured fibroblasts as compared with controls and four patients with a proven defect in the E1a subunit (Table 6.1).

Table 6.1

PDHC activity

$^{14}CO_2$ released with [1-^{14}C]pyruvate (nmol/min/mg protein)	Patient[a]	PDH-E1α-deficient patients[b]	Reference values mean ± SD, $n = 15$
Lymphocytes	1.69	0.353–1.378 $n = 3$	1.86 ± 0.26 (1.48–2.27)
Fibroblasts	1.01	0.348–0.545 $n = 4$	0.88 ± 0.11 (0.63–1.21)

[a] Mean of duplicate in 2 experiments.
[b] Range.

[^{14} C]Pyruvate oxidation rates in digitonin-permeabilized fibroblasts.

A coupled assay based on the oxidation of [$^{2\text{-}14}$ C]pyruvate to [^{14} C]acetyl-CoA and the conversion of the latter to either [^{14} C]acetylcarnitine or [^{14} C] intermediates of the citric cycle was performed on digitonin –permeabilized fibroblasts. Both methods demonstrated that [$^{2\text{-}14}$ C]pyruvate oxidation was severely impaired in the patient'_s fibroblasts whose mitochondrial membranes were intact (Table 6.2). These assays were conducted in the presence of 2 mM DCA. In contrast, production of [^{14} C]acetylcarnitine from [$^{2\text{-}14}$ C]pyruvate was found normal when measured in disrupted fibroblasts from the patient (Table 6.2). Such a discrepancy between permeabilized and disrupted fibroblasts was not observed for patients with PDHE1a deficiency (Table 6.2). Production of ^{14} CO2 was severely decreased with [$^{1\text{-}14}$ C]pyruvate, but normal with [$^{U\text{-}14}$ C]malate, ruling out an electron transport chain disorder in the patient (Table

6.3). Furthermore, with increasing concentrations of pyruvate, the patient'_s fibroblasts behaved like control fibroblasts incubated with alpha-cyano-4-hydroxyncinna-mate, a specific pyruvate uptake inhibitor (Halestrap et al 1974). This inhibition was specifically proven by the inefficiency of the inhibitor on the decarboxylation of [$^{U-14}$C]malate, whose entry into mitochondria is mediated by a dicarboxylate carrier (Table 6.3).

Table 6.2

Oxidation of [2-^{14}C]pyruvate in digitonin-permeabilized fibroblasts vs disrupted fibroblasts

	[^{14}C]Acetylcarnitine (nmol/min/mg protein)	[^{14}C]Citric cycle intermediates (nmol/min/mg protein)
Permeabilized fibroblasts[a]		
Patient	0.08	0.15
	0.09	0.62
PDHE1α-deficient patients (range, $n = 4$)	0.68–1.32	1.28–1.68
Reference values (mean ± SD, $n = 15$)	2.52 ± 0.64 (1.71–3.11)	2.81 ± 0.41 (1.75–3.90)
Disrupted fibroblasts[a]		
Patient	1.02	
	0.98	
PDHE1α-deficient patients (range, $n = 4$)	0.37–0.55	
Reference values (mean ± SD, $n = 15$)	0.88 ± 0.11 (0.63–1.21)	

[a] Mean of duplicate, two experiments.

Table 6.3

Oxidation of [1-^{14}C]pyruvate and [U-^{14}C]malate in digitonin-permeabilized fibroblasts

Release of $^{14}CO_2$ with (nmol/min/mg protein[a])	Patient	Control + inhibitor[b]	Control[c]	Reference values mean ± SD, $n = 15$
1 mM [1-^{14}C]pyruvate + 4 mM malate	0.48		8.47	5.75 ± 0.73 (4.68–8.13)
1 mM [1-^{14}C]pyruvate + 10 mM L-carnitine + 5 mM malonate	0.38	0.17	8.33	6.57 ± 0.78 (5.88–7.55)
10 mM [1-^{14}C]pyruvate + 10 mM L-carnitine + 5 mM malonate	1.51	1.30	8.47	
20 mM [1-^{14}C]pyruvate + 10 mM L-carnitine + 5 mM malonate	2.88	2.65	7.65	
50 mM [1-^{14}C]pyruvate + 10 mM L-carnitine + 5 mM malonate	6.23	6.88	7.58	
10 mM [U-^{14}C]malate + 10 mM L-acetyl carnitine + 5 mM malonate	4.70	5.00	5.03	4.55 ± 0.42 (3.93–5.10)

[a] Mean of duplicate.
[b] 50 μM α-cyano 4-OH cinnamate.
[c] In the same assay.

Prenatal diagnosis

We were asked for a prenatal diagnosis in a subsequent pregnancy involving dichorionic twin fetuses. Cultured trophoblasts derived from each fetus and from one age-matched control fetus were used to measure [$^{2\text{-}14}$ C]pyruvate oxidation by the coupled assay in digitonin -permeabilized cells. Normal pyruvate oxidation rates were observed for one fetus and abnormal rates for the other (Table 5, 6.4). ^{3} H2O production from [9,10-^{3}H]palmitate and [9,10-^{3} H]myristate, assayed in parallel as a control of the overall mitochondrial function (Pande et al 19936), was show normal for the two fetuses, ensuring that a specific defect of pyruvate oxidation was present in the affected fetus (data not show). Ultrasound survey detected severe intrauteri e growth retardation for the affected fetus. Selective pregnancy termination was conducted at 17 weeks of pregnancy. During selective feticide, a cardiac blood sample from the affected fetus was take for an alysis of DNA polymorphic markers by comparison with amniotic fluid cells from the two fe-tuses. The pregnancy course remained uneventful until 37 weeks of gestation whe a healthy baby was delivered by cesarean section . She is currently 2-month old and apparently in good health.

Table 6.4

Oxidation of [2-^{14}C]pyruvate in digitonin-permeabilized trophoblasts

	[^{14}C]Acetylcarnitine (nmol/min/mg protein)	[^{14}C]Citric cycle intermediates (nmol/min/mg protein)
Permeabilized trophoblasts[a]		
Fetus 1 (down-forward)	0.06	0.72
	0.07	0.92
Fetus 2 (top-back)	2.44	3.37
	2.93	3.77
Control[b]	2.88	3.79
	2.65	3.67
Reference values (mean ± SD, $n = 8$)	2.54 ± 0.75 (1.85–3.66)	2.98 ± 0.49 (1.65–3.63)

[a] Mean of duplicate, two experiments.
[b] In the same assay.

4.7- RESULTS OF THE PATIENT WITH HYPOGLYCAEMIAS AND HEPATIC COMPLEX III DEFICIENCY

Biochemical results

A severe decrease of CIII activity was shown in lymphocytes with the impairment of both absolute and relative CIII activities. Accordingly, low rates of substrate oxidation in detergent-permeabilized cells were demonstrated, particularly when decylubiquinol, which feeds electrons directly to CIII, was used as a substrate. CIII activity was also strongly decreased in liver associated with a mild decrease of complex I activity. A seemingly low activity of all measured red cell (RC) enzymes was observed in cultured skin fibroblasts; however, all activities except CIII activity became normal when expressed as a ratio to citrate synthase activity. Because of the low CIII activity measured in all investigated tissues, a low temperature (–196°C) differential

spectrum analysis was further performed on mitochondria isolated from cultured skin fibroblasts by using dithionite as a potent reducing agent. Whereas a normal cytochrome *aa*3 (absorption peaks: 601 and 440 nm) and cytochrome *c* (absorption peak: 550 nm) content was detected in mitochondria from the fibroblasts of the patient when compared with controls, a strongly decreased absorption of cytochrome *b* at 560 nm was demonstrated, together with severe reduction of the absorption peak at 427 nm, representing the mixed absorption of both cytochromes *c* and *b*. Mutation analysis of CIII subunit genes.

Mutation analysis of CIII subunit genes

Since the cytochrome *b* content was found to be very low in these cells, we first looked for mutations in the mitochondrial cytochrome *b* gene, despite consanguinity in this family. However, no divergence from the Cambridge reference sequence (Anderson et

al. 1981) was shown except for four common polymorphisms (nt 14983:TTA → CTA, nt 15043:GGC ⟶ GGA, nt 15301:TTG TTA and nt 15326: ACA → GCA). We further sequenced the ten nuclear-encoded CIII subunits and the BCS1 gene. A 4-bp deletion (AAAA) was uncovered in the QP-C cDNA at the nt 338–341 and three non-conservative amino acid changes were observed in the patient and all controls: G901A and G1078C (reported by Valnot et al. 1999) and A206T for the core1, core2 and HINGE proteins, respectively. Genomic DNA analysis indicated that the patient was homozygous for the deletion located in exon 4 of the gene. Both parents were found to be heterozygous for the deletion. The deletion was not seen in five French controls by direct sequencing and in 50 controls of similar ethnic origin (Turkey) by DHPLC analysis. The resulting protein is predicted to have seven changed amino acid residues plus an additional stretch of 14 amino acids at the C-terminal end (Fig. 2a). Multiple sequence alignment of CIII subunit VII shows a considerable homology across species (Fig. 2b). This holds particularly true for the C-terminus with a large number of both acidic and basic residues, making this part of the protein highly hydrophilic and predicting a conserved helical structure. The 4-bp deletion identified in exon 4 of the QP-C and resulting in an abnormal elongation with a less hydrophilic amino acid chain may be expected to disrupt this helical structure. This might in turn affect the interactions of this subunit within the complex, thus lowering CIII stability and possibly accounting for the observed decrease in cytochrome *b* content accompanying the loss of CIII activity in the fibroblasts of the patient.

Fig. 1a, b Low temperature (–196°C) difference spectra in isolated mitochondria from control (**a**) and patient (**b**) fibroblasts, under the experimental conditions described

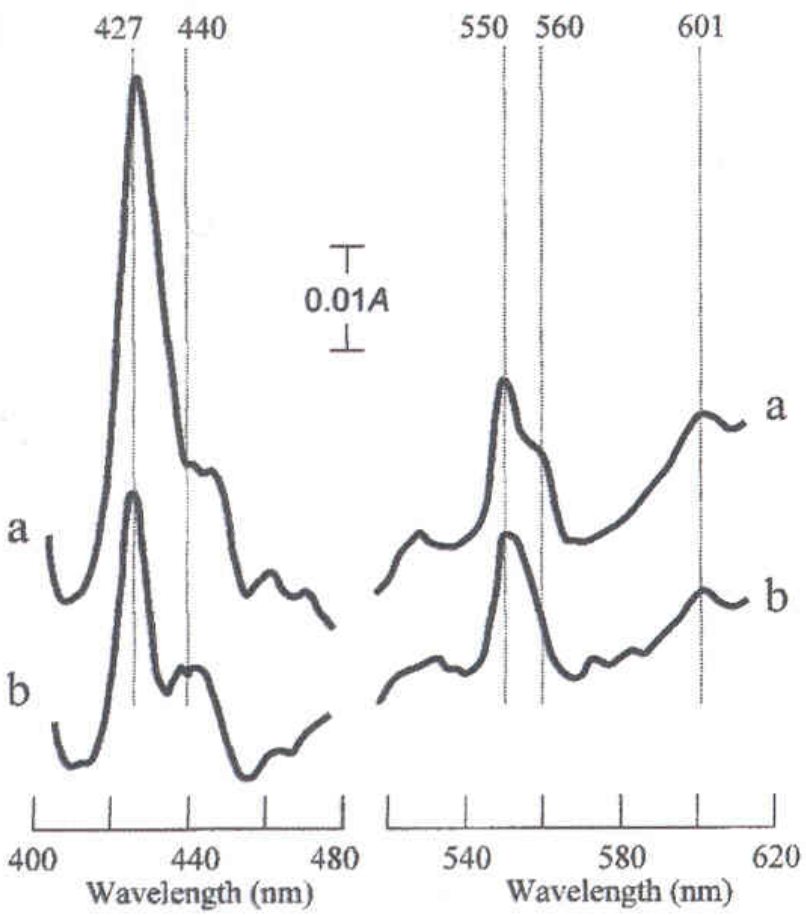
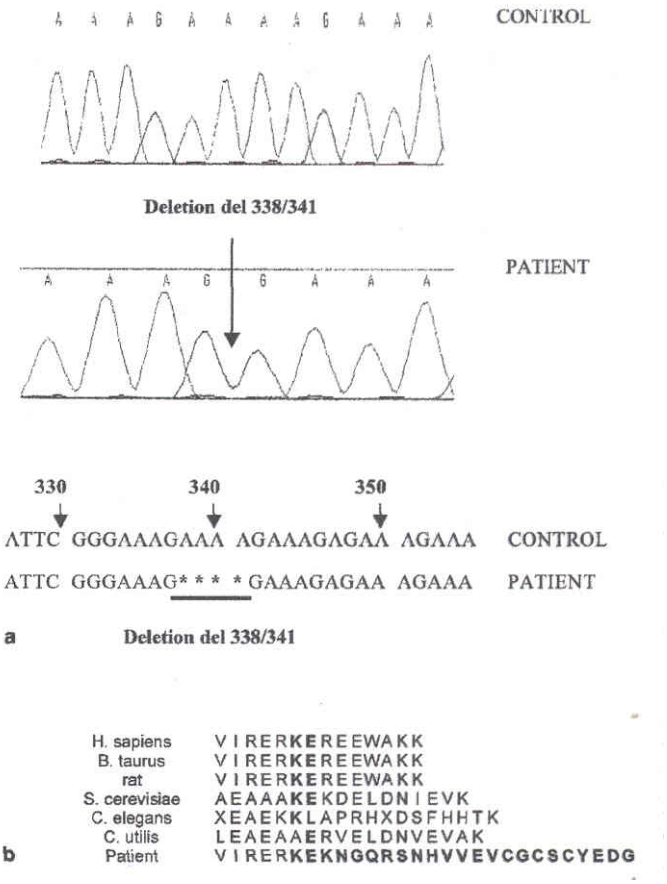


Fig. 2a, b Sequence analysis of the QP-C gene. *Arrows* Location of the AAAA deletion at nt 338–341 (**a**) and sequence alignment of QP-C (subunit VII) from various species (**b**)



5-DISCUSSION

5.1.- POPULATION Nº 1 “TOTAL POPULATION REFERRED TO IN THE THESIS: 241 CHILDREN WITH DIVERSE MITOCHONDRIAL DISORDERS”.

Inherited mitochondrial diseases have been reported in the literature from 1962 (Luft et al, 1962). Although initially described as mitochondrial myopathies the clinical spectrum of these disorders has expanded to multi-system presentations and above all to those organs dependent on high rate aerobic metabolism such as the brain, skeletal and cardiac muscle, sensory organs, liver and kidneys (Donald et al 1995, Sokol 1996, Zeviani et al, 1996, Munnich et al 1996, Nissenkorn et al 1999). Moreover, in most pediatric patients the clinical picture does not fit in one particular enzymatic or genetic defect.

From a biochemical point of view, hyperlactacidemia is used as the most valuable biochemical diagnostic marker of impaired mitochondrial energetic metabolism. Nevertheless, normal lactate value does not exclude a primary mitochondriopathy.

Under physiological conditions the blood level of lactate is lower than 2.5 mmol/l . The lactate/pyruvate (L/P) ratio reflects the NADH/NAD ratio in the cytosol; therefore an accumulation of blood lactate with high L/P ratio could be a result of a hypoxic insult or other conditions with cellular respiration failure. This feature explains that lactic acidosis can be observed in a variety of acquired circumstances including infections, severe catabolic states (chronic diarrhoea, malnutrition), all kinds of hypoxia (local and general), severe dehydration and poisoning (Duke 1999, Zeman et al 1998).

However, many inherited metabolic diseases reflect the enzymatic defect in an abnormally high blood lactate. In fact, disorders of energy mitochondrial production comprises abnormalities of pyruvate and the tricarboxylic acid cycle metabolism,

disorders of fatty acid oxidation, ketogenesis and ketolysis, and defects of the respiratory chain.

In this respect, organic acidurias, urea-cycle defects, fatty acid oxidation defects, multiple carboxylases defects, and disorders of glycogen and gluconeogenesis metabolism are considered causes of secondary hyperlactacidaemia. Conversely, disorders leading to primary or congenital HL are due to abnormalities of lactate-pyruvate oxydation (pyruvate dehydrogenase complex deficiency, and pyruvate carboxylase deficiency), Krebs cycle defects, and deficient activity of the respiratory chain components. They are globally defined as mitochondrial disorders.

Even though the association “mitochondrial disorders-lactate” seems evident, the incidence of hyperlactacidemia, its characteristics (mild, moderate, severe or lactic acidosis) and the association with other clinical or biochemical parameters are not well defined in the different groups of mitochondrial defects.

This work reports, from a series of 241 paediatric patients, the clinical and biochemical presentation, enzymatic and genetic characteristics of primary mitochondrial disorders. Furthemore, the incidence and characteristics of hyperlactatemia and other biochemical parametres as diagnostic marker is developed.

Clinical, biochemical, enzymologic and genetic data of 241 patients affected with mitochondrial disorders were reviewed. Specific enzymatic diagnosis was detected in 160 patients. The distribution of these diagnoses is coherent with the known expected prevalence of each deficit described in the literature (Munnich et al 1996, Di Mauro et al 2003, Munnich et al in “Inborn Errors of Metabolism”, Fernandes et al, 2000); the majority of the patients had respiratory chain disorders, followed by PDHC, PC, and CK; one case of pyruvate carrier deficit (Brivet et al, 2003) is also added. The patients

presented here reflect a broad spectrum of clinical presentations. Nevertheless we found some general characteristics worthy of consideration:

1-Concerning MRC we can distinguish two completely different forms of presentation: on one hand new-borns have multisystem involvement and a common pattern of symptom combination composed of hypotonia, feeding difficulties and tachypnea with a relatively well-preserved level of consciousness. This is frequently associated with neurological and/or hepatic involvement (hepatomegaly and elevated liver enzymes). On the other hand children older than 1 month have predominantly monosystem presentations: non specific psychomotor delay, neurological regression or Leigh manifestations follow neurological symptoms in this order. These findings are similar to those reported by different authors (Munnich et al 1996, Munnich et al in "Inborn Errors of Metabolism", Fernandes et al, 2000, Munnich et al 1992, Munnich et al 1996, Darin et al. 2003, Skladal et al 2003, Di Mauro et al 2002, Sue et al 1999, Bioulac-Sage et al 1993, Rustin P et al 1994, Ronald et al 1999, Bonnet et al 1999, Kirby et al 1999, Isashiki et al 1998, Loeffen et al 2000, Triepels et al 2001, Lombes et al 1989)

2-Concerning the rest of mitochondrial deficiencies (PDHC, PC, CK) we did not find any differences compared with other clinical observations that have been already described (Wexler et al 1998, Bonnefont et al 1992, Rustin et al 1997, Robinson et al 1996, Cross et al 1993, Robinson et al 1984, Saudubray et al 1976, Coude et al 1981).

Newborns presented with hypotonia and tachypnea. 7 out of patients with PC deficiencies corresponded to the French phenotype (Robinson et al 1984, Saudubray et al 1976, Coude et al 1981); we underline the excellent initial level of consciousness and the high incidence of movement disorders as initial symptoms. One special part is dedicated to the patients with this deficiency.

Older patients with PDHC deficiencies presented mainly with intermittent ataxia and peripheral neuropathy.

Biochemical results in MRC show HL in 77.6 % of the cases, revealing a similar percentage of high blood lactate than other reported series (Munnich et al 1996, Poggi-Travert et al 1996, Rubio-Gozalvo et al 2000). Severe HL is the most frequently found due to our high proportion of newborns. This age group shows statistically significant differences compared to older children. L/P ratio is constantly elevated in all age groups; pre-prandial ketone bodies and 3OHB/AcAc ratio are high in half of the cases. AAC and OAC where less frequently altered than HL, revealing that in some cases high lactate is the only biochemical abnormal data (13% of patients in our series).

In patients with normal lactate, AAC was always found to be normal whereas AOC was abnormal in 2 cases. Nevertheless, a strong statistical relation between high lactate and both abnormal amino acids and organic acids was detected. Regarding blood lactate and clinical presentation , newborns presenting with multisystem involvement disclosed severe HL, wich was more marked in those with the combination of hypotonia, tachypnea and failure to thrive, and hepatic forms. We did not find a specific pattern of symptoms in patients with a normal lactacidemia. To our knowledge there is little reported data correlating lactate and clinical presentation.

PC deficiencies presented with the highest HL followed by KC , PDHC and pyruvate carrier deficiency. Hyperpyruvicaemia with normal or low L/P ratio was seen in PDHC and ketone bodies were very elevated in PC and normal in PDH. AAC shows elevated alanine and proline except in PC deficiency where a characteristic profile was found (increased citrulline, lysine and proline). OAC showed Kreb's cycle metabolites (especially elevated in KC deficiencies). Those findings have been already reported (Coude et al 1981, Poggi-Travert et al 1996).

Distribution of enzymatic deficiencies in MRC do not differ from previously published results (Munnich 2000, Di Mauro et al 2002, Di Mauro et al 2003). CI was the most frequent deficit (and the predominant in newborns and infants) followed by CIV (the most prevalent in older children) and the combined form CI+CIV. The combination of hypotonia, feeding difficulties and tachypnea was the most common presentation of CI deficit (36 out of 45). CIV and CI+CIV deficiencies had mainly neurologic symptoms (18/27 and 9/21 respectively) 5 CIII deficiencies were found out (a significant number of patients compared to other reported series (Zeviani et al 1996, Nissenkorn et al 1999, Sue et al 1999, Sciacco et al 2001) with very heterogeneous clinical presentation. Among them two presented with late-onset encefalopathy, which is the most frequently reported phenotype of CIII deficit (Mourmans et al 1997). Four patients had CII deficiencies. Little is known about the clinical expression of this complex though non specific neurological symptoms seem to be the rule (Bourgeron et al 1995, Bourgeois et al 20). Three of our cases had an unspecific combination of hypotonia, thachypnea and failure to thrive with severe HL, whereas only one presented with late-onset cardiomyopathy. Patients with CI+CIV deficiencies had the highest HL (range: 1.8-25.5 mmol/l, mean:10 mmol/l) and this has a statistical significance ($p<00.5$) compared to those with CI deficiency (range:1.4-17.7 mmol/l, mean:5.9 mmol/l). No relevant differences were detected between the other complex deficiencies.

Concerning genetic results we found 8 mitochondrial DNA depletions. The main clinical presentations of mitochondrial DNA depletion are characterised by severe muscle weakness, hepatic failure or renal tubulopathy with fatal outcome, normally associated with lactic acidosis (Donald et al 1995, Elpeleg et al 2002, Hirano et al 2001, Vu et al 1998). In our series, the patients had an equal hepatic (3/8) and neurologic (3/8) presentation followed by cardiac disorders (2/8). HL was severe in most of the cases

(mean: 6.2 mmol/l, range: 3.3-9.4 mmol/l). Other different mitochondrial DNA mutations had a late-onset presentation and were associated with other complex deficiencies in 5 cases. In these cases plasmatic lactate was lower (mean: 2.6 mmol/l, range: 1.7-3.7 mmol/l) than in the groups described above.

5.2- POPULATION Nº 2 "LONG TERM FOLLOW-UP OF NEONATAL MITOCHONDRIAL CYTOPATHIES: A STUDY OF 57 PATIENTS".

Although neonatal presentation of respiratory chain deficiencies is quiet common because of the high energy requirements of the growing newborn, relatively little is known about the long-term clinical and biological outcome. A rapid and fatal course is normally suspected in neonatal presentations of these diseases. Nevertheless no long-term studies are currently reported in the literature.

Here it is reported the clinical and biochemical outcome over a long period of time of a group of patients diagnosed and managed in our hospital. The long-term outcome and its relation with the clinical presentation, enzymatic deficiency and initial plasmatic lactate as possible contributing prognosis factors are examined

This is the first study showing data on the long-term outcome of various types of neonatal onset mitochondrial cytopathies. Some reports have described the outcome of isolated cases (Ishikawa et al 2000, Finsterer et al 2001, Shoubridge et al 2001, Triepels et al 2001) but studies of large series in the medical literature have not been done yet. Neither has been found a systematic study concerning survival or potential risk factors that could modify the natural course of this type of metabolic disorders starting in infancy. By contrast, data dealing with clinical presentation has been widely reported (Munnich et al 1996, Nissenkorn et al 1999, DiMauro et al 2003, 11-DiMauro S, Andreu AL, De Vivo DC. Mitochondrial disorders. J Child Neurol. 2002; 17 Suppl 3: 3S35-45, Smeitink et al 2003, Skladal et al 2003). There are several factors that could explain this lack of information. The high precocious mortality and the important number of patients that are lost in the follow-up are probably the most relevant limiting conditions.

In this series different outcome aspects of these patients using diverse approaches: general description, statistical methods and detailed medical reports of the oldest surviving patients, have been analysed.

A global overview of this work would highlight some main points:

First of all a remarkable mortality rate (33/57 or 57,8 %) that is especially high in the first three months of life (16/33 or 48,4 %) was found. This finding supports the widespread perception of such disorders being fatal in the newborn (Sue et al, 1999). 12/57 or 21% of the patients are lost in the follow-up; this fact implies that some censored cases will constantly appear in the survival statistical analysis. In spite of this limitation, most of these patients have been followed for a relatively long period of time and they actively contribute to the results of our study. The other 21% corresponds to the surviving children. Half of them (10,5%) are now older than 4 years (mean: 9,5 years) and three are in their second decade of life; among these only two are cognitively normal.

Secondly a clear tendency to develop multiple health handicaps in a notable proportion of patients (68%) was observed. Therefore not only survival expectance is low, but the chances of good prospects are very poor.

Finally, the third general trait that is worth of consideration is the great proportion of patients with persistent high lactate over time (31+13=44/57 or 77,1%). This fact makes a remarkable difference compared to late onset mitochondrial disorders, where lactate is lower and tends to normalise in a high proportion of patients (Zeviani et al 1996, Sciacco et al 2001).

These general main features have been analysed using different statistical methods in order to establish possible prognostic factors contributing to the natural course of these disorders.

Survival studies did not disclose significant differences with any selected variable (clinical presentation form, initial lactate or enzymatic deficiency). By contrast, statistical significance when comparing survival time categories with different variables was demonstrated. This apparent contradiction is due to the difficulties in analysing a population with a great age dispersion (from 0 to 18 years at the time of data collection) and 12% of censored cases, using survival Kaplan-Meier curves. Therefore this test was not useful to identify risk factors in our study; however all the survival curves marked a break point around 2,5-3 years. In general, children who survived this "critical" age had more probabilities of surviving for a long period of time. This finding reflects once again the strong sensitivity of very young children to an impaired ATP production in a period of time where energy demands are very high.

Different prognostic factors relating initial and follow-up variables were identified.

Concerning clinical presentation neurological and hepatic symptoms were identified as potential prognostic outcome factors. In fact, in newborns with initial neurological signs a strong risk to develop an established neurological illness over time was detected. No other presentation type could statistically predict the clinical outcome. This finding is logical if we consider that many other neonatal symptoms are more likely to regress because they represent and age-linked manifestation rather than features exclusively caused by the disease. Furthermore the higher division rate of heteroplasmic cells in other organs can minimise the clinical expression as time pass (Andreu et al 2003). Obviously, this cannot be the case of the nervous system. On the other hand initial hepatic symptoms represented a risk factor of persistent severe hyperlactacidemia. Reduced activity of respiratory chain complexes has been associated with liver disease of varying severity, however neonatal presentation predominate and one of the key features to be noted is severe lactic acidemia (Ronald et al 1999, Vilaseca et al 1991,

Treem et al 1998). Complexes I, III and IV as well as mitochondrial DNA depletion syndrome are related to neonatal liver failure presenting with severe hyperlactacidemia (Ronald et al 1999, Vilaseca et al 1991, Treem et al 1998, Bioulac-Sage et al 1993, Cormier-Daire al. 1997, Mazziotta et al 1992, Bakker et al 1996, Maaswinkel-Mooij et al 1996, DiMauro 2004). In our series CI and mitochondrial DNA depletion where the most frequent deficiencies.

Regarding initial lactate, the group of children who did not survive the three months of life had a clear more persistent and severe hyperlactacidemia with significant differences in relation to the other age groups. Furthermore initial severe hyperlactacidemia indicated a significantly high propensity to develop a multiorganic disease. Therefore, initial lactic acidosis in neonatal mitochondrial cytopathies, resulted in a bad prognosis sign, not only due to the shorter survival expectance but to the severity of the disease involving several organs.

If we consider the different type of enzymatic abnormalities, we found that combined CI + CIV forms and generalised deficiencies in very young children (0-3 months) conditioned also a significantly higher mortality. The severity of generalised deficiencies is well known in different reported cases, above all if they are caused by mitochondrial DNA depletion (Rabinowitzs et al 2004 , Carrozzo et al 2003, Elpeleg et al 2002), . It has been also reported that patients with CI deficiency combined forms tend to have a more severe illness when compared with those with isolated CI (Korenge et al 1990). A fatal infantile multisystemic form of CI deficiency has been described in different articles (Loeffen et al 2000, Kirby et al 1999). Although many cases of fatal CI form were present in our work, this group did not present statistical differences compared to the others.

CI and CI+IV deficiencies in the total population represented in our study a negative outcome factor because they evolved towards a multisystemic disease in a high proportion of patients. Nevertheless, this finding could be explained by the important prevalence of these deficiencies in our series associated with the well-known general tendency to develop multiorganic involvement in mitochondrial disorders.

Concerning the 6 oldest surviving patients, only two could be considered as “benign or mild forms” due to normal intellectual level and minor motor limitations without dysfunction of other organs. Other than limitation in some physical activities, these patients can lead a normal life. They are similar in some aspects to the described cases of benign reversible muscle cytochrome c oxydase deficiency (DiMauro et al 1983, Zeviani et al 1987, Servedei et al 1988) but with some discrepancies. One one hand in our patients different degrees of CI deficiency are associated. The great majority of benign mitochondrial myopathies have been related to cytochrome c oxidase deficiency, however at least one case of combined CI and CIII has been described (Castro-Gago et al, 2000). Furthermore in one of them signs of spinal anterior horn involvement are present. Mitochondrial myopathies mimicking early spinal progressive muscular atrophies, although very rare, have also been reported (Pons et al 1996, Salviati et al 2003). Several elements are however atypical in our case such as the late onset, and the mild non-progressive clinical expression.

Regarding the other 4 patients, one has a multiorganic disease with persistent severe lactic acidosis, in spite of which she has a preserved quality of life. This is exceptional as both features, persistent severe hyperlactacidemia and multiorganic involvement are theoretically bad prognostic factors. 2 patients evolved towards a more common clinical expression in these disorders: neurological disease manifested as slight-moderate mental retardation and different motor handicaps. Finally our older patient has a severe

encephalopathy. Some aspects of this late case are worth of consideration: first the normalisation of severe initial liver failure. A similar phenomenon in other patients of our series was observed. A progressive reversion of liver failure and lactic acidosis has been previously reported in a child with liver and mitochondrial DNA depletion (Ducluzeau 2002) and in another one with cytochrome c oxidase deficiency (Lev et al 2002). Secondly, although liver function tended to normalise over time and the expression of the disease is now exclusively neurological, the defect was only found in hepatic tissue.

To conclude , neonatal mitochondrial cytopathies have a high early mortality and many chances to develop complex multiorganic diseases or severe encephalopathies over time. Persistent lactic acidosis and combined enzymatic deficiencies are markers of poor prognosis. Some initial symptoms can gradually improve and later normalise giving rise to less severe forms, that in some exceptional cases evolve to “benign” forms. Exclusively myopathic outcome is the most frequent “benign” clinical type.

5.3- POPULATION Nº 3 "CHARACTERISTICS OF PATIENTS WITH MITOCHONDRIAL RESPIRATORY CHAIN DEFICIENCIES EXPRESSING THE ENZYMATIC DEFICIENCY IN THE HEPATIC TISSUE: A STUDY OF 31 PATIENTS".

Impaired activity of the mitochondrial respiratory chain has been associated with liver disorders of varying severity in infants and older children. Neonatal liver failure with severe hyperlactacidemia, Alpers' disease, mitochondrial DNA depletion syndrome, Pearson's marrow-pancreas syndrome, chronic diarrhoea and intestinal pseudo-obstruction with liver involvement, and MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) are the main primary mitochondrial hepatopathies described until now (Ronald 1999, Morris 1999).

In general, the unexplained association of neuromuscular symptoms in patients with acute or chronic liver disease is the major stimulus to examine the mitochondrial respiratory chain in hepatic tissue. By contrast, this examination is not systematically carried out in patients with no signs of hepatic dysfunction and normal results of respiratory chain activities in muscle. Theoretically, and since defects can be tissue-specific, investigations should be performed on the liver as well as on more standard tissues if the suspicion of mitochondrial cytopathy is high. However, due to the more difficult technique involved and the lack of normal enzymatic hepatic values in the majority of laboratories, this examination is only performed in a few reference centres.

We report a large series of patients with respiratory chain deficiencies, presenting with and without hepatic disease but sharing a common trait: the enzymatic defect is found in the liver. The aim is to describe the clinical and biochemical characteristics of these patients in order to find possible diagnostic guidelines that justify a liver biopsy.

A series of 31 patients in which the respiratory chain enzymatic defect was detected in the hepatic tissue was analysed. Reports concerning numerous groups of patients with mitochondrial cytopathies frequently deal with encephalomyopathies and neuromuscular disorders. In contrast , even though many isolated cases (Uusimaa et al 2003, Sewell et al 1997, Edery et al 1994, Worle et al 1998, Rasmussen et al 2000) and short series (Gauthier-Villars et al 2001, Cormier-Daire et al 1997, Mandel et al 2001) of mitochondrial hepatopathies have been described, we have not identified any studies including a large number of patients. Furthermore there is a lack of data regarding patients without evidence of hepatic dysfunction but with enzymatic deficiency in this organ rather than in muscle. Consequently two main objectives were requested: first to describe the basic clinical and biochemical traits of these patients and second, to suggest possible guidelines that lead metabolic paediatricians to carry out a liver biopsy.

Almost a quarter (24%) of our total population of patients with mitochondrial cytopathies expressed a defect in the liver, reflecting its considerable involvement as a target organ. In apparent conflict with this finding, 20 of these 31 patients (65%) did not present with hepatic disease; moreover, cardinal symptoms were neurological as in other more usual mitochondrial diseases. If we analyse these results in terms of the onset age we observe the following:

Early neonatal onset was most frequent (21/31: 67,7 %) as described in several reports (Ronald et al 1999, Morris et al 1999, Edery et al 1994, Cormier-Daire et al 1997, Mandel et 2001, Ruiz Escusol et al 2000, Valnot et al 2000). Interestingly, only 9 of these 21 newborns (42,8%) presented initially with signs of hepatic disease. Acute early neonatal liver disease displayed predominantly as liver failure, has been reported primarily in association with mitochondrial DNA depletion (Rabinowtz et al 2004, Bakker et al 1996, Elpeleg et al 2002), but also with deficiency of CIV in several

infants (Cormier-Daire et al 1997, Biolac-Sage et al 1993, Vilaseca et al 1991), and with CI, CIII and generalised (Cormier-Daire et al 1997, Biolac-Sage et al 1993) in several others. Our newborns shared these same deficiencies with a marked dominance of CI and only 1 case of mitochondrial DNA depletion. Complex deficiencies in our series are similar regardless whether the newborns first exhibited hepatic dysfunction . Therefore, enzymatic deficiencies did not predict the early or late development of hepatic disease in our cases. The very low incidence of mitochondrial DNA depletion could partly explain the fact that initial hepatic symptoms are not predominant in our group of patients. Beyond the neonatal period only one patient (1/31: 3%) presented with liver failure and cholestasis (CIII deficiency with rapid fatal outcome). Hence, although neonatal hepatic onset is more frequent than in other periods of life, and is classically considered a hallmark of liver mitochondriopathies, its absence does not exclude it. Besides, this result suggests that the main form of presentation is multiorganic and neurological.

On the other hand, in considering the appearance of hepatic disease over time, a total of 22 (70,9%) patients who exhibited this disorder at some point in their lives were detected. 11 patients (35,4%) who did not initially manifest liver dysfunction developed it later (between 2 and 10 months; mean: 5,9 months). The majority of them (16/31: 52%) presented neurological and liver involvement. Curiously, the prevalence of this form of outcome was high independently of the initial symptoms. The best recognized clinical presentation of neurohepatic disorder of mitochondrial origin is Alper's syndrome. It is characterized by a progressive encephalopathy of early onset that presents rapid and severe developmental delay, intractable seizures and liver involvement . The clinical tetrad that is often used in diagnosis is: 1-refractory seizures, 2-episodic psychomotor regression, 3-cortical blindness and 4-liver disease (liver

failure, micronodular cirrhosis, pharmacogenic sensitiviy to valproic acid toxicity) (Harding et al 90, Naviaux et al 2004). According to this, 4 out of 16 patients with neurohepatic outcome can be diagnosed with Alper's syndrome. The other 12 never developed epilepsy; however, the follow-up has not proceeded long enough (up to 3 years of age) to rule out the appearance of seizures later. Despite this we should consider other clinical pictures with neurohepatic manifestation in addition to Alper's syndrome (Umer et al 2002, Simonati et al 2003). 10/16 patients in this group disclosed CI deficiency.

Predominant neurological outcome (CI, CIV and combined deficiency) was expressed in the form of mainly as Leigh's disease, as occurs in many other mitochondrial encephalopathies (Medina et al 1990, Munnich et al 1996, Nissenkorn et al 1999, DiMauro et al 2003). Although we discovered 6 cases with a primarily hepatic outcome, only one patient with Pearson's syndrome and a combined deficiency (CI, CIII, CIV) has been followed up for a sufficient period of time to provide relevant data to determine the development in this subgroup.

Among the patients who did not present with hepatic disease (9/31: 29%), two cases are of special interest. Both had a late neurological presentation and an exclusively non-specific neurological outcome (mental retardation, regression and cerebellar dysfunction). Signs indicating mitochondrial disorder were initial hyperlactacidemia in one of them and neurological regression preceded by dystonia in the other. The lack of enzymatic deficit in muscle indicated to the hepatic biopsy. These are examples of how the study of this tissue can be useful in the absence of clinical liver involvement.

With respect to biochemical results, hyperlactacidemia was the rule. Furthermore it was severe and constant in fatal cases. Those findings have already been reported (Edery et al 1994, Cormier-Daire et al 1997, Mandel et al 2001, Ruiz Escusol et al 2000, Valnot

et al 2000, Rabinowitz et al 2004, Bakker et al 1996) and are consonant with the already known greater incidence of hyperlactacidemia in newborns.

Enzymatic deficiencies found in our patients were dominated by the presence of CI deficiency. In addition, a significant difference depending on the onset-age was demonstrated. In fact, CI and CI+CIV were more prevalent in newborns whereas CIV was more prevalent in older children. Isolated complex I deficiency is probably the most common enzyme defect among the group of OXPHOS disorders (Loeffen et al 2000, Kirby et al 1991); however, although it has been linked to fatal infantile lactic acidosis (11% approximately), it is not established as a major cause of liver disease except in the context of multi-system disorders. COX deficiency has been related to fatal congenital lactic acidosis and hepatopathy in early chilhood (Cormier-Daire et al 1997, Biolac-Sage et al 1993, Vilaseca et al 1991).

The principal differences between our series and the general population of mitochondrial cytopathies were: the very rare presentation in children older than 1 year, the significantly lower survival probabilities in patients with liver deficiencies, and finally the greater prevalence of hepatic diseases (as an initial or an evolving manifestation).

Taking into account the results discussed above some recommendations to help determine the need for a liver biopsy are proposed. Liver biopsy is indicated when:

1- The clinical expression of the disease is hepatic.

2- When there are “neurohepatic” outcomes, even when they do not fit exactly into the diagnosis of Alper’s syndrome.

3 - In early onset-age cases: mainly neonatal but also early childhood even in the lack of initial or later liver dysfunction.

4-When there is a suggestive clinical picture with normal respiratory chain activities in muscle, even without hepatic disorder for a very long period of time, especially if the expression is dominated by neurological disease.

5.4- POPULATION Nº 4 "PYRUVATE CARBOXYLASE DEFICIENCY: METABOLIC AND NEUROLOGICAL CHARACTERISTICS OF NINE PATIENTS".

Pyruvate carboxylase deficiency is a rare autosomal recessive inborn error of metabolism with three different clinical presentations. Robinson et al , 1984 defined two forms according to the severity of clinical and biochemical manifestations: The B or "French" phenotype is a neonatal form with severe biotin unresponsive lactic acidosis, hyperammonemia, hypercitrullinemia and fatal outcome in the first few months of life (Saudubray et al, 1976, Coude et al, 1981, Wong et al, 1986). The A form or "North American phenotype" is characterised by infantile onset, mild to moderate hyperlactacidemia and longer survival but severe clinical sequelae.

On the other hand some benign forms with nearly normal development have also been described (Van Coster et al, 1991, Hamilton et al, 1997, Arnold et al, 2001, Vaquerizo-Madrid et al, 1997).

It has been argued that the severity of PC deficiency is related to the degree of residual enzyme activity (Robinson et al, 1984) as well as the specific molecular phenotypes, although it is controversed (Wexler et al, 1994, Robinson et al, 2000, Robinson et al, 1987). B and A phenotypes can not be distinguished by complementation studies (Augereau C et al, 1985)

The reported neurological abnormalities in most French forms comprise: hypotonia, spasticity, seizures, coma, macrocephaly and periventricular cysts (Saudubray et al, 1976, Coude et al, 1981, Wong et al, 1986, Pineda et al 1995, Brun et al, 1999). By contrast there is little data concerning other neurological features such as movement disorders (other than dystonia when presenting as Leigh's syndrome) or abnormal

ocular behaviour. On the other hand, only isolated cases or short series have been described concerning both clinical and biochemical features.

The possibility of treating PC deficiency with triheptanoin and citrate has been introduced recently (Mochel et al, 2004 In press). Both therapeutic drugs should be administered to the patient as soon as possible in order to ensure maximum benefit. Therefore it is essential to have an early and appropriate diagnosis approach.

In order to design the main clinical and biochemical guidelines that could orientate the diagnosis before determining enzymatic activity, the clinical and biochemical presentation, together with the outcome of 9 patients with PC deficiency of our initial series are reviewed.

The neurological manifestations, especially in some poorly documented aspects such as epilepsy and abnormal movements are focused on.

Although numerous isolated cases of this inborn error of metabolism have been reported since its first description in 1968 (Hommes FA et al 1968) large series in the medical literature have not been reported. This work reviews nine neonates with the severe French form of PC deficiency. All patients had similar clinical and biochemical findings. Hence the profile of the ideal newborn candidate to receive triheptanoid and citrate as soon as possible was attempted to be defined.

This type of patient is born after a normal pregnancy and delivery. In some cases prenatal ultrasonography examinations reveal choroidal plexus cysts or ischemia-like brain lesions (Brun N et al, 1999). Apgar test and initial neonatal examination including birth weight, are normal except for the presence of macrocephaly in some patients (Brun N et al 1999 , Pineda et al 1995, Wong et al 1986, Pollock et al 1986). Symptoms

usually start during the first day of life (around 10 hours according to our study) with hypotonia and tachypnea. At this moment an excellent level of consciousness is still present.

Laboratory exams would probably be performed due to the symptoms of the child. The initial rapid first line biochemical tests would disclose severe lactic acidosis with high L/P ratio, hyperammoniemia (approximately 100 umol/l in our study), severe hypoglycemia (around 1,5 mmol/l), and in some cases hypernatremia with hypertransaminemia that could be associated with low coagulation factors. At that point the diagnostic strategy would focus on some type of congenital lactic acidosis like respiratory chain deficiency and organic aciduria. Hyperammoniemia in conjunction of severe lactic acidosis is a rare condition . Some causes of secondary hyperlactacidemia (HL) such as fatty acid beta-oxidation defects or organic acidurias may display slight to moderate hyperammoniemia but HL is usually mild. On the other hand, the constant association of hypoglycaemia could once again point towards inborn errors of fatty acid beta-oxidation. However, the measurement of plasmatic ketone bodies would disclose a high synthesis of these, which is not seen in beta-oxidation.

The next element that would be of great value in carrying out the diagnostic process would be the plasmatic amino acid study. Nevertheless, in some hospitals the result of this test is not obtained immediately. Thus, the time required to receive it could represent a negative delay for starting the therapy. In that case, other clinical manifestations may be worthy of consideration in order to guide the metabolic paediatrician.

Some special neurological signs such as abnormal movements and bizarre ocular behaviour appear during the first days of life. Abnormal movement contrasting with severe axial hypotonia in the context of severe lactic acidosis is strongly evocating of

PC deficiency contrary to respiratory chain deficiency. The most common pattern of abnormal movements involves high amplitude tremor of the limbs aggravated by external stimuli, in a child who is otherwise paradoxically hypomimic and discloses slow and few spontaneous movements. Pendular nystagmus, rapid conjugated ocular movements and rolling eyes (Pineda et al 1994) are usually associated with the previously described uncommon limb movements. Although abnormal movements are seen in newborns with different metabolic disorders, this combination of symptoms seems very specific because it simultaneously includes the two broad groups in which movement disorders are clinically divided (Fernandez-Alvarez, 2000) : hypokinetic-rigid syndrome and dyskinesias (which in this case are restricted to tremor). Some conditions such as organic acidurias, non ketotic hyperglycinemia, congenital hyperekplexia and neurotransmitter deficiencies may present similar abnormal movements (Gascon et al 1994, Blau et al 2001, Tijssen et al 2002, Kure et al 2004, Saudubray et al 2002). In particular, biogenic amine deficiencies display almost equivalent anomalies : hypokinesia, rigidity and a wide range of dyskinesias associated with abnormal ocular movements (Blau et al 2001, Hyland et al 2004). Interestingly, PC plays an important role in the formation of the neurotransmitters acetylcholine and gamma-aminobutyric acid (Wallace et al, 1998) . Hence, the low production of these metabolites could form the basis of these movement disorders.

Seizures were not one of the main clinical hallmarks of the disease. In fact there are no specific epileptic characteristics described in this disease. Epilepsy seems more likely to appear when children survive metabolic decompensation. They are described as myoclonic and generalised tonic-clonic. (De Meirleir et al 2002)

Due to the complex neurological symptoms, brain MRI is usually performed. Cystic periventricular leukomalacia at birth is the most frequent finding in our series and in the

revised literature (De Meirer et al 2002). Other observations have described subdural effusions, ischemia-like brain lesions and periventricular hemorrhagic cysts (De Meirer et al 2002, Brun et al 1999). Pyruvate dehydrogenase deficiency may disclose similar prenatal lesions, but microcephaly and high L/P ratio are usually associated (Wada et al 2004, De Meirer et al 2002).

At this stage of the diagnostic process, we should have the results of the plasmatic amino acids analysis. Increased citrulline is constant in our patients and in all the other reported cases (Saudubray et al 1976, Coude et al 1981, Charpentier et al 1982) due to a decreased mitochondrial oxaloacetate and consequently a defective synthesis of aspartate. Proline and Lysine also frequently increase (the later due to the decrease of alpha-ketoglutarate formation, Kamoun et al 2002) By contrast and in spite of the presence of both hyperammoniemia and hyperlactacidemia, glutamine and alanine are low or normal. Therefore the amino acids profile is very suggestive and highly useful in order to perform the diagnosis.

Another striking finding is the contrast between a high L/P ratio (reflecting a high NADH/NAD ratio in the cytosol) and a low 3OHB/AA ratio (reflecting a low NADH/NAD ratio in the mitochondria). Unfortunately due to numerous pitfalls in measuring acetoacetate, this quite pathognomonic finding is not available in many centres.

Different treatments to restore the Krebs cycle were performed in our patients without obtaining a significant clinical impact on the associated neurological deterioration and the fatal outcome.

To conclude, the clinical, biochemical and neuroimaging features of the French form of PC deficiency are highly characteristics. The careful evaluation of these should lead the paediatrician to perform a correct diagnostic approach and therefore an early treatment.

The new treatment proposed in PC deficiency associating triheptanoin and citrate should permit to restore the metabolic failure in the first hours then weeks of life and to thwart the usual fatal outcome of this severe metabolic disease.

5.5-IMPAIRED MITOCHONDRIAL PYRUVATE IMPORTATION IN A PATIENT AND FETUS AT RISK.

It is well known that defects of pyruvate dehydrogenase and respiratory chain complexes are the most frequently recognized causes of neurodegeneration associated with congenital lactic acidosis. Rare causes, such as mitochondrial transmembrane carrier defects, must also be considered to explain the disturbed energy metabolism in patients in whom no enzyme deficiency has bee found (Huizing et al, 1996). The direct unambiguous diagnosis of a carrier defect is difficult, since conventional assays based on uptake or ef flux of metabolites are unsuitable for clinical work, but indirect approaches by functional assays and or immunological studies have prove their efficiency (Trijbels et al, 1997). The presence of a mitochondrial pyruvate carrier (PyC) defect was hypothesized for a neonate presenting with severe hyperlactacidemia and yperpyruvicemia, normal lactate/pyruvate molar ratios and normal PDHC activity. PyC, which mediates the proton symport of pyruvate across the inner mitochondrial membrane, supplies the pyruvate dehydrogenase complex and the pyruvate carboxylase with their substrate and plays a key role in glycolysis and gluconeogenesis. In an attempt to demonstrate the PyC defect in this case, functional assays to compare the oxidation rates from [2-14 C]pyruvate or [1-14 C]pyruvate in disrupted and digitonin - permeabilized fibroblasts were carried out.

Our patient presented with neonatal encephalopathy associated with severe hyperlactacidemia and normal lactate/pyruvate ratios. These data were suggestive of PDHC deficiency but such a defect in both lymphocytes and fibroblasts was not found. An alternative hypothesis was that pyruvate transport across the mitochondrial membranes was impaired. PyC mediates the proton symport of pyruvate (or exchange with OH) across the inner mitochondrial membrane. It plays a key role in glycolysis and

gluconeogenesis supplying the pyruvate dehydrogenase complex and the pyruvate carboxylase. PyC shows fairly broad specificity for short chain monocarboxylates substituted in the 2- or 3-position by an oxo group (including acetoacetate, 3-hydroxybutyrate, and branched chain oxoacids) or by a halide (as in dichloroacetate or 2-chloropropionate) (Halestrap et al 1975). The mitochondrial pyruvate carrier was purified from bovine heart, rat liver, and rat brain (Nalecz et al, 1992), but the chromosomal localization of the PyC gene in humans and the cDNA sequence of this carrier are currently unknown . Functional assays were carried out ex-vivo in an attempt to establish the impairment of mitochondrial pyruvate transport in our patient. Parallel methods in disrupted or digitonin -permeabilized fibroblasts were designed to measure the oxidation of [^{14} C]pyruvate: digitonin allows pyruvate to bypass the plasma membrane and directly reach the mitochondrial membrane. [$^{2\text{-}14}$ C]Pyruvate oxidation was normal in disrupted fibroblasts and severely impaired in permeabilized fibroblasts whose mitochondrial membranes were intact. The defect in pyruvate oxidation can be overcome at high concentration of pyruvate: an on–carrier mediated diffusion , in a manner that is identical to normal fibroblasts in the presence of a specific inhibitor of PyC, was observed. The high values of oxidation rates reached at 50 mM pyruvate demonstrate that PDHC could be fully activated in the patient. Hyperlactacidemia was found to be resistant to 2-chloropropioate administration , a therapy currently used to decrease blood lactate levels; 2-chloropropionate (an analog of dichloroacetate) is a PDHC kinase inhibitor increasing pyruvate oxidation in patients with either a PDHC defect or a respiratory chain disorder (Stacpoole et al 1997); it must be stressed that these products are mono-carboxylates that share the same mitochondrial carrier as pyruvate. The presence of DCA in the incubation medium was ineffective to restore a normal [2-14 C]pyruvate oxidation in permeabilized fibroblasts of the patient despite

normal PDHC activity. Failure of DCA to activate pyruvate oxidation in vitro and ineffectiveness of 2-chloropionate in our patient are therefore related to the pyruvate carrier dysfunction.

There are currently few attempts made to demonstrate a deficiency of PyC by indirect functional studies. Selak et al., 1997 described four patients presenting with hypotonia, developmental delay, seizures, severe headaches, and ophthalmologic abnormalities. Mitochondria isolated from skeletal muscle of these four children had a high respiratory control index for all substrates tested except for pyruvate. Respiratory rates with glutamate, succinate, alpha-glycerophosphate, acetyl, octanoyl, and palmitoylcarnitine were normal. Activity of all respiratory chain enzymes and of pyruvate dehydrogenase complex was also in the normal range. From these investigations, the authors concluded that the rate-limiting step in pyruvate oxidation in these four patients appeared to reflect a decreased entry of pyruvate into the mitochondrial matrix. Naito et al. 1988 reported another approach in a 5-month-old boy with mental and developmental retardation , muscle hypotonia, and seizures. This patient was also found to have lactic acidosis and considered to have Leigh's disease because computed tomography revealed low density in the bilateral basal ganglia. The authors measured ^{14} CO2 production from [$^{1\text{-}14}$ C]pyruvate and [$^{1\text{-}14}$ C]acetate in monolayers of intact fibroblasts. Decarboxylation of [$^{1\text{-}14}$ C]pyruvate was decreased despite normal PDH activity and normal decarboxylation of [$^{1\text{-}\ 14}$ C]acetate. It is currently not possible to confirm a defect of PyC by mutational analysis at a molecular level. Only monocarboxylate carriers of the plasma membrane have been identified until now but the rapidly evolving characterization of yeasts mitochondrial carriers (Palmieri et al, 2000) will hopefully allow the recognition of the pyruvate carrier in the near future. Several mitochondrial transmembrane carriers have been cloned and sequenced. It appears that they have similarities in their aminoacids

sequences, particularly in the membrane spanning regions, indicating that they are encoded by a gene family and therefore, have evolved from a common ancestor (Belenkiy et al, 2000).

In conclusion , that functional assays based on [^{14} C]pyruvate oxidation in digitonin-permeabilized fibroblasts can provide a convenient mean to investigate a presumptive PyC deficiency, in patients with intractable hyperlactacidemia, normal lactate/pyruvate molar ratios and normal PDH activity. This approach also enables prenatal diagnosis in pregnancies at risk for this unusual disorder.

5.6-A DELETION IN THE HUMAN GP-C GENE CAUSES A COMPLEX III DEFICIENCY RESULTING IN HYPOGLYCAEMIA AND LACTIC ACIDOSIS.

Respiratory chain complex III (CIII; ubiquinol-cytochrome *c* reductase; EC 1.10.2.2) deficiency is a comparatively rare condition in humans (Mourmans et al 1997, Von Kleist-Retswoz et al 1998). CIII channels electrons from the ubiquinone pool to cytochrome *c*, simultaneously extruding protons from the mitochondrial matrix space to the intermembrane space. CIII contains four redox centers, i.e. two haem groups (*b*h and *b*l of the *b*-type cytochromes), cytochrome *c*1 and the iron-sulphur cluster of the Rieske protein (Schägger et al 19953). In mammals, CIII is made up of 11 subunits, all of which are encoded in the nucleus with the exception of the mitochondrially encoded cytochrome *b*. Because of its dual genetic origin, CIII deficiency can be subject to either an autosomal or a maternal mode of inheritance. To date, about 20 different mutations have been identified in cytochrome *b* (Marin Garcia et al 1995, Marin Garcia et al 1996, Dumoulin et al 1996, Bouzidi et al 1996, Andreu et al 1999, Andreu et al 2000, Andreu et al 1999, De Coo et al 1999, Valnot et al 1999, Mitomap (2003)), mostly in patients with skeletal muscle weakness and exercise intolerance. Myoglobinuria has been less frequently observed (Andreu et al 1999). Recently, mutations have been reported in the nuclear BCS1 gene encoding an essential factor for CIII assembly in a series of patients with early liver failure associated with tubulopathy and/or encephalopathy (De Lonlay et al 2001) or in patients with GRACILE syndrome (Visapaa et al 2002).

In an attempt to identify additional nuclear genes responsible for CIII deficiency in humans, direct sequencing of the genes encoding the eleven struc-tural subunits of CIII and the BCS1 assembling gene in a patient with isolated CIII deficiency were performed.

It is reported here for the first time a respiratory chain complex deficiency in which a mutation in a nuclear-encoded complex III subunit has been demonstrated. A 4-bp deletion (nt 338–341; AAAA del) has been found in the exon 4 of the QP-C gene and is homozygous in the proband and heterozygous in both parents. The identification of the molecular defect accounting for CIII deficiency in this family offers the possibility of a confident prenatal diagnosis. The molecular change in the QP-C gene predictably results in a protein with seven modified amino acids plus an additional 14-amino-acid-long stretch at the C-terminal end. The pathogenicity of this abnormal elongation, which was absent in 55 controls and which occurs in a highly conserved region of the protein (Suzuki et al. 1988, 1989), is highly likely in view of the important function conferred by this helical domain on CIII subunit VII in the maintenance (or assembly) of the complex. Indeed, previous experiments on the yeast *Saccharomyces cerevisiae* have shown that various deletions in the helical domain of the C-terminal part of the yeast counterpart of QP-C result in reduced residual CIII activities associated with decreased cytochrome *b* and other CIII subunit content (Hemrika et al. 1994). Both the experimental data obtained on the yeast and the reduced cytochrome *b* content measured in the cultured skin fibroblasts from the patient described in this study suggest that this particular domain of CIII subunit VII plays a prominent role in the maintenance (or assembly) of complex III. Multisystem disorders and tissue-specific diseases, such as myopathy or cardiomyopathy, have been shown to result from complex III deficiency (Marin Garcia et al. 1995, 1996; Dumoulin et al. 1996; Bouzidi et al. 1996; Andreu et al. 1999a, 1999b, 2000; De Coo et al. 1999; Valnot et al. 1999). Here, it is shown that such a deficiency can also result in hypoglycaemia and lactic acidaemia expending the spectrum of symptoms possibly associated with this defect. Mutations in the gene encoding the BCS1 protein involved in CIII assembly trigger severe liver CIII defi-

ciency and have been shown to cause early hepatic failure in humans (de Lonlay et al. 2001). The case reported here is one of severe CIII deficiency expressed in liver but with no evidence of permanent liver dysfunction, except for metabolic crises episodes occuring after fasting, with hepatomegaly, hypoglycaemia and mild elevation of liver enzymes.

As observed in the cases of deficiencies affecting the other RC complexes (Zhu et al. 1998; Papadopoulou et al. 1999; Valnot et al. 2000; Benit et al. 2001), it is difficult to provide an explanation for these highly variable clinical presentations. So far, too few patients with proven molecular defects have been reported for a correlatation to be made between the nature of the gene involved and a given clinical phenotype.

Whereas the BCS1 gene mutation appears to be a frequent cause of CIII deficiency, we have found the first molecular abnormality in the QP-C gene. A systematic study attempting to identify mutations in nuclear genes encoding CIII structural subunits in another cohort of five patients has failed to identify any mutations (Valnot et al. 2000). This supports the view that, in addition to mutations in mitochondrial cytochrome *b*, mutations resulting in CIII deficiency most often lie in genes involved either in the assembly or, more generally, in the maintenance of the complex. At present, only one such gene, BCS1, is known. However, this is likely to change as the search for the molecular bases of human mitochondrial diseases takes advantage of the ever increasing functional data available for yeast and other organisms.

SUMMARY AND CONCLUSIONS

This study gives a broad overview of primary mitochondrial defects (pyruvate and Krebs cycle metabolism defects, and respiratory chain deficiencies) through the analyse of different aspects of a large series of patients. In general, it has fulfilled three main purposes:

1-To describe common traits of clinical and biochemical presentation, as well as enzymatic characteristics of these disorders.

2-To report poorly or non documented facets of these diseases such as long-term follow-up of neonatal mitochondrial respiratory chain deficiencies, characteristics of patients expressing the defect in the hepatic tissue, and neurological and metabolic features of pyruvate carboxylase deficiency.

3-To describe new aspects not documented until the realisation of this work such as functional assays in a case of impaired pyruvate import and a patient with a CIII deficiency presenting with hypoglycemias in wich a new nuclear mutation is found.

Concerning GENERAL CHARACTERISTICS

Since the report of the first mitochondrial abnormality causing disease in humans (Luft et al 1962), numerous clinical descriptions as well as large biochemical and genetic studies have been outlined. Nevertheless, one of the main problems nowadays remains the clinical recognition of patients with these disorders.

In this respect our series shows similar results than many other reports in the literature. However we highlight some particular points that provide additional information:

- Newborns present in most of the cases with unspecific multiorganic involvement that is indistinguishable in all the primary mitochondrial disorders analysed in

our study. In spite of that, patients with pyruvate carboxylase deficiency have some common different specific traits that, from a clinical point of view, may be useful in the diagnostic orientation. These characteristics are movement disorders (high-amplitude tremulations) affecting limbs and eyes mainly. Movement disorders in mitochondrial energy metabolism impairment, are basically referred to dystonia in Leigh's syndrome, by contrast little data about any other kind of abnormal movements have been reported.

- Children older than one month presents in the majority of the cases with psychomotor delay. This is also a very unspecific symptom, that does not lead the clinician to consider mitochondrial disorders in first intention. Clinical follow-up as well as the result of intermediary metabolism tests in some cases, will finally sustain the diagnosis.
- Initial hyperlactacidemia was found in more than three quarters of MRC (77.6%) and in all the patients with other mitochondrial defects. The highest hyperlactacidemias were found in PC deficiency followed by MRC, PDH and KC defects. Severe HL is the most frequently found due to our high proportion of newborns.; furthermore, this age group shows statistically significant differences compared to older children.
- Newborns with MRC deficiencies presenting with multisystem involvement disclosed severe HL, wich was more marked in those with the combination of hypotonia, tachypnea and failure to thrive , and hepatic forms. The association between severe HL and neonatal hepatic disease is currently well known (Ronald et al 1999).
- The results of L/P ratio, ketone bodies, 3OHB/AcAc ratio, AAC and OAC are consonant with other observations previously published (Poggi-Travert et al

19965). We propose a diagnostic flow-chart based in these findings and in the onset-age.

- The distribution of enzymatic complex deficiencies in respiratory chain disorders of our patients is in congruence with the global expected prevalence (Di Mauro et al 2003). The statistically significant hyperlactacidemia observed in the combined CI+CIV deficiency is highlighted. This fact could be explained by the higher severity of multiple complex defects.
- The results of the genetic studies do not appoint commentaries of special consideration. Many of the patients were studied too long ago to underwent complete molecular tests. On the other hand the study of the nuclear genes in the latest patients was not systematically performed. Therefore no discussion or conclusions can be attired in this sense.
- Another weak point of the study is the lack of participation of morphological and inmunohistochemical tests . This information was not fully used due to its recollection in another centre.

Concerning LONG-TERM FOLLOW-UP OF NEONATAL MITOCHONDRIAL CYTOPATHIES

This is an original aspect of our series due to the lack of information concerning the long-term follow-up of these patients.

Neonatal presentation in respiratory chain disorders is very frequent (44% in our series). Although we have consistent information about the clinical presentation, the outcome has been mostly reported in isolated cases (Ishikawa et al 2000, Finsterer et al 2001, Triepels et al 2001) rather than in large series.

The main hallmarks of this study are exposed as follows:

1-There is an important mortality rate (33/57 or 57,8 %) that is especially high in the first three months of life (16/33 or 48,4 %). This finding agrees with the general perception of such disorders being fatal in the newborn (Sue et al, 1999).

2-There is a globally poor quality of life due on one hand to a clear tendency to develop multiorganic involvement (68% of the patients) and on the other hand to the almost constant neurological handicap (only two of the surviving children are cognitively normal). Only two out of our 57 patients developed a “clinically bening form” with signs and symptoms of myopathic disease.

3-We found a great proportion of patients with persistent high lactate over time (77,1%). This fact markedly differs from late onset mitochondrial disorders, where lactate is lower and tends to normalise in a high proportion of patients (Zeviani et al 1996, Sciacco et al 2001).

4-Survival studies disclosed a break point around 2,5 - 3 years. In general, children surviving this “critical” age had more probabilities of surviving for a long period of time.

5-We detected different prognostic factors that could modify the course of these diseases:

- Initial neurologic and hepatic presentation conditioned more probabilities to develop neurological disease and severe persistent hyperlactacidemia respectively. Hepatic mitochondrial deficiencies have been associated with liver

disease of varying severity, however neonatal presentation predominate and one of the key features to be noted is severe lactic acidemia.

- Initial severe hyperlactacidemia (>6 mmol/l), CI+CIV and multiple deficiencies are negative prognostic factors due to the great probability to develop multiorganic diseases and a high early mortality (< 3 months)

6-Concerning the patients surviving more than 4 years of age:

- Interestingly, these 6 patients survive in spite of negative prognostic factors such as multiple deficiencies and severe initial hyperlactacidemia
- "Myopathic" outcome forms could be considered "benign forms" even combined enzymatic deficiencies and one case of late-onset spinal atrophy. These cases share some similarities with the described benign reversible muscle cytochrome c oxydase deficiency (Di Mauro et al 1983, Zeviani et al 1987, Servedei et al 1988) even though our patients associate different degrees of CI deficiency. With respect to the patient with signs of spinal atrophy, she is exceptional due to the late onset and the mild non-progressive clinical expression. Previous reports dealing with spinal atrophy in mitochondrial respiratory chain deficiencies refer to early progressive forms (Pons et al 1996, Salviati et al 2003)
- Some initial symptoms may gradually improve and later normalise giving rise to less severe forms.

Concerning CHARACTERISTICS OF PATIENTS WITH MITOCHONDRIAL RESPIRATORY CHAIN DEFICIENCIES EXPRESSING THE ENZYMATIC DEFICIENCY IN THE HEPATIC TISSUE

The hepatic tissue is an important target of mitochondrial disorders. Although several reports concerning isolated cases and short series of mitochondrial hepatopathies have been described, we have not identified any studies including a large number of patients. The aims were to describe the basic clinical and biochemical traits of these patients and furthermore to suggest possible guidelines that orientate metabolic paediatricians to carry out a liver biopsy.

The following aspects of the study are emphasised:

1-The majority of the patients (65%) did not present with hepatic disease; moreover, cardinal symptoms were mainly neurological. Therefore, initial nervous system involvement seems to be the rule whatever the tissue specificity of the disease.

2- Concerning Onset age/Enzymatic deficiency:

- Early neonatal onset was the most frequent (67,7 %), as described in several reports, but only 9 of these 21 newborns (42,8%) presented initially with signs of hepatic disease.

- Enzymatic deficiencies did not predict the early or late development of hepatic disease due to the fact that complex deficiencies in our series are similar regardless whether the newborns first exhibited hepatic dysfunction . Mitochondrial DNA depletion, CIV , CI, CIII and generalized deficiencies have

been described to be responsible of neonatal hepatic disease. Our newborns shared these same deficiencies with a marked dominance of CI and only 1 case of mitochondrial DNA depletion. The very low incidence of mitochondrial DNA depletion could partly explain the fact that initial hepatic symptoms are not predominant in our group of patients.

CIV was more prevalent in older children although it has been related to fatal congenital lactic acidosis and hepatopathy in early chilhood .

4- With respect to the outcome:

- The appearance of hepatic disease over time was detected in 22 (70,9%) patients. Thus, hepatic involvement is more likely to be found in the follow-up than in the clinical presentation.
- Neurological and liver involvement was the most common outcome form (16/31: 52%) whatever the initial symptoms. The majority of them had CI deficiency. Only 4 of these patients can be considered as Alper's syndrome .
- There are two patients with normal muscle biopsies, and an exclusively neurological presentation and outcome. Therefore, the study of this tissue can be useful even in the absence of liver disease.

5- Regarding plasmatic lactate:

- Hyperlactacidemia was the rule. This fact is consonant on one hand with the young age of our patients and on the other with the known relationship between high lactate and hepatic mitochondrial disease .
- Additionally, we could determine a positive statistical relation between severe and constant hyperlactacidemia and fatal outcome.

6- Some basic differences between patients expressing the defect in the liver and those with muscle deficiencies (all of them belonging to our series) were detected:

- The very rare presentation in children older than 1 year.
- The significantly lower survival probabilities in patients with liver deficiencies.
- The greater prevalence of hepatic diseases (as an initial or an evolving manifestation).

7- It is suggested to perform a liver biopsy when:

1- The clinical expression of the disease is hepatic.

2- When there are “neurohepatic” outcomes, even when they do not fit exactly into the diagnosis of Alper’s syndrome.

3- In early onset-age cases: mainly neonatal but also early childhood even in the absence of initial or later liver dysfunction.

4- When there is a suggestive clinical picture with normal respiratory chain activities in muscle, even without hepatic disorder for a very long period of time, especially if the expression is dominated by neurological disease.

Concerning PYRUVATE CARBOXYLASE DEFICIENCY (METABOLIC AND NEUROLOGICAL CHARACTERISTICS OF NINE PATIENTS).

The aim here was to describe the main clinical and biochemical characteristics of nine patients with the French form of pyruvate carboxylase deficiency in order to draw up some guidelines that allow an early treatment with triheptanoid and citrate.

9 patients with the severe neonatal French form of pyruvate carboxylase deficiency diagnosed in our hospital were retrospectively studied. We describe the clinical presentation focusing on some poorly documented aspects. Brain imaging, biochemical characteristics and global outcome were also reported.

All the patients presented with axial hypotonia and tachypnea during the first hours of life. The initial level of consciousness was preserved in most of the cases. Abnormal movements (high amplitude tremor and hypokinesia) and bizarre ocular behaviour were the most common findings (6 and 5 cases respectively) whereas epilepsy was infrequent. Brain MRI mostly disclosed cystic periventricular leukomalacia. Hypoglycemia, severe lactic acidosis and hypercitrullinemia were invariably found. Hepatic involvement was present in 4 patients. Hyperammoniemia, hypernatremia, and high proline and lysine were frequently detected. A rapid fatal outcome was observed in the great majority of them.

To conclude, clinical and biochemical characteristics of this deficiency seem to be highly suggestive. Thus, an appropriate therapy should be rapidly considered. The frequency and intensity of abnormal movements which may orientate to PC deficiency when associated with severe lactic acidosis are underlined.

Concerning IMPAIRED MITOCHONDRIAL PYRUVATE IMPORTATION

The main characteristics of this rare condition as well as the diagnostic procedures are here exposed:

- The clinical, biochemichal and brain neuroimage traits may be identical to those seen in a neonatal onset of PDH deficiency.
- Persistent congenital lactic acidosis with normal L/P ratio but normal PDH activity is the clue to suspect this entity.
- The diagnosis is here confirmed by functional assays in digitonin-permeabilized fibroblasts to measure oxidation rates from radiolabeled pyruvate and malate. The production of [^{14} C]acetylcarnitine or [^{14} C]citric cycle intermediates derived from [$^{2\text{-}14}$ C]pyruvate as well as the release of ^{14} CO2 from [$^{1\text{-}14}$ C]pyruvate was severely impaired, whereas decarboxylation of [$^{U\text{-}14}$ ^{C}]malate was normal. With increasing concentrations of [1-14 C]pyruvate, the patient's fibroblasts behave like control fibroblasts incubated in the presence of alpha-cyano-4-hydroxycinamate, an specific inhibitor of mitochondrial pyruvate uptake: a progressive increase in ^{14} CO2 production was observed, likely due to passive diffusion of [$^{1\text{-}14}$ C]pyruvate through the mitochondrial membranes. These results are consistent with a defect of mitochondrial pyruvate transport in the patient. An affected fetus was recognized in a subsequent dichorionic twin pregnancy using the coupled assay measuring [2-14 C]pyruvate oxidation rates of digitonin-permeabilized trophoblasts.
- This work provides an useful new method to detect and perform prenatal studies of this rare and probably underdiagnosed disease.

Concerning "A DELETION IN THE HUMAN QP-C GENE CAUSES A COMPLEX III DEFICIENCY RESULTING IN HYPOGLYCAEMIA AND LACTIC ACIDOSIS"

Two relevant points should be highlighted in this study:

- A new presentation of mitochondrial diseases is here exposed. This is the presence of recurrent long fasting hypoglycaemias associated with hyperlactacidemia, mimicking a neoglucogenesis defect. The coincident low ketogenesis was determining to rule out a fatty acid oxidation deficiency also.
- The final diagnosis was a complex III hepatic deficit
- Mitochondrial respiratory chain complex III (ubiquinol-cytochrome *c* reductase) consists of 11 subunits,only one (cytochrome *b*) being encoded by the mitochondrial DNA. Disorders of complex III are comparatively rare but are nevertheless present as a clinically heterogeneous group of diseases.
- Before the results of this work, no mutation in any of the nuclear encoded subunits had been described . A deletion in the nuclear gene UQCRB encoding the human ubiquinone-binding protein of complex III (QPC subunit or subunit VII) in a consanguineous family with an isolated complex III defect is here reported. In the proband, a homozygous 4-bp deletion was identified at nucleotides 338–341 of the cDNA predicting both a change in the last seven amino acids and an addition of a stretch of 14 amino acids at the C-terminal end of the protein. Both parents were found to be heterozy-gous for the deletion, which was absent from 55 controls. Low temperature (–196°C) spectral studies performed on isolated mitochondria from cultured skin fibroblast of the proband showed a decreased cytochrome *b* content sug-gestive of a role for the QP-C subunit in the assembly or maintenance of complex III structure.

GENERAL CONCLUSIONS

Mitochondrial disorders form a group of heterogeneous diseases that can be studied from different points of view.

Clinical presentation is frequently common to other pediatric diseases, for that reason the clinician needs useful clues to reach the correct diagnosis. Other than the well known multiorganic involvement associated with severe hyperlactacidemia in young children, there are some guideline signs that may indicate a particular defect. Movement disorders in pyruvate carboxylase deficiency or recurrent isolated hypoglycaemias in complex III deficiency are examples of these rare but very orientative signs.

Biochemical tests may be of great help in deficiencies that do not concern the respiratory chain. Consequently, some “redox cycle”, amino acids or organic acids profiles are more likely to correspond to defects in pyruvate or Krebs cycle metabolism. In particular, HL with normal L/P ratio and normal pyruvate dehydrogenase activity would point towards impaired mitochondrial pyruvate carrier.

Other diagnostic dilemma in MRC is the tissue of choice. It is unusual to perform a liver biopsy if the results of the activities in muscle have been normal but there is no apparent hepatic disease. The results of this study show that the analyse of the hepatic tissue could be very helpful, especially in patients with predominant neurological disease.

Follow-up studies in inborn errors of metabolism are very infrequent. However, they are crutial to have a global and accurate idea about the future of these children as well as to establish possible prognostic factors. In this respect, newborns with respiratory chain deficiencies have a very high mortality and a poor quality of life, although some “reversible” and “benign” forms could be found.

Little data about nuclear genes is here reported. Genetic characteristics or trying to establish phenotype-genotype correlations, are not among the aims of this study. Nevertheless a new nuclear gene is also reported.

In summary, the study of mitochondrial disorders is a big challenge. The great complexity in their clinical, biochemical and genetical traits is understandeable if we take into account that mitochondria was a single and autonomous cell one billion years ago. Therefore studying “mitochondrial diseases” can be as complicated as studying “cellular diseases”.

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