European Review for Medical and Pharmacological Sciences 2013; 17(Suppl 2): 72-81

Breath tests with novel ^{13}C-substrates for clinical studies of liver mitochondrial function in health and disease

I. GRATTAGLIANO[1], L. BONFRATE[2,6], P.J. OLIVEIRA[3], L. CASTORANI[2], V. RUGGIERO[2], A.T. VALENZANO[2], A. ASCENSÃO[4], A. BUZOIANU[5], P. PORTINCASA[2,6]

[1]Italian College of General Practitioners, Florence, Italy
[2]Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari Medical Faculty, Bari, Italy
[3]CNC – Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[4]Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Porto, Portugal
[5]Department of Clinical Pharmacology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania
[6]European Society for Clinical Investigation (ESCI)

Abstract. **Mitochondrial dysfunction determines the onset and progression of chronic deleterious conditions including liver diseases. The in vivo assessment of mitochondrial function, by providing more insight into the pathogenesis of liver diseases, would be a helpful tool to study specific functions and to develop diagnostic, prognostic and therapeutic strategies. The application of breath tests in the clinical setting to evaluate mitochondrial fitness may elegantly and noninvasively overcome the difficulties due to previous complex techniques and may provide clinically relevant information, i.e the effects of drugs presenting mitochondrial liabilities. Substrates meeting this requirement include alpha-ketoisocaproic acid and methionine, both decarboxylated by mitochondria. Long and medium chain fatty acids that are metabolized through the Krebs cycle and benzoic acid, which undergoes glycine conjugation, may also reflect the mitochondrial performance.**

This review focuses on the utility of breath tests to assess mitochondrial function in humans, thus contributing to unravel potential mechanisms associated with the dysfunction of this organelle network in the pathophysiology of liver diseases.

Keywords:

Ketoisocaproate breath test, Octanoate breath test, Methionine breath test, Mitochondria, Liver disease.

Abbreviations: AdoMET, S-adenosylmethionine; KICA, alpha-ketoisocaproic acid; HCC, hepatocellular carcinoma; MeBT, ^{13}C-Methionine breath test; mtDNA, mitochondrial DNA; NASH, nonalcoholic steatohepatitis; RFA, radiofrequency ablation.

Introduction

Mitochondria play a key role in the metabolism of carbohydrates, proteins, lipids and xenobiotics, providing the major cellular source of energy. These organelles have also a very critical role in the modulation of cell death signaling pathways and in the regulation of cytosolic calcium homeostasis[1]. Mitochondrial dysfunction contributes to the onset of chronic liver diseases[2], to the progression of fatty liver to steatohepatitis, and to drug-induced liver injury[3]. The assessment of mitochondrial function *in vivo* provides insights into the pathogenesis of liver diseases and is a helpful tool to study specific functions in the development of diagnostic, prognostic and therapeutic strategies.

Why obtaining a snapshot of liver mitochondrial function in patients?

Although mitochondrial processes are easily investigated in isolated mitochondrial fractions or cell lines, the complexity of an intact biological

Corresponding Author: Piero Portincasa, MD, Ph.D; e-mail: piero.portincasa@uniba.it

system makes sometimes direct comparisons between the different models and what occurs *in vivo* difficult. Furthermore, when investigating the effects of several xenobiotics, including clinically used drugs with known liabilities[4,5], whole-body drug metabolism and conversion into more or less reactive metabolites must be considered. By considering that impaired mitochondrial function alters the acetoacetate: β-OH-butyrate ratio in plasma or decreases intracellular ATP concentrations, this may impact the development of different diseases[6,7]. In fact, the widespread use of metabolomics has led to the search of other markers of mitochondrial function[8]. This is based on metabolite network experiments and aims to quantify all metabolites in a cellular system under defined states so that the dynamics of any perturbation can be accurately assessed[9]. However, abnormal steady state concentrations of mitochondrial metabolites are difficult to understand because they reflect the activity of the metabolic processes including the influx of precursors.

This problem can be bypassed if known quantities of traced exogenous substrates are administered and their metabolism is quantitatively and qualitatively followed. This is the basis of dynamic tests to investigate mitochondrial function. Mitochondrial activity can be followed when a specific substrate is catabolized, with the resulting metabolite being determined[10]. Alpha-ketoisocaproic acid (KICA) and alpha-ketobutyrate, a metabolite of methionine, fulfill this requirement as they are both decarboxylated in mitochondria. Long and medium chain fatty acids, metabolized ultimately through the Krebs cycle, and benzoic acid, which undergoes glycine conjugation, may also reflect mitochondrial function. With this, and considering the relationship between disease stage or severity and mitochondrial fitness, the clinician can have an idea of the current state of mitochondrial capacity in the patient and whether a certain drug treatment or pathology is worsening hepatic mitochondrial function. In the opposite side, one can estimate how well mitochondrial function in the liver is improving resulting from a pharmacological and/or non-pharmacological treatment.

In vivo mitochondrial function during chronic liver diseases and drug-induced damage

Nonalcoholic fatty liver disease (NAFLD)

NAFLD is a multifactorial condition associated with several risk factors[11,12]. Here, we present evidence that mitochondria are not only an important component in the early NAFLD, but are also a mediator of liver injury at later stages. In fact, lipid accumulation and impaired hepatic lipid turnover can result from the alteration of the intracellular fatty acid trafficking and mitochondrial beta-oxidation[13].

Lipid accumulation and insulin resistance increase reactive oxygen species (ROS) generation by different sources, including mitochondria, which results in damage to different hepatocyte substructures with special emphasis on mitochondria[14]. Major redox changes occur markedly in mitochondria with ongoing steatosis[15,16]. Mitochondrial GSH depletion sensitizes hepatocytes to inflammatory cytokines and TNF-α-induced death pathways. Additional mechanisms of mitochondrial damage during NAFLD include increased angiotensin II that contributes to generate oxidative stress and fat accumulation in hepatocytes by impairing mitochondrial fatty acid beta-oxidation (FAO). In conclusion, progressive mitochondrial damage during NAFLD may decrease the ability of the hepatocyte to restore its normal function, leading to bioenergetic collapse and mitochondrial-mediated cell death[17].

If a C atom of a test compound is labeled, the extent and rate of mitochondrial metabolism of this molecule resulting in the formation of CO_2 may reflect the mitochondrial clearance of the compound, which can be assessed by performing a breath test (BT)[18]. A number of substrates have been used in research although none of them has been currently approved for clinical application. In patients with mitochondrial impairment and microvesicular steatosis caused by valproic acid intoxication, results of methionine BT paralleled with the extension of liver damage, suggesting that the test indeed reflects mitochondrial function in patients[19]. In another study, KICA BT appeared to discriminate between patients with non-alcoholic from those with alcoholic steatosis[20], although no information on the extent of fat accumulation was provided. Knowledge on the extent of steatosis may be important, as in patients with biopsy-demonstrated severe nonalcoholic macrovesicular steatosis, a decreased decarboxylation of methionine was observed[19]. Accordingly, KICA decarboxylation was impaired in patients with advanced nonalcoholic steatohepatitis (NASH) but not in those with simple steatosis[21]. Also, obesity appeared to contribute to the decrease in the KICA BT in the presence of complicated steatosis (Figure 1). These observations suggest that although obesity *per se* does not alter KICA BT results, KICA decarboxy-

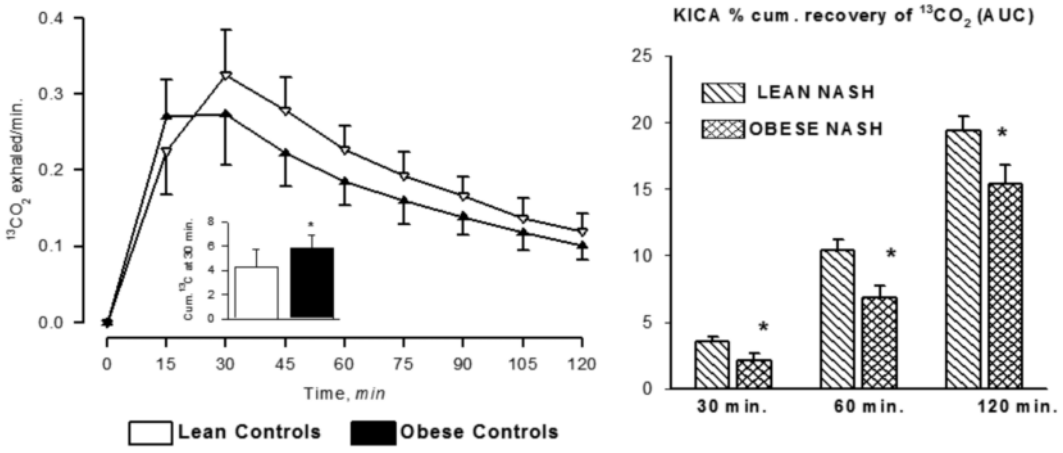

Figure 1. LEFT PANEL: time course and 30 min cumulative exhalation of $^{13}CO_2$ in breath after ^{13}C-KICA administration in healthy volunteers with normal (lean) or high body mass index (large and tall athletic individuals). In the inlet the asterisk above the black bar indicates a significant difference at 30 min between the two groups. RIGHT PANEL: cumulative recovery of $^{13}CO_2$ with ^{13}C-KICA in breath in lean and obese NASH patients. A significant decrease of KICA metabolism is detected in obese patients at 30, 60, and 120 min (asterisks). Adapted from ref. 21

lation increases after dieting, possibly reflecting adaptive modifications in mitochondrial function in response to caloric restriction[22].

Alcoholic liver disease (ALD)

ALD is a major cause of chronic liver disease worldwide, leading to fibrosis and cirrhosis but also including simple steatosis and alcoholic hepatitis.

Early studies[23] indicated that alcohol consumption increases the reduced/oxidized nicotinamide adenine dinucleotide ratio in hepatocytes, which disrupts mitochondrial FAO and results in steatosis[24]. In hepatocytes, ethanol is primarily metabolized into acetaldehyde by alcohol dehydrogenase in the cytosol, cytochrome P450 in microsomes, and catalase in peroxisomes. Ethanol metabolism also generates high levels of ROS and causes lipid peroxidation, mitochondrial GSH depletion and *S*-adenosylmethionine depletion.

Drug-induced liver damage

Drug-induced liver injury (DILI) is an open issue in pharmaceutical field because DILI is a frequent cause for the failure of drug approval or for withdrawal of an already marketed medicine[4,25]. By severely altering mitochondrial function, a number of drugs can induce microvesicular steatosis, which can be associated with profound hypoglycaemia and encephalopathy. Different classes of drugs can also trigger hepatic necrosis and/or apoptosis, causing cytolytic hepatitis, which can evolve into liver failure[26]. Milder mitochondrial dysfunction, sometimes combined with an inhibition of triglyceride egress from the liver, can induce macrovacuolar steatosis, a rather benign lesion in the short term. However, in the long term, this lesion can evolve towards steatohepatitis with a risk evolution to fibrosis and cirrhosis. As liver injury caused by mitochondrial dysfunction can induce the premature end of clinical trials, or drug withdrawal after marketing, it should be detected early during the preclinical safety studies[27]. *In vitro* and *in vivo* investigations can be performed to determine if newly developed drugs disturb mitochondrial FAO and oxidative phosphorylation (OXPHOS), deplete hepatic mitochondrial DNA (mtDNA), or trigger the opening of the mitochondrial permeability transition (MPT) pore[28]. As well-known examples, drugs including amiodarone or valproate inhibit mitochondrial FAO[29,30]. Valproate, an anti-seizure drug, inhibits mitochondrial FAO directly through accumulation of the metabolite 2,4-diene-valproyl-CoA[31]. Other drugs such as aspirin and ibuprofen, both largely used in the treatment of pain and fever, can also inhibit beta-oxidation enzymes leading to microvesicular steatosis[30]. Also, it is likely that initial disruption of mitochondrial FAO may be caused not by one particular drug, but rather by a cocktail of drugs taken for different purposes. One consequence is a progressive loss of mitochondrial oxidative capacity in the liver, causing a negative feedback in many other mitochondrial processes leading to bioenergetic rupture. Examples of widely prescribed non-steroid anti-inflammatory drugs causing liver mitochondrial dysfunction seen in OXPHOS decay and MPT vulnerability include salicylates and diclofenac[32].

In fact, several xenobiotics, including clinically relevant drugs have been described to cause liver injury mediated by excessive MPT activation. These include acetaminophen[25], N-nitrosofenfluramine[33] or salicylate[34], among others. Nimesulide has also been described to cause MPT induction *in vitro*[35-37], although *in vivo* studies failed to detect any effects in this regard[38]. Over-activation of the MPT during drug-induced toxicity can quickly lead to exhaust of mitochondrial capacity to generate ATP, compromising cell survival[39].

At clinically relevant doses, tacrolimus decreases mitochondrial ATP production and inhibits the decarboxylation of KICA, decreasing the resting energy expenditure, and the respiratory quotient in a dose-dependent manner, suggesting an inhibition of mitochondrial respiration[40]. The effect is moderate and its clinical relevance is still not clear.

The principle of the breath test

Breath tests for "dynamic" liver function evaluation have been proposed several years ago. A variety of carbon-labeled compounds has been used for the assessment of mitochondrial, microsomal and cytosolic functions with the aim to obtain data regarding liver disease staging, prognosis and response to therapy. A great interest has arisen in the latest years about the use of BT for the evaluation of mitochondrial function, since abnormalities in those tests appear in a wide range of liver diseases either of genetic or acquired origin. KICA and methionine are so far the best studied carbon-labeled substrates to be used to detect alterations in mitochondrial oxidative metabolism. Although these tests are simple, cost-effective and safe, there is still not a general consensus about their usefulness in a clinical setting since several requirements to overcome the drawbacks of traditional quantitative tests must still be fulfilled. On the other hand, this field of work is relatively recent and further studies are needed in order to assess the suitable substrates for the evaluation of the complex mitochondrial metabolism both in healthy subjects and in patients with liver disease[18].

Breath tests are usually performed by administering the labeled substrate by mouth or intravenously to subjects overnight fasted. The subject should stay at rest in order to minimize variations in endogenous CO_2 production mainly caused by muscle contraction during physical activities. Samples of expired air are collected just before substrate administration to obtain a baseline and thereafter at different time points. The carbon isotope enrichment of expired CO_2 is then analyzed by isotope ratio mass spectrometry or by infrared spectroscopy in the case of $^{13}CO_2$ (Figure 2). Results are then expressed as percentage of the administered dose recovered per hour, cumulative percentage of administered dose recovered over time, and time to peak of exhalation of labeled CO_2. The rate of exhalation of $^{13}CO_2$ at each time point is calculated from the measured increment in the isotopic abundance of $^{13}CO_2$, the known purity of the labeled compound and an assumed constant endogenous CO_2 production. The use of the stable isotope ^{13}C has the advantage that the test can be repeated several times to monitor the course of a disease and can be performed also in infants and pregnant women. Drawbacks are the duration of the test procedure during which complete physical rest is recommended and the availability and cost of the needed equipment. It is important to note that, since all the substrates used to assess mitochondrial function are naturally-occurring compounds and are administered at very low doses, toxicity is unlike to occur and indeed this has been reported so far. Labeled KICA, methionine and octanoic acid (Figure 3) have been used in clinical research.

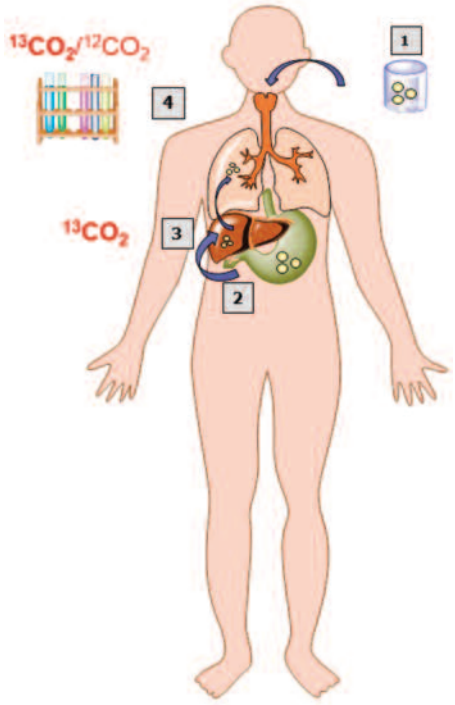

Figure 2. General principles of oral ^{13}C-breath test for liver mitochondrial function. 1, Oral ingestion of the given 13C-substrate. 2, Duodenal jejunal absorption. 3, Liver mitochondrial metabolism. 4, Lung exhalation, measurement.

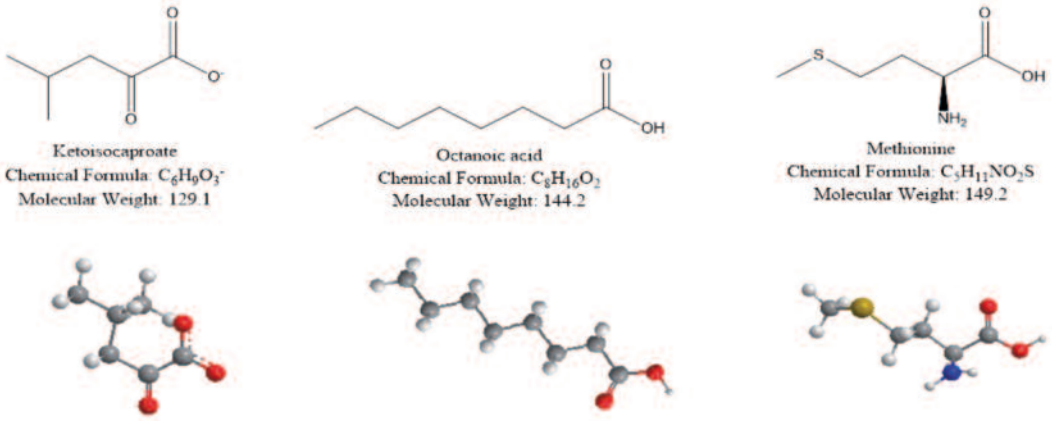

Figure 3. Chemical structure, molecular formula and molecular mass of substrates used in clinical studies for the assessment of mitochondrial function.

Substrates

^{13}C-KICA BT

^{13}C-KICA BT noninvasively assesses mitochondrial KICA decarboxylation by following the exhalation of labeled CO_2 after the administration of labeled KICA[10] (Figure 4) with the major competing pathway, the transamination to leucine, being suppressed by the concomitant administration of leucine. A correspondence between test results and mitochondrial capacity has been shown in experimental models[10], in isolated mitochondria[41], and in healthy subjects: therapeutic doses of acetyl salicylic acid, which should decrease the availability of NADH, increased the decarboxylation of KICA, whereas a 0.5 g/kg dose of ethyl alcohol, which should increase the availability of NADH, decreased its decarboxylation[42]. Decarboxylation of ^{13}C-KICA is significantly higher in females than in males (Figure 5), which must be accounted for during routine and more widespread assessments. Ethanol seems to decrease KICA decarboxylation particularly in women[18,22,43], although when corrected for body composition, no gender differences were observed[44]. Recent studies showed that liver mitochondrial function, as assessed by KICA BT, was decreased in cirrhotic patients with hepatocellular carcinoma (HCC), suggesting mitochondrial and metabolic remodeling in that type of tumors[45]. This will be better described below.

^{13}C-Methionine breath test (MeBT)

Methionine is an essential amino acid that plays a key role in various metabolic processes,

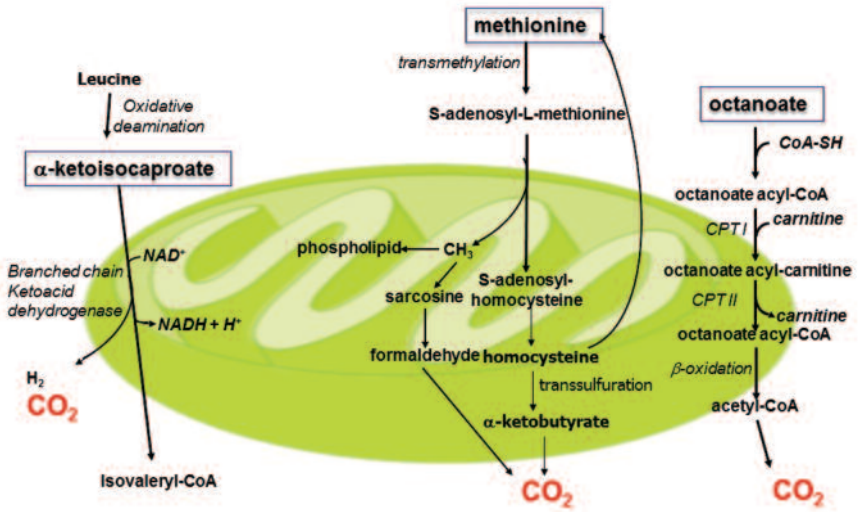

Figure 4. Breath tests employing specific 13C-labeled substrates (i.e. ketoisocaproate, methionine, octanoate) may investigate major mitochondrial pathways and their limiting steps (enzymes). Adapted from ref. 18.

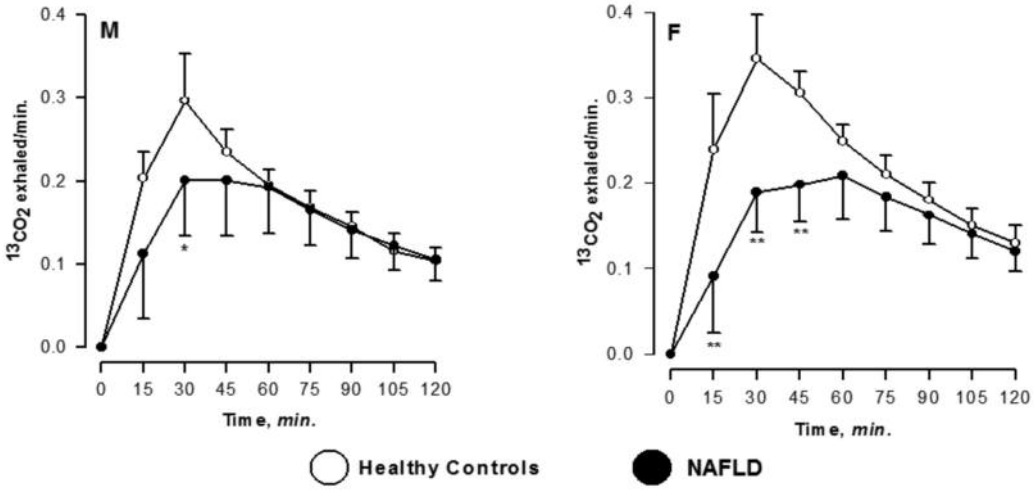

Figure 5. Time course of $^{13}CO_2$ exhaled in breath after ^{13}C-KICA administration in healthy volunteers and in NAFLD patients. M=males (left panel), F=females (right panel). Asterisks indicate significant decrease in NAFLD patients compared to healthy controls. (Grattagliano I. and Portincasa P., unpublished observations).

such as protein synthesis[46]. Exogenous methionine is either used by cells for protein synthesis or undergoes the methionine cycle in which it is first transformed into the principal biologic methyl donor S-adenosylmethionine (AdoMET). After donating its methyl group, the resulting S-adenosyl homocysteine is hydrolyzed to homocysteine, which either undergoes transsulfuration or is re-methylated to methionine. The transsulfuration pathway finally leads to the formation of α-ketobutyrate, which undergoes decarboxylation within mitochondria and results in labeled CO_2 if L-(1-^{13}C) methionine is administered as substrate (Figure 3). If [methyl-^{13}C]-methionine is used as substrate, transmethylation of methionine results in the removal of the labeled methyl group which, in turn, may be in part used for the synthesis of sarcosine which is oxidized to formaldehyde and finally CO_2 in mitochondria. To assess mitochondrial function, either L-(1-^{13}C) methionine or [methyl-^{13}C]-methionine are used[18]. Unfortunately, clinical and research laboratories use differently labelled methionine, making the comparison of results difficult, particularly since more than one labelled CO_2 is formed from L-(1-^{13}C) methionine than from [methyl-^{13}C]-methionine[47]. Furthermore, some investigators have used intravenous rather than oral methionine, which makes comparisons even harder[48]. As for KICA, an acute ethanol consumption in healthy volunteers impairs the decarboxylation of ^{13}C-methionine[49]. During valproate intoxication, the MeBT was impaired and recovered in parallel to the liver damage, suggesting that the test indeed reflects mitochondrial function in patients[19].

^{13}C-octanoate breath test (OBT)

Octanoic acid is a medium-chain fatty acid. Octanoate is absorbed promptly from the intestinal lumen and transported rapidly to the liver through the portal venous system, enters hepatic mitochondria independently of the carnitine transport system and undergoes beta-oxidation with production of acetyl coenzyme A (CoA). Acetyl coenzyme A enters the Krebs cycle and is oxidized to CO_2, generating also substrates for mitochondrial respiratory chain. OBT is a noninvasive test that can be performed routinely not only to assess gastric emptying[50] but also mitochondrial function. However, to what extent these changes reflect specific mitochondrial damage rather than liver injury *per se* is unclear.

Utility and applicability

To study specific metabolic pathways, the hepatic clearance of a variety of exogenous substances can be used to predict drug metabolism, to estimate the prognosis of liver disease and to assess the outcome of treatment[51]. Therefore, BT have several advantages including the noninvasiveness, the use of safe and naturally occurring ^{13}C and substrates, plus easy and reproducible procedures, making this approach useful to stage liver diseases and to monitor patients[52]. A number of studies have explored how

useful assessing mitochondrial function by BT is in the context of liver disease. Since liver mitochondria abnormalities are a characteristic and early feature in humans consuming excessive amounts of ethanol for a longer period of time[53] and single doses of moderate amounts of ethanol decrease KICA decarboxylation[42], the focus has initially been on the ability of the BT to add to the diagnostic accuracy in identifying patients with NAFLD and ALD. In fact, KICA decarboxylation was found to be decreased in alcoholic patients compared to patients with non-alcoholic liver disease and controls[54,55]. In contrast, no difference was observed between healthy controls and chronic alcoholics in a subsequent study[43]. Also, in a small study, KICA BT discriminated between patients with NAFLD and those with ALD[20]. In another study, KICA decarboxylation was impaired in patients with advanced NASH but not in those with simple steatosis[56] and was inversely related to the extent of fibrosis. Also, KICA BT was superior to serum hyaluronate for staging purposes. The value of KICA BT in assessing mitochondrial function is growing in importance due to the increased prevalence of NAFLD in many countries, although standardization of patients before testing (i.e. high fat vs low fat diet, current physical activity/habitual physical activity and fitness levels and drugs) may provide a better predictive value for clinical investigation (Figure 4). Also, the variability in results may be controlled by repeated testing of patients and larger sample sizes. In contrast to results obtained with either the MeBT or the KICA BT, the oxidation of octanoate was found to be unchanged in patients with different stages of NASH[56] or was even increased[57]. Octanoate metabolism was also not impaired in patients with early stage and advanced cirrhosis with and without porto-systemic shunt[58]. The different results obtained with octanoate might be explained by subtle differences in the metabolic pathways probed by the different substrates or by oxidation of octanoate by extra-hepatic mitochondria. Unfortunately, the various BTs have not been compared in the same subjects, which is clearly a limitation and should be further subject of study.

In a recent study, cirrhotic patients with and without HCC were tested using the KICA BT. The decarboxylation of KICA was significantly lower in patients with HCC than in patients with identical Child-Pugh score without HCC[45]. One day after radiofrequency ablation (RFA) of HCC foci, KICA decarboxylation had decreased by almost 30%, returning to baseline over the subsequent 6 months. In contrast, one day after transarterial chemoembolization, KICA decarboxylation had increased by 18% and returned to baseline over the subsequent 6 months. In patients whose KICA BT did not return to baseline within 6 months following RFA, a recurrence of the tumor was found. The findings are difficult to explain assuming that the BT only reflects the function of hepatic mitochondria. Indeed, mitochondria isolated from HCC exhibit a defective ATP synthesis, indicating mitochondrial alterations, which may be part of a larger metabolic remodeling process[59]. Destroying tumor cells by either method employed in the study would be expected to decrease KICA decarboxylation, concomitant with the amount of destroyed liver cell mass. Why KICA decarboxylation increased after one procedure and decreased out of proportion to the destroyed tissue in the other remains open to speculation. In this direction, the search for a correlation between mitochondrial function assessed by BT with markers of liver mitochondrial biogenesis in patients with HCC before/after treatment might add important information.

Uncertainties

BT interpretation is still not straightforward. Confounding variables must be carefully considered when assessing the pathophysiological relevance and quantitative implications of test results. With orally administered substrates, gastric emptying, bioavailability and hepatic first pass metabolism alter the kinetics of the administered substrates. Competing pathways of elimination and metabolism of the test compounds to metabolites other than CO_2 and 'competing' mitochondria, i.e. mitochondrial metabolism in organs other than the liver, may also influence the test results. When using substrates that also occur endogenously, these will have an impact on the concentration of the tracer and thereby influence the amount of tracer that is metabolized by mitochondria. The exogenous labeled methionine will be diluted in a larger pool of unlabelled methionine, thus resulting in a lower enrichment of the amount metabolized by mitochondria[47]. Even if hepatic mitochondria on such patients decarboxylate the same amount of methionine, a BT based on exogenous methionine will result in an erroneously lower exhalation of labeled CO_2 due to

the dilution of the tracer. Finally, the concentration of labeled CO_2 in breath will depend on the amount of CO_2 that is excreted in breath or ending up excreted as bicarbonate in urine, plus how unlabelled CO_2 varies from subject to subject[60]. These variables influencing the test result are difficult to control. Disease- or intervention-related changes in BT results are therefore not necessarily reflecting changes in mitochondrial function and conclusions as to the pathophysiology of observed changes in the results of the BT must be drawn with caution. Nevertheless, BTs may still have clinical utility in regard to diagnosis, prognosis or control of treatment effects if they can be adequately validated.

Perspectives

A considerable variability of the results is common to all clinical studies using BTs. Statistically significant differences can be observed between groups formed by established criteria such as histology or ethanol exposure, but the predictive value of a single test result is likely to be limited in the individual patient. This is not surprising considering the many variables other than mitochondrial function that will influence the test result and that are difficult to control such as rate and extent of absorption, the endogenous pool of substrate and competing pathways of metabolism. Overall, the data confirm that liver steatosis results in a decreased decarboxylation of KICA and methionine, pointing to a mitochondrial dysfunction in all pathogenic steps of fatty liver[61,62]. Independently of the underlying pathogenetic mechanisms, the predictive value of BT so far actually does not allow to make clinical decisions based on the test results. Thus, one challenge to the clinician is an early detection of mitochondrial dysfunction in liver pathologies, making further studies needed for the potential implication of easily performed BT to select outpatients requiring consultation.

Conflict of interest

The Authors declare that they have no conflict of interests.

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