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Master's Thesis in Medicine No 352

The Unsolved Pathogenesis of Idiopathic Ketotic Hypoglycemia:

Involvement of the Pyruvate Dehydrogenase Kinase Isoenzyme 4 Gene?

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ABBREVIATIONS

A: adenine
ACC: acetyl-CoA carboxylase
AMP: adenosine monophosphate
AMPK : adenosine monophosphate activated protein kinase
ALAT: alanine aminotransferase
ASAT: aspartate aminotransferase
BCAA : branched-chain amino acid
C: cytosine
CBF: CCAAT box binding factor
cDNA: complementary DNA
Co: control
CPT1 : carnitine palmitoyltransferase 1
ddNTP: dideoxynucleoside triphosphate
dNTP: deoxynucleotids triphosphate
EDTA : ethylene-diamine-tetra-acetic acid
EER: estrogen related receptor
FA : fatty acid
FFA : free fatty acid
Fox01: forkhead transcription factor
G: guanine
GH: growth hormone
GR: glucocorticoid receptor
GRE: glucocorticoid response element
GRU: glucocorticoid response unit
IGF-1: insuline-like growth factor 1
IGFBP-3: insuline-like growth-factor binding protein 3
IKH : idiopathic ketotic hypoglycemia
kbp: kilo base pair
MAPK: mitogen-activated protein kinase
mRNA: messenger RNA
PCR: polymerase chain reaction
PDC : pyruvate dehydrogenase complex
PDK : pyruvate dehydrogenase kinase
PDK2: pyruvate dehydrogenase kinase isoenzyme 2
PDK4 : pyruvate dehydrogenase kinase isoenzyme 4
PDP: pyruvate dehydrogenase phosphatase
PGC-1α: peroxisome proliferator activated receptor γ coactivator
PPAR: peroxisomal proliferator-activated receptor
PRL: prolactin
RA: retinoic acids
RARα: retinoic acid receptor α
RARE: retinoic acid response element
Rb: retinoblastoma
RT: reverse transcriptase
RXRα: retinoid X receptor α
T: thymidine
T_3 : thyroid hormone
TBE: tris-borate
TCA : tricarboxylic acid
TR: thyroid hormone receptor
TRE: T_3-response element
TSA : trichostatin A

1. INTRODUCTION

1.1. Idiopathic Ketotic Hypoglycemia

Idiopathic ketotic hypoglycemia (IKH) is the most common cause of hypoglycemia in childhood, among non-diabetic children (1). IKH is defined by episodes of hypoglycemia with high ketone levels in plasma and urine, provoked by periods of fasting, often in combination with an intercurrent illness (2). IKH usually begins between 18 months and 5 years of age (3) and typically spontaneously remits by the age of 8 or 9 (2). Patients with IKH are predominantly male. They are often less than 50th percentile for height and weight (3).

The most common symptom leading to evaluation in an emergency department is lethargy, expressed as sleepiness, fatigue, decreased energy, or inability to awake normally from sleep. Vomiting is also a frequent symptom. Some patients even present with a first seizure (1).
Symptoms of hypoglycemia can be divided in two categories: neurovegetative and neuroglycopenic symptoms (4).
The autonomic response causes the early symptoms of hypoglycemia, which include sweating, weakness, tachycardia, pallor, nausea, vomiting, tremor, nervosity and hunger. These symptoms usually occur at higher blood glucose concentrations than the symptoms of neuroglycopenia (4).
The neuroglycopenic symptoms result from brain glucose deprivation. They are more severe than autonomic symptoms and usually start at lower blood glucose levels, when hypoglycemia occurs rapidly and/or is prolonged in time. These symptoms include lethargy, irritability, confusion, uncharacteristic behavior, hypothermia, hypotonia, headache, visual troubles, dysarthria, aphasia, hemiplegia, paresthesia, amnesia, dizziness, seizure and coma (4).
However, neuroyglycopenic symptoms can occur without a warning autonomic response after repeated or prolonged episodes of hypoglycemia, when the threshold of neurovegetative symptoms is decreased to that for neuroglycopenic signs. Permanent central nervous system damage and even death can occur due to severe and repeated episodes of hypoglycemia (4).
The symptoms usually appear in the morning, after a fasting period of 10-20h (1,3). The diagnosis of IKH can be difficult because the symptoms and clinical signs are unspecific (3).
Hypoglycemia due to IKH improves after oral or intravenous glucose administration (5). Dietary interventions to prevent new episodes include avoiding fasting, frequent snacks and meals rich in complex carbohydrates (3).

The pathophysiology of IKH has not been fully understood. Investigations have searched for disturbances in gluconeogenesis, glycolysis, glycogenolysis, regulation of insulin response to fasting states and brain glucose utilization (1).
Children with IKH only present symptoms after a relatively long fasting period. This suggests that their glycogenolysis pathway is intact. Moreover, these patients have a normal response to glucagon in the fed state and patients with IKH do not have inappropriately increased insulin levels in the starved state (1), thus excluding hyperinsulinism as a pathogenic mechanism.
Endogenous glucose production is decreased in IKH: glycogenolysis decreases physiologically without the expected compensatory increase in gluconeogenesis, despite all hormonal stimuli to gluconeogenesis are activated. However the gluconeogenic pathway has been proven to be intact. Thus, an adequate glycemic response following administration of gluconeogenic precursors can be observed. It has been shown that IKH patients have lower alanine levels, which implicates a substrate deficiency for gluconeogenesis as a factor in the pathophysiology of IKH. Alanine

production may be limited in IKH (2). Alanine sources may be limited in certain children, perhaps because of a lean body mass. This could explain why the disorder remits with age and growth (1,2).
Young children have a higher brain mass to body mass ratio and cerebral glucose utilization is the major contributor to peripheral glucose uptake. Therefore, there is a relatively high glucose demand and hepatic glycogen is more rapidly depleted during fasting, increasing the dependency in gluconeogenesis. This could explain the spontaneous remission of IKH with increasing age (2).
Fatty acid (FA) mobilization has an important role in the maintenance of glucose homeostasis. Free fatty acids (FFAs) and ketone bodies can be used by body tissues and reduce their demands for glucose. However, the brain cannot use FFAs directly because they do not cross the blood-brain barrier. It can only use ketone bodies to partially replace glucose. Ketone bodies are derived from oxidation of leucine among other amino acids or fatty acids (4). In a study, data have shown a lower leucine oxidation in IKH children, while energy expenditure is significantly increased. This may be of pathophysiological relevance. Impaired ketone body utilization seems unlikely because neither extreme supra-physiological levels of plasma ketone bodies nor an increased peripheral glucose uptake have been found (5).
Some authors even postulate that IKH is not a true disease but represents the lower tail of the Gaussian distribution of fasting tolerance in children (1,2). However, familial recurrence of IKH is often observed, suggesting a genetic basis of glucose homeostasis dysregulation.

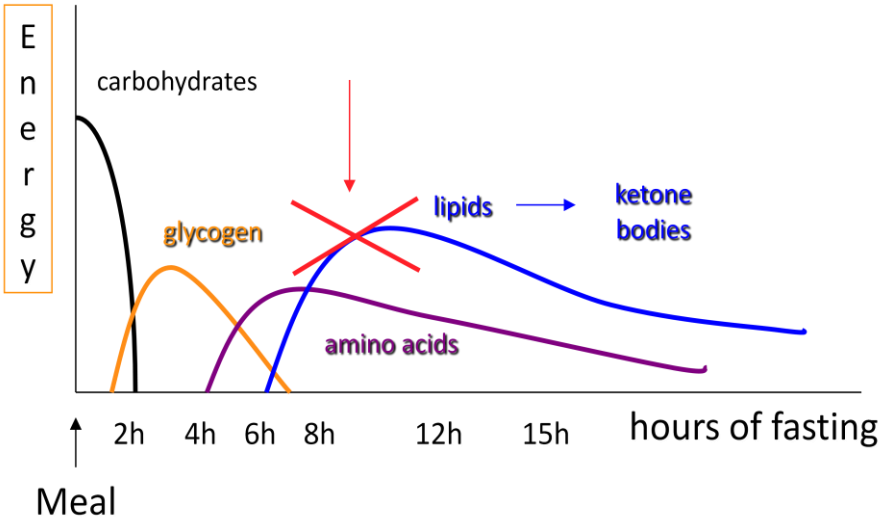

Figure 1: Substrates utilization during fasting

The hypothesis of this Master project is that variations in genes involved in the glucose homeostasis could be the cause of IKH. In this study, we have a particular interest for the pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) gene (ENST00000005178), located on chromosome 7q21.3 (6).

1.2. Pyruvate Dehydrogenase Kinase 4

The mitochondrial pyruvate dehydrogenase complex (PDC) catalyses the conversion of pyruvate to acetyl-CoA, linking the glycolytic degradation of glucose and the degradation of glycogen to the tricarboxylic acid (TCA) cycle. The result of this reaction is the loss of glucose carbon as CO_2 and the generation of NADH (7–9).
The entry of acetyl-CoA into the TCA cycle allows ATP generation from glucose. The combination of acetyl-CoA with oxaloacetate produces citrate, which enters the TCA cycle. Citrate exits the

mitochondrion and is then cleaved to yield cytoplasmic acetyl-CoA, which is converted in malonyl-CoA. Malonyl-CoA serves as precursor for FA synthesis. Malonyl-CoA is also an inhibitor of carnitine palmitoyltransferase 1 (CPT1), required for the import of acyl-CoAs derived from FA into mitochondria. Therefore, increased rates of glucose utilization block FA oxidation (7,9) and ketone bodies production.
The conversion of pyruvate into acetyl-CoA is irreversible, therefore inhibition of PDC is crucial to conserve glucose when glucose supply is insufficient (7–9).

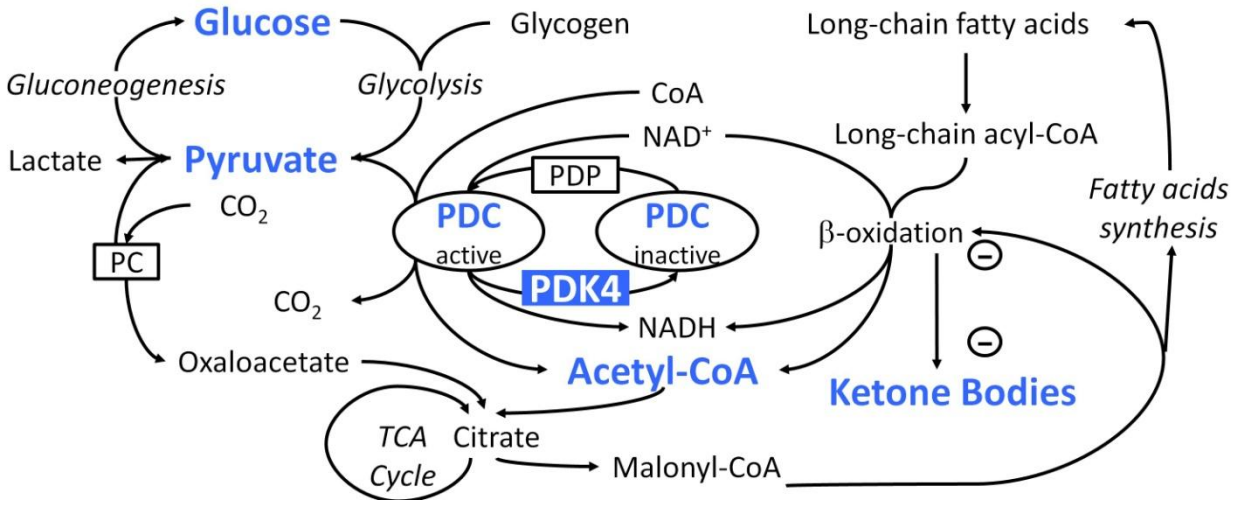

Figure 2: PDK4, PDC and interlinking metabolic pathways

The regulation of PDC includes three mechanisms: 1) the reversible phoshorylation or dephosphorylation of PDC, 2) levels of acetyl-CoA and NADH and 3) the transcriptional regulation of the regulatory enzymes. (7,9)
Short-term regulation of PDC involves the pyruvate dehydrogenase kinases (PDKs), which phosphorylate and inactivate the PDC, and the pyruvate dehydrogenase phosphatases (PDPs), which dephosphorylate and activate the PDC. During starvation, the PDC is inactivated to maintain glucose levels by conserving substrate for gluconeogenesis (7–10).

The PDC is composed of multiple copies of three subunits: E1 - pyruvate dehydrogenase, E2 – dihydrolipoamide acetyltransferase and E3 – dihydrolipoamide dehydrogenase. E1 catalyses the conversion of pyruvate into acetyl-CoA and is regulated by PDKs and PDPs. Each PDC contains two or three PDK molecules bound to E2. The phosphorylation can occur subsequently at three different sites on E1: serine 293 – site1, serine 300 – site2 and serine 232 – site 3. Phosphorylation of one site is sufficient to inactivate the PDC. Site 1 phosphorylation is the most rapid and site 3 phosphorylation is the slowest (7,9).

PDK4 as candidate gene for IKH

The present knowledge about the regulation of PDC, the central enzyme in energy metabolism homeostasis, in human and animal models, points to PDK4 as an important player in glucose homeostasis during fasting. Here in summary the main reasons for which we have we have chosen PDK4 as candidate gene for IKH:

Tissue expression of PDK4 indicates that this isoform is active in the main tissues involved in glucose homeostasis.

Four PDK isoforms have been identified: PDK1, PDK2, PDK3 and PDK4. PDK1 can be found in heart, pancreatic islets and possibly skeletal muscle. PDK2 is expressed ubiquitously, with high expression in the heart, liver and kidneys. PDK3 is detected in the testis, kidneys and brain. PDK4 is found in the heart, skeletal muscle, the liver, kidneys and the pancreatic islets. All tissues in which PDK4 is expressed are involved in glucose homeostasis: high rates of glucose utilization, glucose production or production of glucoregulatory hormones (7,9).

PDK4 phosphorylates all three phosphorylation sites of PDC, whereas other PDK isoformes act only on two of them.

All four PDKs phophorylate site 1 and 2, but only PDK1 phosphorylates site 3. Moreover, PDK4 shows a higher activity towards site 2 compared with PDK1, PDK2 and PDK3 (9).

Thus, PDK2 may account for short-term inhibition of PDC through phosphorylation of site 1, whereas PDK1 and PDK4 may be more important for allowing multisite phosphorylation, which has been postulated to retard reactivation of PDC by PDP (7).

Low intracellular glucose availability promotes PDK4 expression.

Acetyl-CoA and NADH directly inhibit the PDC and indirectly promote inactivation of PDC by stimulation of PDK activity. Pyruvate is an inhibitor of PDK activity.

These short-term mechanisms are supplemented by a long-term regulation consisting in a stable increase of PDK activity due to an increased expression of PDK genes.

Starvation and diabetes increase the amount of PDK isoenzyme 4 in rat heart. The amount of PDK4 is important in long-term regulation of the pyruvate dehydrogenase complex in rat heart. It has been shown that the increase in the amount of rat heart PDK4 protein correlates with the stable increase in PDK activity that occurs in the heart of starved and diabetic rats. Furthermore, starvation and diabetes induce a PDK isoenzyme shift in rat heart: PDK4 increased from less than one-third of the total PDK protein to more than 50% in the starved state and to 75% in the diabetic state. Therefore, it seems that PDK4 protein may contribute more than any other PDK to the increase of PDK activity. This suggests that an increase in the amount of PDK activity caused by an increase in the amount of PDK4 protein leads to greater phosphorylation and lower activity of the PDC. This suppresses glucose oxidation and favors FA oxidation in the heart of starved and diabetic rats in order to spare glucose, which is advantageous in the starved state but exacerbates the diabetic state because glucose is spared instead of being oxidized to face high levels of glucose in blood (11).

PDK4 knockout mice have fasting relative hypoglycemia because of inappropriately high flow of glucose through PDC.

Studies have shown that blood glucose levels are lower in $PDK4^{-/-}$ mice than in wild type after starvation. This suggests that PDK4 is important to maintain a normal glycemia during fasting.

In the liver of starved $PDK4^{-/-}$ mice, the concentration of intermediates of the gluconeogenic pathway are lower, which is consistent with a lower rate of gluconeogenesis due to lack of substrates. The induction of a lower concentration of precursors is a consequence of higher PDC activity in $PDK4^{-/-}$ mice. In mice lacking in PDK4, concentrations of lactate, pyruvate and alanine are lower because oxidation of pyruvate is stimulated. The decreased pyruvate availability inhibits transamination of branched-chain amino acids (BCAAs), which are a source of amino groups for alanine formation. This results in an elevation in BCAAs and a reduction in alanine.

Starvation induces a greater increase in non-esterified FA and ketone bodies in PDK4$^{-/-}$ mice. But it is not known whether this is due to a greater hepatic ketogenesis or less ketone body utilization by peripheral tissues (10).

Low energy status induces PDK4 expression either through AMP sensing or through free fatty acid and PPAR-α signaling.

Adenosine monophosphate activated protein kinase (AMPK) and FA synergistically induce PDK4 expression and decrease cellular glucose oxidation at the same time. Adenosine monophosphate (AMP), which is a signal for low energy status, activates the AMPK. Activated AMPK phosphorylates acetyl-CoA carboxylase (ACC), which converts acetyl-CoA in malonyl-CoA. Malonyl-CoA is an inhibitor of CPT1, required for the import of acyl-CoAs into mitochondria. AMPK activation relieves repression of CPT1 by malonyl-CoA by phosphorylation and inactivation of ACC and so increases energy production by FA oxidation. AMPK and FA play a role in the energy homeostasis by sparing glucose (12).

FFAs can activate the peroxisomal proliferator-activated receptor (PPAR)-α and activation of PPAR-α can enhance PDK4 expression. Starvation can increase PDK4 expression in tissues of wild-type mice but not in PPAR-$\alpha^{-/-}$ mice. This indicates the importance of PPAR-α for PDK4 expression during starvation (13).

Hormonal control of PDK4 expression: PDK4 is up-regulated by contro-insular hormones and thyroid hormones.

Exercise, fasting and epinephrine increase PDK4 messenger RNA (mRNA) expression in epididymal adipose tissue. In contrast to PDK4, PDK1, 2 and 3 are not responsive to these perturbations. The phosphorylation of the PDC on serine 293 and 300 is also increased, which suggests an increase in PDK4 activity, since PDK4 phosphorylates these residues. Treatment with epinephrine increases PDK4 mRNA levels. Studies suggest that p38 mitogen-activated protein kinase (MAPK) and PPAR-γ may be involved in this pathway (14).

Prolactin (PRL) and growth hormone (GH) can induce the expression of PDK4 in 3T3-L1 adipocytes. This effect is mediated by a STAT5 binding site in a hormone sensitive region in the murine PDK4 promoter. PRL and GH potently activate STAT5 proteins. Moreover, insulin pretreatment attenuates the ability of these hormones to induce PDK4 expression (15).

Transcription of the PDK4 gene is elevated by glucocorticoids and inhibited by insulin. This is mediated by the glucocorticoid receptor (GR). The GR binds to glucocorticoid response elements (GREs) within the gene and interacts with other transcription factors and coactivators to form glucocorticoid response units (GRUs), which control the gene expression. Studies have identified two distal GREs in the PDK4 promoter that are located more than 6000 base pairs 5' to the start site of transcription. Subtle changes in the GRE alter transcriptional activation. Studies have also shown that forkhead transcription factor (Fox01) participates in the glucocorticoid induction of PDK4. Fox01 binding sites have been identified in the PDK4 promoters of mouse, human and rat.
Insulin acts at multiple sites in the promoter to decrease PDK4 transcription. The GR and Fox01 are dissociated from the PDK4 promoter by insulin. Moreover, mutation of the 3' estrogen related receptor (ERR) site reduces the ability of insulin to inhibit PDK4 transcription. This suggests that EER contributes to the insulin inhibition of PDK4 (16).

ERRα and ERRγ are orphan nuclear receptors which have a role in the induction of fatty acid oxidation in heart and muscle. Both ERRs stimulate the PDK4 gene in hepatoma cells and recruit peroxisome proliferator activated receptor γ coactivator (PGC-1α) to the PDKs promoter. FoxO1 binds to the promoter of PDK4 and contributes to its induction by ERRs and PGC-1α. Insulin suppresses PDK4 expression through the dissociation of FoxO1 and PGC-1α from the PDK4 promoter (17).

Recent studies demonstrate that thyroid hormone (T_3) stimulates PDK4 gene expression in vitro in hepatoma cells and in vivo in hyperthyroid rats, via the thyroid hormone receptor (TR). TRs are nuclear hormone receptors, which are a class of ligand-activated transcriptional regulators. TRs bind to specific DNA sequences called T_3-response elements (TRE). Two binding sites for thyroid hormone receptor have been identified in the promoter of the rat PDK4. Mutations of TRE reduce the ability of T_3 to induce the PDK4 gene. It has also been shown that the PGC-1α plays an important role in the T_3 stimulation of PDK4 expression. After T3 administration, there is an increase in the association of PGC-1α with the proximal rat PDK4 promoter, through interactions with the orphan nuclear receptor- ERRα. This results from an increase in the abudance of the PGC-1α by T_3(18).

Other transcriptional regulators of PDK4.

Retinoic acids (RA) and Trichostatin A (TSA), an inhibitor of histone deacetylase regulate PDK4 gene expression. Retinoid X receptor α (RXRα) and retinoic acid receptor α (RARα) bind to and activate two retinoic acid response elements (RAREs). The two RAREs are present in the human PDK4 proximal promoter. Sp1, a transcription factor, and CCAAT box binding factor (CBF) bind to the region between two RAREs. Mutation of Sp1 or the CBF site decreases basal expression, transactivation by RXRa/RARa/RA, and the ability of TSA to stimulate PDK4 gene transcription (19).

E2F1 is a transcription factor. It controls the cell cycle by promoting cellular growth during S phase. E2F1 is a component of the retinoblastoma (Rb)-E2F tumor suppressor complex. The Rb tumor suppressor binds E2F1 and represses its activity. A study demonstrates that E2F1 regulates the gene encoding PDK4, which is chronically elevated in obesity and diabetes and induced by starvation. It has shown that loss of E2F1 in mice results in lower blood glucose, improved plasma lipid profile and increased sensitivity to insulin stimulation. Inactivation of Rb induces PDK4 and increases the E2F1 occupancy onto the PDK4 promoter. Two overlapping E2F binding sites have been identified on this promoter. When E2F sites are mutated, the PDK4 promoter does not response to E2F1 (20).

Conclusion

All the above mentioned studies show that PDK4 has an important role in glucose homeostasis during starvation and point to this enzyme as a key regulator of fasting tolerance. From the present knowledge on PDK4, we hypothesized that genetic variations reducing PDK4 activity, could be disease-causing in some IKH patients.

2. METHODS

2.1. Review of the medical records of IKH patients

Clinical features

The medical records of ten IKH patients were reviewed. Patients' characteristics taken into account were:

- sex
- age of onset
- weight and height
- diet, nutritional habits
- triggers of hypoglycemic crises
- symptoms
- presence/absence of hepatomegaly
- psychomotor development

Biochemical features

The biochemical features chosen are those that are important for the differential diagnosis of hypoglycemia. They allow the identification of the cause of hypoglycemia.

We characterized the episodes of IKH of each patient according to the following biochemical criteria:

1. plasma glucose level
2. plasma lactate, pyruvate, lactate/pyruvate ratio
3. plasma keton bodies and urine strip tests
4. serum free fatty acid
5. plasma amino acid concentrations (alanine level, branched-chain amino acid levels)
6. plasma free carnitine and acylcarnitine levels, acylcarnitine profile
7. plasma transaminase levels
8. cortisol, insulin, IGF1, IGFBP-3
9. urinary organic acids

Ad 1) Blood glucose concentrations were determined to define the degree of hypoglycemia.

Ad 2) Pyruvate is the end product of the glycolysis. It can be converted to lactate, the end product of anaerobic glycolysis. Elevation of lactate level can indicate liver damage, impaired glycogenolysis or impaired gluconeogenesis. It is also elevated after a seizure or difficult blood sampling. Pyruvate is never measured without lactate. It is used to determine the lactate/pyruvate ratio, which is elevated in respiratory chain disorders (24).

Ad 3) Ketosis is a physiological response to fasting (24). The ketone bodies include β-hydroxybutyrate, acetoacetate and acetone, but only β-hydroxybutyrate and acetoacetate are transported in blood. FFAs are the precursors for ketogenesis in the liver, when glucose supply is lacking to form ATP (25). Urine test strips are used to detect and measure the level of ketone bodies in urine among other features of urine. Hypoglycemia associated with ketonuria suggests others diagnosis than hypoglycemia without ketonuria (24).

Ad 4) Elevation of FFAs suggests active lipolysis and that hypoglycemia is associated with fasting (24).

Ad 5) Amino acids analyses in blood samples are helpful when disorders of energy metabolism or aminoacidopathy are suspected. Prolonged fasting induces increase of BCAA level leucine, isoleucine and valine (24).

Ad 6) Carnitine allows the transport of long chain FAs in the mitochondrial matrix, where β-oxidation occurs. The long chain acyl-CoA is transformed in acylcarnitine and can go through the mitochondrial membranes (25). Free and total carnitine in plasma and acylcarnitine profile analysis are diagnostic tests for most fatty acids oxidation disorders, carnitine deficiency, carnitine transporter defect and various organic acidurias that could cause hypoglycemia. Generally, total carnitine values are normal, free carnitine values are decreased and acylbound carnitine values are increased after fasting (24).

Ad 7) Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) tests were performed to detect a liver disease that may affect glucose homeostasis, FAs oxidation or ketogenesis.

Ad 8) Cortisol, insulin, insuline-like growth factor 1 (IGF-1) and insuline-like growth-factor binding protein 3 (IGFBP-3) values were also assessed. Cortisol is a glucocorticoid hormone stimulating neoglucogenesis to reform glycogen so that the blood concentration of glucose can be maintained normal between meals by glycogen degradation. Insulin is a hormone promoting the storage of glucides, lipids and proteins. Their blood concentrations are then decreased (26). IGF-1 is a hormone whose structure is similar to insulin. It has its own receptor but it can also bind to insulin receptor. Actions of IGF-1 are then insulin-like. IGF-1 binds to IGFB-3. IGFB-3 increases half-life of IGF-1 and limits its availability. Fasting lowers IGF-1 level (27).

Ad 9) Organic acids are analyzed in urine (24). They are derived from dietary protein, fat and carbohydrate and are used by the body to generate cellular energy. They are intermediary compounds of many biochemical pathways (28). Urinary organic acids test can detect inherited disorders of metabolism such as organic aciduria, aminoacidopathy, fatty acid oxidation defect and disorder of energy metabolism among others. A specific profile of urinary organic acids can suggest a particular metabolic disease (24).

2.2. Genetic analyses in IKH patients

To study the PDK4 gene, we collected DNA samples from ten patients with previous episodes of IKH. Written informed consent was obtained from all participating individuals. The study has been approved by the ethics committee of the CHUV.

The PDK4 gene is composed of 11 exons (6). The gene was divided into 11 fragments according to the size of the exons. Each fragment contains one exon with the following exeptions: Fragment 4+5 contains exons 4 and 5, fragment 8+9 contains exons 8 and 9 and the huge exon 11 is divided in three fragments (11-1, 11-2, and 11-3).

PCR-Fragment	1	2	3	4+5	6	7	8+9	10	11-1	11-2	11-3

Size (in bp)	1020	734	667	695	391	599	940	300	998	844	996

The cDNA of PDK4 has a length of 1’236 bp. For RT-PCR the cDNA was devided into 3 fragments overlapping each other and allowing the sequencing of the whole cDNA in both directions.

RT-PCR Fragment	1	2	3
Size (in bp)	900	960	900

The following steps, explained below in detail, were applied for each patient and each fragment:

- DNA extraction from EDTA blood
- Primer design and preparation
- Polymerase Chain Reaction (PCR)
- Agarose gel electrophoresis
- Purification of PCR product
- Sequencing reaction (SR)
- Purification of SR product
- Sequencing on an ABI PRISM® 3100 Genetic Analyzer
- Lecture and interpretation of sequencing results

For patients the following steps were added:

- RNA extraction from fibroblasts
- Primer design and preparation
- Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)
- Agarose gel electrophoresis
- purification of RT-PCR product
- Sequencing reaction (SR)
- Purification of SR product
- Sequencing on an ABI PRISM® 3100 Genetic Analyzer
- Lecture and interpretation of sequencing results

2.2.1. DNA and RNA extraction

DNA was extracted from leucocytes isolated from blood samples. Blood samples from the 10 IKH patients were taken in tubes with Ethylene-diamine-tetra-acetic acid (EDTA). EDTA prevents the blood from coagulation. RNA was extracted from fibroblasts of patients 1, 2, 3 and 6. Fibroblasts were obtained by skin biopsy and tissue culture. DNA and RNA were extracted with the AllPrep DNA/RNA Mini Kit from Qiagen.

DNA extraction procedure:

- The cells were lysed and homogenized in Buffer RLT. The Buffer RLT inactivates DNases and RNases so that intact DNA can be isolated.

- The lysate was transferred to an AllPrep DNA spin column placed in a collection tube. The lysate was passed through the AllPrep DNA spin column by centrifugation. The column and the high-salt buffer allow selective binding of DNA.
- The column was then placed in a new collection tube and centrifugated. The spin column membrane was washed with Buffer AW1 and AW2.
- The column was placed in a new collection tube and centrifugated. DNA was eluted with Buffer EB.

RNA extraction procedure:

- The cells were lysed and homogenized in Buffer RLT. The Buffer RLT inactivates DNases and RNases so that intact RNA can be isolated.
- The lysate was transferred to an AllPrep DNA spin column placed in a collection tube. The lysate was then passed through the AllPrep DNA spin column by centrifugation. The column and the high-salt buffer allow selective binding of DNA.
- Ethanol was added to the flow-through collected in the collection tube after the centrifugation.
- The sample was transferred to an RNeasy spin column placed in a collection tube. The sample was passed through the RNeasy spin column by centrifugation. RNA binds selectively to the membrane of the column.
- The column was placed in a new collection tube and centrifugated. The spin column membrane was washed with Buffer RPE.
- The column was finally placed in a new collection tube and centrifugated. RNA was eluted with RNase free water.

2.2.2. Primer design, order and preparation

Primers are oligonucleotides that are complementary to sequences that flank the DNA segment of interest. The primer pairs are composed of a forward primer and a reverse primer. The forward primer is complementary to the 3' end of the anti-sense strand of the DNA target, whereas the reverse primer is complementary to the 3' end of the sense strand (21).

The PCR primers were designed with the free software Primer3 (http://frodo.wi.mit.edu/primer3/). Primers were ordered from Microsynth, Switzerland.

The lyophilized primers delivered by Microsynth were hydrated with H_2O MilliQ in order to obtain a stock solution with a concentration of 1 μg/μl. The stock solutions are conserved at a temperature of -20°C. To prepare 100 μl of working solution at a final concentration of 50 ng/μl, 5 μl of the stock solution were added to 95 μl of H_2O MilliQ. The primers sequences used for the PCR or RT-PCR are listed in the following tables.

Primers for the PCR

Fragment	Primer name F=forward	Primer Sequence

	R=reverse	
1	1F	GTT CTT CCC ACC CTT TTT CC
	1R	GAG CCT AGC CCT CCC TCT AC
2	2F	AAT CGC CCC TGA ATT ATC G
	2R	CAT TCA GAT CAC CTG TTT TCC A
3	3F	TGA ACA TAA TGC TTA CTT TCT CTT TTC
	3R	TTC AGA GCT GAA TTC CTT TAA TTT
4+5	4+5F	TGT TCA GAT GGG TAA TGC AAA
	4+5R	TCA AAA GCA AAG GAC CTT GAA
6	6F	TTG CTA AAA CTG CAA TGC AAA
	6R	TGT GTA CAA ACT TTA GGC AAG AGA A
7	7F	TGC CAA CCC TCT TGA AGT CT
	7R	GCT TCT GTT TTG TGG CGA AT
8+9	8+9F	GAA CTT TGA ATT ACC CTC TGT GC
	8+9R	TCA GAC ATC CCT TGT TTC ACC
10	10F	AGG ACA TTA GTA TCA GTG AGG ACA G
	10R	TTG GGG AAT TCA CTC CAG AC
11-1	11-1F	TGA ATG TTT GGA TAC TTA CCT CTG A
	11-1R	TCA CTC AAG ACT AGG CAC CTG A
11-2	11-2F	GCA TTG TAT TCA AAG TTG CAG TG
	11-2R	TGC TTT CAA AAC ACC AGT TCT T
11-3	11-3F	CAG ACC AGT TGC GCT GAT TA
	11-3R	TTG TAG GCA AAC ACA CCA TTG

Primers for the RT-PCR

Fragment	Primer name F=forward R=reverse	Primer Sequences
1	1F	GACTCTGCCCAGACTCTTCACT
	1R	GGTTCATGTAAAATCGATCCAA
2	2F	CCAGATGACCAGAAAGCATTATC
	2R	AATAATTCTCAGGGGAACACCA
3	3F	CAGTTGAACACCAGGAAAATCA
	3R	CACTGGTGTAGACCCACTTTGA

2.2.3. Polymerase Chain Reaction

The PCR is a process used to amplify a segment of DNA. It allows the generation of large amounts of DNA for sequencing and the analysis of genetic variations. It consists in a series of synthetic reactions. A synthetic reaction can be divided in three steps: denaturation, annealing and DNA chain extension. The reaction mixture is composed of forward and reverse primers, the four deoxynucleotids triphosphates (dNTPs) in excess, a buffer system and the Thermophilus Acquaticus (Taq) DNA polymerase.

In the first step, the template DNA is denatured by heating. The double-stranded DNA becomes single-stranded. The reaction mix is then cooled. The primers can so anneal to their complementary sequences. The forward primer anneals to the 3' terminus of the anti-sense strand and the reverse primer anneals to 3' terminus of the sense strand. After having annealed to their

target sequences, the primers are extended with the Taq DNA polymerase. As the Taq DNA polymerase is a thermostable enzyme, it can survive the heat denaturation step. Moreover, annealing and extension can then be carried out at elevated temperatures. In this way, the result of the amplification reaction is improved.
As the product of each cycle is used as template for the next one, the amount of DNA doubles at each cycle. The DNA chains produced by the first cycles of amplification are heterogeneous in size. However, the segment between the two primers is preferentially amplified and will become dominant (21).

PCR protocol

The PCR for fragment 1 was carried out at a volume of 24 μl with the following components:

- 4.8 μl of DNA at a concentration of 20 ng/μl
- 12 μl of Premix Fail Safe Buffer J (Epicentre Biotechnologies)
- 1.92 μl of forward primer at a concentration of 50 ng/ μl
- 1.92 μl of reverse primer at a concentration of 50 ng/ μl
- 0,39 μl Expand Long Template (Roche)
- 2.97 μl of H_2O

PCRs for fragments 2 to 11-3 were carried out at a volume of 25 μl with the following components:

- 5 μl of DNA at a concentration of 20 ng/μl
- 12,5 μl of Taq PCR Master Mix kit 250U from Qiagen. The Taq PCR Master Mix is a solution containing Taq DNA Polymerase, PCR Buffer, and dNTPs.
- 0,75 μl of forward primer at a concentration of 50 ng/ μl
- 0,75 μl of reverse primer at a concentration of 50 ng/ μl
- 6 μl of H_2O

These components were mixed in reaction tubes. The PCR was performed in a Biometra thermocycler. The PCR programs chosen for our experiments are shown below. The annealing temperature was the same for exons 2 to 11 (54^o) but it is different for exon 1 ($61,2^o$).

PCR program for Fragment 1

Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*	*Step 6*
94^o	94^o	$61,2^o$	72^o	72^o	4^o
10’	30’’	1’	1’ 30’’	7’	∞

The steps 2 to 4 were repeated 35 times.

PCR program for Fragments 2 to 11-3

Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*	*Step 6*
94°	94°	54°	72°	72°	4°
10'	30''	1'	1'	10'	∞

The steps 2 to 4 were repeated 35 times.

2.2.4. Agarose gel electrophoresis

We performed an agarose gel electrophoresis to check the molecular size of the amplified DNA fragments. We wanted to know if the size in base pairs (bp) of the amplified DNA fragments corresponded to the calculated size of the DNA fragments we wanted to amplify, if the PCR product was clean enough (no smear) and if the amount of amplified DNA was sufficient to perform the sequencing reaction.

Q-Biogene 2% agarose gel with GelRed preparation protocol

The agarose gels were prepared by melting 3.2 g of Agarose TM Molecular-Biology Grade from Q-Biogene in the presence of 160 ml of Tris-borate (TBE) 0.5x as buffer. The mix was heated in the microwave until the agarose dissolved and a transparent solution was achieved. When the mix had cooled down, we added 16 µl of GelRed Nucleic Acid Gel Stain 10'000x in DMF 0.5 ml from Biotium. The GelRed is a fluorescent nucleic acid gel stain. The solution was then poured into a mold and allowed to harden. If the agarose gels were not used immediately, they were covered by plastic foil and stocked at 4°C.

Electrophoresis procedure

We mixed 4 µl of PCR-product with 2 µl of loading dye from Qiagen. The loading dye is a solution containing marker dyes. It allows the assessment of the DNA migration and it weighs down the DNA so that it sinks into the bottom of the wells. The gel must be submerged in the buffer. We then loaded the mixture into the slots of the gel using a micropipette. 1.3 µl of GeneRuler® 1000bp DNA ladder from Fermentas was loaded into the first slot. DNA ladder is a solution containing DNA molecules of different sizes. It is used as reference to evaluate the size of DNA fragments. We applied a tension of 135 V for 30 minutes. The DNA fragments migrated and were sorted by size in the electrical field.

2.2.5. PCR purification

The aim of a PCR purification is to desalte the PCR product and to remove excess dNTPs and primers.

PCR purification protocol:

- Add MilliQ H_2O water in each reaction tube with PCR product to obtain a volume of 100 µl.

- Load the 100 µl in the multiscreen $PCR_{\mu 96}$ Filter Plate from Millipore. The multiscreen PCRm96 Filter Plate is composed of a size-exclusion membrane that allows the selective binding of DNA fragments.

- Filter with Millipore Vacuum Mannifold for 5 to 10 minutes or until the wells are dry. The Millipore Vacuum Mannifold is a filtration system using vacuum.

- Blot excess liquid from the bottom of the multiscreen PCRm96 Filter Plate on an absorbent material like Kimberly-Clark® KIMTECH® KimWipes. KimWipes are cleaning tissues.

- Add 25 µl of MilliQ H_2O to each well.

- Put the plate on the plate shaker for 10 to 15 minutes to resuspend the samples.

- Purified PCR products are transfered in new reaction tubes.

2.2.6. Sequencing Reaction

We use the enzymatic method of DNA sequencing, which is the sequencing by chain termination. The principle of the sequencing reaction is the same as for the PCR. It also consists in three main steps: denaturation, annealing and DNA synthesis. The reaction mixture is composed of primers, dNTPs, Taq DNA polymerase and dideoxynucleoside triphosphates (ddNTPs).
ddNTPs are analogues of normal dNTPs. However, they lack a hydroxyl-residue at the 3' position of deoxyribose. DNA polymerases can incorporate them into a DNA chain through their 5' triphosphate groups. But no phosphodiester bond can be formed with the next dNTP because of the lack of 3'-hydroxyl residue. The DNA chain cannot be extended. Competition between dNTPs and ddNTPs results in production of DNA fragments with different sizes. The size is determined by the distance between the primer and the site of premature termination. ddNTPs are fluorescently labelled. Each ddNTPs emits a different fluorescent color: ddATP green, ddCTP blue, ddGTP black, and ddTTP red (22).

Sequencing reaction protocol:

The sequencing reaction is carried out in a final volume of 5 µl. The following components are required:

- 0.5-0.8 µl of purified PCR products, the quantity of product depends on the intensity of the band obtained on the agarose gel after electrophoresis.

- 0.8 µl of BigDye Terminator v.3.1/v.1.1 Ready Reaction Mix, part of the BigDye Terminator Cycle Sequencing Kit from Applied Biosystems. The BigDye Terminator v.3.1/v.1.1 Ready Reaction Mix is a solution containing the dye terminators. The dye terminators are the marked ddNTPs.

- 0.4 µl of Buffer BigDye Terminator v.3.1/v.1.1 Sequencing 5x from Applied Biosystems. The buffer is a solution of Tris-HCL, pH 9.0 and $MgCl_2$. It provides the pH and magnesium optimal for the reaction.

- 0.8 μl Oligo dNTPs (50 ng/μl)

- 0.8 μl of forward or reverse primers. Two sequencing reactions will be run for each DNA fragments, one with the forward primer and the other with the reverse. We will obtain in this way the complete sequence of the DNA fragments.

- 2.2 to 2.5 μl of H_2O to obtain a final volume of 5 μl.

All these components are mixed together in tubes. Then the sequencing reaction is performed in a Biometra thermal cycler with the following program:

Sequencing reaction program

Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*	*Step 6*
96^o	96^o	50^o	60^o	4^o	4^o
1’	10’’	5’	4’	7’	∞

The steps 2 to 4 are repeated 25 times.

2.2.7. Sequencing reaction purification

The unincorporated dye terminators, excess dNTPs and contaminating salts must be removed to obtain high quality sequencing data. This increases the intensity of the fluorescence signal.

Sequencing reaction purification protocol:

- Add 20 μl of injection solution in each reaction tube with 5 μl of sequencing reaction product.

- Transfer the 25 μl in the SEQ96 plate from Millipore. The SEQ96 plate is composed of a size-exclusion membrane.

- Filter with Millipore Vacuum Mannifold for 3 to 5 minutes or until the wells are dry.

- Blot excess liquid from the bottom of the SEQ96 plate on an absorbent material (Kimwipes).

- Add 25 μl of injection solution to each well and repeat the vacuum step.

- Blot excess liquid from the bottom of the SEQ96 plate on an absorbent material (Kimwipes).

- Add 25 μl of injection solution and put the plate on the plate shaker for 10 to 15 minutes to resuspend the samples.

- Transfer the 25 μl of purified sequencing reaction product in a sequencing plate.

- Add 5 μl of HiDi-formamide in each sample to avoid the evaporation of the samples during the plate analysis in the sequencer.

2.2.8. Sequencing on an ABI PRISM® 3100 Genetic Analyzer

In the ABI PRISM® 3100 Genetic Analyzer, DNA fragments migrate through a capillary containing a polyacrylamide electrophoretic gel, where they are separated according to their size. This method is the DNA sequencing by capillary electrophoresis. In the capillary, DNA chains pass in front of a laser beam focused on a fixed position, which induces a distinct fluorescent signal depending on the ddNTPs. A computer analyzes the data and the sequence is visualized as alternating peaks of one of the ddNTPs colors, according to the nucleotides sequence of the DNA of interest (22).

2.2.9. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

The basic principle of RT-PCR is the same as for PCR starting from mRNA as template. The cDNA is synthesized by the enzyme reverse transcriptase (RT). The RT is an enzyme present in retroviruses that can synthesize DNA from RNA. The cDNA is single-stranded DNA that does not contain any introns and derives from a coding DNA segment (22). The obtained DNA was then purified and sequenced as described previously.

RT-PCR protocol:

For the RT-PCR the Qiagen one step RT-PCR kit was used. The reactions were carried out in a final volume of 20 μl containing the following components:

- 2 μl of RNA at a concentration of 20 ng/μl
- 4 μl of Qiagen one step RT-PCR Buffer 5x. The buffer prevents inhibition of PCR amplification by reverse transcriptases, allows specific primer annealing over a wide range of temperatures and efficient RT-PCR from any RNA template.
- 0,8 μl of dNTPs mix, 10mM each
- 1,8 μl of forward primer and reverse primer at a concentration of 50 ng/ μl
- 0,8 μl of Qiagen one step RT-PCR Enzyme mix. The enzyme mix contains reverse transcriptases and Taq DNA polymerases for reverse transcription and PCR. The two reactions can efficiently occur in one tube.
- 1 μl of Q-Solution 5x. The Q-Solution facilitates reverse transcription and amplification of GC-rich templates or those with a high degree of secondary structure. RT-PCR is then optimized for difficult templates.
- 7,8 μl of RNA free water

The reactions were carried out in a Biometra thermocycler with the following program:

RT-PCR program for fragments 1 to 3

Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*	*Step 6*	*Step 7*
50°	95°	94°	58°	72°	72°	10°
30'	15'	30''	30''	1'	10'	∞

The steps 3 to 5 are repeated 39 times.

2.2.10. Nomenclature for the description of sequence variations at DNA level

To describe the sequence variations of PDK4, we followed the guidelines of the Human Genome Variation Society (www.hgvs.org). The subsequent recommendations were taken from *den Dunnen JT and Antonarakis SE (2000). Hum.Mutat. 15: 7-12.* (23)

The nucleotides are designated by the bases: A (adenine), C (cytosine), G (guanine) and T (thymidine).

The nucleotide numbering is proceeded as follows:

- There is no nucleotide 0.
- The nucleotide 1 is the A of the ATG-translation initiation codon.
- The nucleotide 5' of the ATG-translation initiation codon is -1, the previous -2 and so on.
- The nucleotide 3' of the translation stop codon is *1, the next *2 and so on.

The intronic nucleotides are described according to their position in the intron:

- If the nucleotide is at the beginning of the intron, we use the number of the last nucleotide of the preceding exon, a plus sign and the position in the intron, like c.77+1G.
- If the nucleotide is at the beginning of the intron, we use the number of the first nucleotide of the following exon, a minus sign and the position upstream in the intron, like c.78-1G.
- In the middle of the intron, numbering changes from "c.77+…" to "c.78+…". For introns with an uneven number of nucleotides, the central nucleotide is the last described with a plus sign.

The sequence variations of PDK4 include substitutions, deletions, duplication and variability of short sequence repeats. They are described as follows:

- Single nucleotide substitutions are designated by a ">"-character, indicating "changes to". c.76A>C denotes that at nucleotide 76 an A is changed to a C.
- Deletions are designated by "del" after an indication of the first and last nucleotide(s) deleted. c.7_8del or c.7_8delTG denotes a TG deletion in the sequence ACTTTG**TG**CC to ACTTTGCC.
- Duplications are designated by "dup" after an indication of the first and last nucleotide(s) duplicated. c.7_8dup or c.7_8dupTG or c.7_8dup2 denotes a TG duplication in the sequence ACTTTGTGCC to ACTTTGTG**TG**CC.

- Variability of short sequence repeats are described as c.123+74T(3_6). c.123+74 indicates the start of the first nucleotide of the variable repeat and T indicates the sequence of the repeat unit. The underscore is used to indicate the range (3 to 6 times).

3. RESULTS

3.1 Review of the medical records of 10 IKH patients

Ten patients with 24 IKH episodes were included in this study. The medical records of the patients were reviewed and each episode of IKH was characterized (see Methods).

Clinical features

1. Sex:

Our group of patients is composed of five girls and five boys. The proportion of female and male is thus equal.

2. Age of onset:

The mean age at first crisis of our patient group was 37,5 months. 3 of our 10 patients had an age under 18 months (patients 3, 4 and 7) and 1 patient an age over 5 years (patient 6) when they presented with the first IKH at the hospital.

Patient	Sex	IKH episode	Age	Height	Weight	Trigger
1	M	1	4y 9m	P10-25	P10-25	Appetite loss
2	F	2.1	2y 5m	P25-50	P3	Infection
		2.2	2y 9m	P25-50	P50	Febrile infection
		2.3	3y 7m	P50	P50	?
		2.4	3y 8m	P75-90	P50	Febrile infection
		2.5	3y10m	?	P10-25	?
		2.6	4y10m	?	P50	Fever
		2.7	5y10m	P50-75	P75-90	Infection
		2.8	6y10m	?	P25-50	Infection
3	F	3.1	1y 4m	P50-75	P25-50	Appetite loss
		3.2	2y	?	?	Infection
4	M	4.1	1y4m	P-50-75	P10	Appetite loss Infection
		4.2	1y6m	P3	P3-10	?
5	F	5.1	3y2m	?	P25	Infection
		5.2	3y2m	?	P25	Infection
6	F	6	10y11m	P<3	P<3	Appetite loss
7	M	7	1y2m	?	?	Infection
8	F	8.1	1y10m	P50	P50-75	Appetite loss Infection
		8.2	3y7m	P75-90	P75-90	Appetite loss Infection

9	M	9	2y10m	?	?	Appetite loss Infection
10	M	10.1	1y6m	?	?	Appetite loss Fever
		10.2	1y11m	?	P10	Appetite loss Fever
		10.3	2y4m	?	P3-10	Appetite loss Fever
		10.4	6y3m	P25-50	P3	Appetite loss Fever

3. Weight and height:

In our cohort we find 4 out of 7 patients (=57%, data missing for patients 5, 7 and 9) with a height below the 50th percentile corresponding to 5 out of 13 (=39%) IKH episodes (height not documented for 11 episodes). 7 out of 8 patients (=88%, data missing for patients 7 and 9) have a weight below the 50th percentile corresponding to 13 out of 20 (=65%) IKH episodes (weight not documented for 4 episodes).

4. Diet, nutritional habits:

The available information on our patient group revealed little appetite, frequent intake of small snacks (lack of structured meals allowing glycogen and fat storage) and a preference for high fat snacks that can favor ketosis.

5. Triggers of hypoglycemic crises:

The triggers of the crisis in the 24 documented IKH episodes were loss of appetite, fever and infection due to an intercurrent illness. These infections are predominantly gastroenteritis, rhinitis, bronchitis and pharyngitis.

6. Symptoms of hypoglycemic crises:

The symptoms of hypoglycemia presented by our patients were vomiting, nausea, pallor, tremor, hypotonia, sleepiness, fatigue, decreased energy, apathy, altered level of consciousness, loss of consciousness and seizures. The most frequent symptom presented by our patients was vomiting. Lethargy presented as sleepiness, fatigue, decreased energy, apathy, altered level of consciousness and loss of consciousness was also a frequent symptom among our patients.

Patients 1, 2, 3, and 9 presented neurovegetative and neuroglycopenic symptoms. Patient 2 only showed symptoms of neuroglycopenia at one of the eight reported crisis. Patient 3 had no autonomic response before lethargy at the second crisis. Patients 4 and 7 did not show any neurovegetative symptoms, while patients 5 and 10 did not show any neuroglycopenic symptoms. Patient 8 had one episode with only autonomic response and a second episode during which he presented both kinds of symptoms.

A blood glucose level of 1.8 mmol/l is the lowest value documented among our data. With the same blood glucose, patient 1 presented seizures and loss of consciousness, patient 4 had an altered level of consciousness whereas patient 2 only presented vomiting and nausea.

7. Varia:

No hepatomegaly or delayed psychomotor development has been reported in our patient cohort.

Biochemical features

Levels of pyruvate, lactate, lactate/pyruvate, β-hydroxybutyrate and acetoacetate in blood can only be determined in a medical center with a specialized laboratory, like the CHUV. The blood samples must be analyzed as quickly as possible to determine these values. This explains why these levels are often not available for our patients as most episodes of hypoglycemia were treated in regional hospitals.

1. Blood glucose:

Documented blood glucose levels of our patients ranged from 1.8 to 5.4 mmol/l.

2. Lactate and pyruvate:

Two pyruvate values were available for patient 8 and both were slightly elevated. Lactate levels were normal for patients 1, 2, 3, 8 and 10. Patient 4 had the first time a normal lactate level and during another crisis a slightly elevated lactate at 3 mmol/l. This could be an artifact due to a difficult blood take. The lactate/pyruvate ratio could only be calculated for the two IKH episodes of patient 8 and were normal.

3. Keton bodies in blood and urine strip tests:

β-hydroxybutyrate and acetoacetate values were only available for patient 8. During one crisis, both values were elevated and during another one, only the acetoacetate level was elevated. Urine test strips were negative for keton bodies in patient 8 (timepoint of urine collection?), but most patients showed +++ or ++++.

4. Free fatty acids (FFA):

FFA levels have only been documented in 5 episodes. They were elevated in patients 1, 3 and 4. Patient 8 had one normal and one elevated measurement.

5. Plasma amino acids:

Patient 4 did not show any significant alteration of amino acid profile in plasma. Patient 1 had an elevated level of leucine and isoleucine. Patients 2, 3 and 5 had increased levels of leucine, isoleucine and valine. Patients 6 and 9 had elevated levels of glutamine. Patient 8 had once increased values of glutamine, proline, alanine, valine and leucine and once elevated levels of glutamine, proline, alanine and taurine. Patient 10 had elevated levels of glutamine, alanine, threonine, serine, lysine and glycine, but the blood was not taken during an IKH episode. The changes in amino acid profiles of patients 6, 8, 9 and 10 might be of nutritional origin. No amino acid analysis was available for patient 7.

6. Free and total carnitine/acylcarnitine profile:

Data on total and free carnitine levels were available for 5 out of 10 patients. They were normal for patients 2, 3 and 5, increased for patient 6 and decreased for patient 9. Free carnitine levels were normal for patient 5, increased for patient 6 and decreased for patients 2, 3 and 9. For exclusion of a primary carnitine deficiency, levels of free carnitine were controlled in patients 2, 3 and 9 after the IKH episode. Nearly all patients had altered acylcarnitines profiles compatible with ketosis and lipolysis, but without signs indicating a primary metabolic disorder. Only patient 9 had a normal acylcarnitine profile, whicht could be explained by a low total carnitine level at this moment.

7. Plasma transaminases:

Data on plasma transaminases was available in 6 out of 10 patients (not documented for patients 4, 5, 7 and 8). ASAT was increased for patients 3 and 10 and normal for patients 1, 2, 6 and 9. ALAT value was normal for all 6 patients.

8. Cortisol, insulin, IGF1 and IGFBP-3:

Patients 1, 3, 4, and 7 had elevated levels of cortisol while patients 9 and 10 had levels in the normal range. Cortisol was not documented for patients 2, 5, 6 and 8. Insulin levels have been determined in only 4 patients. It was normal in patients 3 and 4 and decreased in patients 1 and 7. IGF-1 has been measured in patient 1 (normal) and patient 7 (decreased). IGFBP-3 levels were available for patients 1, 4 and 7 with a normal result in all of them.

9. Urinary organic acids:

Available data concerning patients 1 and 7 are limited. All others patients present ketonuria, dicarboxyluria, 3-OH-dicarboxyluria and lactaciduria at various degrees of severity. The two crises of patient 8 had different urinary organic acids profiles: low or normal ketonuria, dicarboxyluria present or not, but no lactaciduria. Patient 3 also had various profiles: the dicarboxyluria and 3-OH-dicarboxyluria were one time important compared to the ketosis and the other time they were only mild. However, patient 10 had several similar profiles. In patients 4, 8 and 10 other pathological organic acids were also present during an IKH episode, but were not detectable in control samples.

3.2 DNA sequencing

The genomic DNA of our ten IKH patients and of a control (Co) was sequenced according to the methods described in the previous chapter. The ten patients were numbered from 1 to 10 to guarantee the confidentiality and interpretation of results independently from the clinical presentation. The results of sequence analysis are summarized in the following tables and in Figure 3. Polymorphisms already described in the literature are written in blue.

Substitution, duplication, deletion and variability of short sequence repeat were found. Patients 1 and 4 had no special features in their PDK4 sequences. No changes have been revealed in fragments 3 and 7.

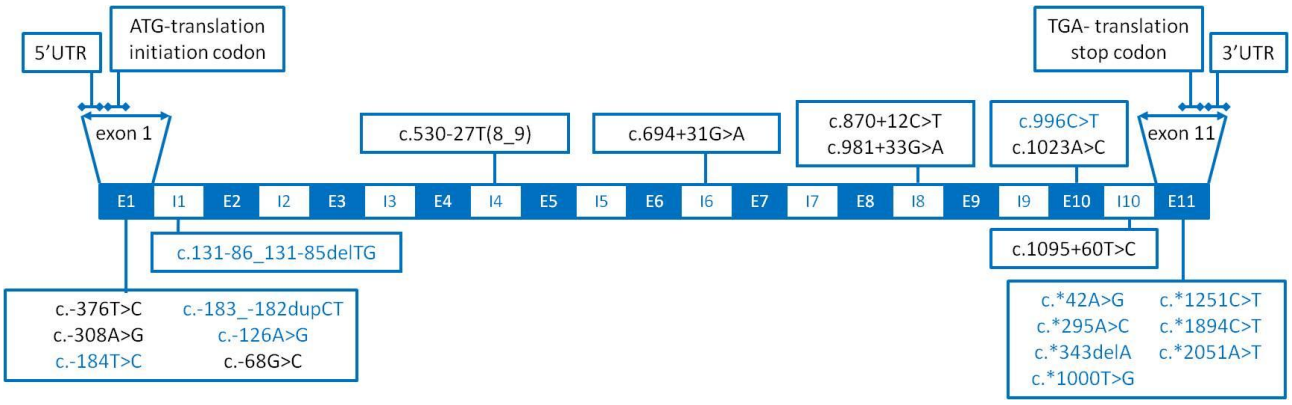

Figure 3: Results of PDK4 gene sequencing

In fragment 1, five substitutions and a CT-duplication are located upstream the ATG-translation initiation codon. All patients were heterozygous for those variations. These changes were also

found in the control DNA, except the c.-184T>C and the c.-68G>C. The findings c.-184T>C, c.-183_-182dupCT and c.-126A>G are known polymorphism, the others not.

Only patient 10 had the c.131-86_131-85delTG in the intron 2. The patient was heterozygous for this deletion which was not present in the control DNA and is not a known polymorphism.

Patient 3 is heterozygous for a stretch of eight T-residues instead of nine T-residues in intron 4. This variable stretch was not found in the control DNA and is not a known polymorphism.

In fragment 6, a G to A substitution located in intron 6 was detected in patients 2, 3, 5, 6, 8, 9, 10 and the control DNA. This polymorphism has not yet been described. Patient 3 was homozygous for this variation, while the other patients were heterozygous.

Intron 8 of patients 3 and 10 contained a C to T substitution and intron 9 of patient 7 revealed a G to A substitution. Both substitutions were not found in the control DNA and are not known as polymorphisms. Patients were all heterozygous for these changes.

A C to T and a A to C substitution were found in exon 10. A T to C substitution was located in intron 10. The c.996C>T is a known polymorphism. The other changes have not been described as polymorphisms. Patient 10 was homozygous for these variations, the other patients were heterozygous.

Changes found in fragments 11-1, 11-2 and 11-3 are all located downstream the TGA-translation stop codon and are known polymorphisms, except c.*2148G>A. Patients 3 and 10 were homozygous for those variations, the other patients were heterozygous.

Results for fragments 1 to 4+5

	Fragment 1	Fragment 2	Fragment 3	Fragment 4+5
1	-	-	-	-
2	c.-376T>C c.-308A>G c.-183_-182dupCT c.-126A>G heterozygous	-	-	-
3	c.-376T>C c.-308A>G c.-184T>C c.-183_-182dupCT c.-126A>G heterozygous	-	-	c.530-27T(8_9) heterozygous
4	-	-	-	-
5	c.-376T>C c.-308A>G c.-184T>C c.-183_-182dupCT c.-126A>G heterozygous	-	-	-
6	c.-376T>C	-	-	-

	c.-308A>G c.-184T>C c.-183_-182dupCT c.-126A>G c.-68G>C heterozygous			
7	-	-	-	-
8	c.-376T>C c.-308A>G c.-184T>C c.-126A>G heterozygous	-	-	-
9	c.-376T>C c.-308A>G c.-183_-182dupCT c.-126A>G heterozygous	-	-	-
10	c.-376T>C c.-308A>G c.-184T>C c.-183_-182dupCT c.-126A>G heterozygous	c.131-86_131-85delTG heterozygous	-	-
Co	c.-376T>C c.-308A>G c.-183_-182dupCT c.-126A>G heterozygous	-	-	-

Results for fragments 6 to 10

	Fragment 6	Fragment 7	Fragment 8+9	Fragment 10
1	-	-	-	-
2	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous
3	c.694+31G>A homozygous	-	c.870+12C>T heterozygous	c.996C>T c.1023A>C c.1095+60T>C heterozygous
4	-	-	-	-
5	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous
6	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous
7	-	-	c.981+33G>A	-

			heterozygous	
8	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous
9	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous
10	c.694+31G>A heterozygous	-	c.870+12C>T heterozygous	c.1023A>C c.1095+60T>C homozygous
Co	c.694+31G>A heterozygous	-	-	c.1023A>C c.1095+60T>C heterozygous

Results for fragments 11-1 to 11-2

	Fragment 11-1	Fragment 11-2	Fragment 11-3
1	-	-	-
2	c.*42A>G c.*295A>C c.*343delA heterozygous	c.*1000T>G c.*1251C>T heterozygous	c.*1894C>T c.*2051A>T heterozygous
3	c.*343delA homozygous	c.*1000T>G c.*1251C>T homozygous	c.*1894C>T c.*2051A>T homozygous
4	-	-	-
5	-	c.*1000T>G heterozygous	-
6	c.*343delA heterozygous	c.*1000T>G c.*1251C>T heterozygous	c.*1894C>T c.*2051A>T heterozygous
7	-	-	-
8	c.*343delA heterozygous	c.*1000T>G c.*1251C>T heterozygous	c.*1894C>T c.*2051A>T heterozygous
9	c.*42A>G c.*295A>C c.*343delA heterozygous	c.*1000T>G c.*1251C>T heterozygous	c.*1894C>T c.*2051A>T heterozygous
10	c.*343delA homozygous	c.*1000T>G c.*1251C>T homozygous	c.*1894C>T c.*2051A>T c.*2148G>A homozygous
Co	c.*343delA heterozygous	c.*1000T>G c.*1251C>T heterozygous	c.*1894C>T c.*2051A>T heterozygous

3.3 cDNA sequencing

In patients 1, 2, 3 and 6 cultivated fibroblasts deriving from a former skin biopsy were available for RNA extraction. As previously explained, RNA was extracted from fibroblasts and cDNA was obtained by RT-PCR. Two transcripts of PDK4 are described (PDK4-001 and PDK4-201). We sequenced the longer transcript (PDK-001, ENST00000005178) of patients 1, 2, 3 and 6 and the control in order to examine if the variations found by DNA sequencing affect protein synthesis.

In fragment 1 of the cDNA, a G to C substitution located upstream the ATG-translation initiation codon has been found in patient 6 and the control.

The sequencing of fragment 2 did not reveal any sequence alteration.

Patient 3 had a known polymorphism in fragment 3. Patients 2, 3 and 6 had a A to C substitution. The codon GCA becomes GCC. However, this does not lead to an amino acid change. Both codons are coding for an alanine.

Results for fragments 1 to 3

	Fragment 1	Fragment 2	Fragment3
1	-	-	-
2	-	-	c.1023A>C
3	-	-	c.996C>T c.1023A>C
6	c.-68G>C	-	c.1023A>C
Co	c.-68G>C	-	c.1023A>C

4. DISCUSSION

IKH is defined by episodes of hypoglycemia with presence of ketone bodies in plasma and urine, provoked by periods of starvation, often in combination with an intercurrent illness (2).
PDK4 phosphorylates and inactivates the PDC to conserve glucose when glucose supply is lacking during fasting (10). The PDK4 gene has be selected for our Master project because current knowledge supports its central role in glucose homeostasis in fasting and fed states.

The analysis of clinical data of our patients mostly confirms the features of IKH reported in the literature. Children with IKH were reported to be predominantly male. In our study with a very small patient group we find an equal relation between both genders. Regarding larger patient groups, IKH rarely begins before the age of 18 months or after the age of 5 years (3). This disorder typically spontaneously remits by the age of 8 or 9 years (1). In our cohort, the mean age at first crisis is 37,5 months. This value is situated between the age of presentation and the age of remission of IKH. However, we find 3 out of 10 patients with a first crisis before the age of 18 months and one patient beyond the age of 5 years (= 40% of initial presentation outside the reported range). Children with IKH often have a height and weight below the 50^{th} percentile for age (3). In our cohort this seems to be particularly true for the weight with 88% of documented patients below the average weight for age in contrast to only 57% of patients with a height below average. This supports the hypothesis of low availability of gluconeogenetic substrates. The type of diet could be a precipitating factor for hypoglycemia. Frequent fat rich little snacks were dominating in our patients thus supporting the production of ketone bodies. Intercurrent illnesses, particularly infections of the upper airways and gastroenteritis, are frequently the trigger of an IKH episode accompanied by vomiting and lethargy. For the same level of glycemia, one patient only presents autonomic response and the other presents directly neuroglycopenic symptoms. With repeated or prolonged episodes of hypoglycemia, the threshold of neurovegetative symptoms is decreased to that for neuroglycopenic signs. Besides that, patients might have individual thresholds. Some patients present mainly with symptoms of autonomic response during hypoglycemia, some others mainly with neuroglucopenic symptoms.

The biochemical phenotype of our group of patients was quite homogeneous: low blood glucose, high blood/urine ketones, moderate and usually comptensated metabolic acidosis, appropriate increase of contro-insular hormones with suppressed insulin, absence of pathologic metabolites in urine indicating the absence of an underlying aminoacidopathy or organic aciduria. Some patients showed, beside ketosis, a significant increase of dicarboxylic acids in urine. These metabolites witness the activation of alternative (microsomal) fatty acid oxidation when mitochondrial fatty acid oxidation is saturated by an intense lipolysis. The extent of dicaboxilic aciduria varied between patients, with no correlation with severity of symptoms and/or level of glycemia. Some of the reported IKH episodes showed results that were not conform to this biochemical phenotype. In these cases it seems to be most likely that blood and/or urine sampling has not been performed during hypoglycemia. Hypoglycemia is an emergency. Children with severe presentation of hypoglycemia often receive intravenous glucose even before blood is taken. This explains documentation of normal glycemia, lack of ketone bodies, not suppressed insulin levels and not elevated cortisol values.

The sequencing of PDK4 gene in genomic DNA has shown multiple changes. The majority of them are known polymorphisms and/or are also found in the control DNA. The variations that are not known polymorphism or not found in the control DNA are mostly situated in introns, upstream the

ATG translation start codon or downstream the translation stop codon. They are not predicted to interact with the gene transcription or translation. Unlike intronic variations, exonic changes may affect the protein synthesis. However, the exonic changes found in PDK4 are known polymorphisms also present in controls or do not lead to an amino acid change in the protein. Thus, they do not seem to be pathogenic. However, to be sure that intronic changes did not generate splicing errors, we sequenced the cDNA. Only fibroblasts of patients 1, 2, 3 and 6 were available. The sequencing of the longer PDK4 transcript has not revealed any altered cDNA form. Variations of PDK4 sequence found in patients and in the control DNA are probably not yet described polymorphisms of the PDK4 gene. Because of their presence in controls with no IKH, they are not supposed to affect PDK4 activity.

This study has certainly some limitations: a) the size of the patient group is small, not allowing any statistical analysis of the clinical and biochemical parameters analyzed; b) biochemical data were not complete at each episode of hypoglycemia in each patient, because hypoglycemic episodes took place at different moments in different hospitals; c) the study is retrospective and data were not collected with a well defined protocol.

Conclusions

IKH is the most common cause of hypoglycemia in children between 1 and 5 years of age among non-diabetic children. It is a cause of multiple hospitalisations and of extensive investigations in affected children. IKH has a potential risk for life threatening events if not treated promptly. Given the frequency and the observation of heritability of this condition, more research effort should be done in understanding the molecular and biochemical basis of this benign, but potentially dangerous condition.

No pathogenic changes in the open reading frame of PDK4 related to IKH were found in our cohort. However, our study has not completely excluded a possible role of the PDK4 gene in the pathogenesis of IKH. Further work should concentrate on the promoter of the PDK4 gene. Recent studies have shown that mutations on the PDK4 promoter affect the regulation of PDK4 gene expression (15–20). The other PDK isoforms could also be worth to be investigated.

Even with the mentioned limitation of our study, our results seem to show no correlation between the clinical presentation, the biochemical features and the changes in PDK4 sequence.

5. REFERENCES

1. Daly LP, Osterhoudt KC, Weinzimer SA. Presenting features of idiopathic ketotic hypoglycemia. J Emerg Med. 2003 juill;25(1):39–43.
2. Huidekoper HH, Duran M, Turkenburg M, Ackermans MT, Sauerwein HP, Wijburg FA. Fasting adaptation in idiopathic ketotic hypoglycemia: a mismatch between glucose production and demand. Eur. J. Pediatr. 2008 août;167(8):859–65.
3. Matthieu J-M, Boulat O. [Ketotic hypoglycemia in children]. Rev Med Suisse Romande. 2002 déc;122(12):640–4.
4. Agneta Sunehag, Morey W. Haymond. Approach to hypoglycemia in infants and children. Dans: UpToDate. Waltham, MA: Denise S. Basow; 2011.
5. Bodamer OA, Hussein K, Morris AA, Langhans C-D, Rating D, Mayatepek E, et al. Glucose and leucine kinetics in idiopathic ketotic hypoglycaemia. Arch. Dis. Child. 2006 juin;91(6):483–6.
6. Rowles J, Scherer SW, Xi T, Majer M, Nickle DC, Rommens JM, et al. Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human. J. Biol. Chem. 1996 sept 13;271(37):22376–82.
7. Sugden MC, Holness MJ. Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. Arch. Physiol. Biochem. 2006 juill;112(3):139–49.
8. Patel MS, Korotchkina LG. Regulation of the pyruvate dehydrogenase complex. Biochem. Soc. Trans. 2006 avr;34(Pt 2):217–22.
9. Holness MJ, Sugden MC. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem. Soc. Trans. 2003 déc;31(Pt 6):1143–51.
10. Jeoung NH, Wu P, Joshi MA, Jaskiewicz J, Bock CB, Depaoli-Roach AA, et al. Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homoeostasis during starvation. Biochem. J. 2006 août 1;397(3):417–25.
11. Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, Harris RA. Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. Biochem. J. 1998 janv 1;329 (Pt 1):197–201.
12. Houten SM, Chegary M, Te Brinke H, Wijnen WJ, Glatz JFC, Luiken JJFP, et al. Pyruvate dehydrogenase kinase 4 expression is synergistically induced by AMP-activated protein kinase and fatty acids. Cell. Mol. Life Sci. 2009 avr;66(7):1283–94.
13. Wu P, Peters JM, Harris RA. Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor alpha. Biochem. Biophys. Res. Commun. 2001 sept 21;287(2):391–6.
14. Wan Z, Thrush AB, Legare M, Frier BC, Sutherland LN, Williams DB, et al. Epinephrine-mediated regulation of PDK4 mRNA in rat adipose tissue. Am. J. Physiol., Cell Physiol. 2010 nov;299(5):C1162–70.
15. White UA, Coulter AA, Miles TK, Stephens JM. The STAT5A-mediated induction of pyruvate dehydrogenase kinase 4 expression by prolactin or growth hormone in adipocytes. Diabetes. 2007 juin;56(6):1623–9.
16. Connaughton S, Chowdhury F, Attia RR, Song S, Zhang Y, Elam MB, et al. Regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) gene expression by glucocorticoids and insulin. Mol. Cell. Endocrinol. 2010 févr 5;315(1-2):159–67.
17. Zhang Y, Ma K, Sadana P, Chowdhury F, Gaillard S, Wang F, et al. Estrogen-related receptors stimulate pyruvate dehydrogenase kinase isoform 4 gene expression. J. Biol. Chem. 2006 déc 29;281(52):39897–906.
18. Attia RR, Connnaughton S, Boone LR, Wang F, Elam MB, Ness GC, et al. Regulation of

pyruvate dehydrogenase kinase 4 (PDK4) by thyroid hormone: role of the peroxisome proliferator-activated receptor gamma coactivator (PGC-1 alpha). J. Biol. Chem. 2010 janv 22;285(4):2375–85.
19. Kwon H, Huang B, Jeoung N, Wu P, Steussy C, Harris R. Retinoic acids and Trichostatin A (TSA), a histone deacetylase inhibitor, induce human pyruvate dehydrogenase kinase 4 (PDK4) gene expression. BIOCHIMICA ET BIOPHYSICA ACTA-GENE STRUCTURE AND EXPRESSION. 2006 avr;1759(3-4):141–51.
20. Hsieh MCF, Das D, Sambandam N, Zhang MQ, Nahlé Z. Regulation of the PDK4 isozyme by the Rb-E2F1 complex. J. Biol. Chem. 2008 oct 10;283(41):27410–7.
21. Zschocke J, Hofmann GF. Vademecum Metabolicum. Schattauer Gmbh; 2011. 184 p.
22. Granner D-K, Mayes P-A, Murray R-K, Rodwell V-W. Biochimie de Harper. De Boeck; 2003. 933 p.
23. Sherwood L. Physiologie humaine. De Boeck Supérieur; 2006. 774 p.
24. Lecture Notes — School of Medicine at the University of Virginia [Internet]. [cité 2011 déc 9];Available from: http://www.medicine.virginia.edu/basic-science/departments/pharmacology/dab_lab/lecture_notes
25. Urinary organic acid analysis: a powerful clinical tool [Internet]. 2004 [cité 2011 déc 9];Available from: http://findarticles.com/p/articles/mi_m0ISW/is_255/ai_n6211964/?tag=content;col1
26. Ausubel FM. Current protocols in molecular biology. John Wiley & Sons; 2010. 1286 p.
27. Passarge E. Color Atlas of Genetics, Third Edition. 3e éd. Thieme; 2006. 496 p.
28. Dunnen JT den, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. Human Mutation. 2000 janv 1;15(1):7–12.