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^{13}C-Breath Tests in the study of mitochondrial liver function

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Abstract. – **Breath tests for "dynamic" liver function evaluation have been proposed several years ago.**

A variety of carbon-labelled breath tests for the assessment of mitochondrial, microsomal and cytosolic liver function have been described with the aim to increase data on liver disease staging, prognosis, and response to therapy. In the last years a great interest is developed about the use of breath test for liver mitochondrial function evaluation since it results impaired in a wide range of liver diseases either of genetic or acquired origin. In these cases mitochondrial oxidative metabolism of some substrates, as far as recovery of the hepatic energy state after a metabolic insult, results impaired because of the disfunction of the electron transport chain and/or ATP synthesis.

Ketoisocaproic acid and methionine are the best studied carbon-labelled substrates for the investigation of mitochondrial functional damages related to structural alterations that occur in many liver diseases. Although these tests are simple, cost-effective and safe, to date there is still not general approval for their usefulness in clinical settings since they should fulfill several requirements to overcome the drawbacks of traditional quantitative tests.

On the other hand, this field is relatively young and further studies are needed in order to assess the suitable substrate for the evaluation of the complex mitochondrial metabolism both in healthy subjects and in patients with liver disease.

Key Words:

Liver, Mitochondrial function, Breath test.

Introduction

The assessment of liver function is usually performed using tests that provide accurate informations about diagnosis, severity estimation, prognosis and therapy evaluation in patients with liver disease[1]. Since the liver accomplishes a huge number of functions of paramount importance, such as carbohydrate, protein and lipid metabolism, xenobiotics detoxification, synthesis and bile excretion, it is probably impossible to obtain a single test able to evaluate these different biochemical pathways. Even if several biochemical tests can be used, none of these has enough specifity and sensibility to be considered as an "ideal" test and, for this reason, the search for the best test has been ongoing for years[2].

Conventional static biochemical liver tests, such as aminotransferase, bilirubin, alkaline phosphatase and albumin plasma levels or prothrombin time, cannot be regarded as a reliable marker of global liver function and, therefore, a tool to quantify functional hepatic reserve and patients prognosis or to reflect life-threatening complications of acute and chronic liver diseases. Portal hypertension, fluid disturbances, hemodynamic abnormalities, renal failure and hepatic encephalopathy, complications that can determine the outcome of the disease, are only unspecifically reflected by the alterations of liver function tests. As demonstrated by the Child-Turcotte and Pugh scoring system, currently the most widely employed tool to discriminate liver disease severity, only a concerted evaluation of clinical and biochemical data, can assess disease severity and patients prognosis in hepatology[2,3]. Each of Child's classes, however, includes fairly heterogeneus patients population with a wide range of individual risk.

To improve these shortcomings, over recent decades have been proposed several dynamic tests that, although reflecting a specific function, can provide useful information

about the functional liver cell mass. The opportunity to do serial measurements that can monitorize the course of chronic liver disease would provide a valid tool to establish disease severity and to assess prognosis.

Liver dynamic tests are founded on the measurement of the concentration in plasma or metabolite formation of a given exogenous substance, which is mostly, or better exclusively, metabolized or eliminated by the liver. Liver's capacity to metabolize or eliminate the exogenous substance, evaluated at a given time, reflects the concept of "hepatic functional mass"[4-9].

It is possible to obtain tests providing information on liver blood flow ("flow-limited" test compounds) and tests that explore hepatic metabolic capacity ("enzyme-limited" test compounds)[10].

An "ideal" substrate able to assess global human hepatic function need to fulfill the following criteria: rapid and consistent absorption if administered orally, exclusive hepatic metabolism, simple pharmacokinetics, low hepatic extraction, well-known metabolic pathway, safe, minimal interaction with extra-hepatic pathologies, environmental and genetic factors, fairly rapid metabolite appearance in blood, breath, saliva or urine, easy and cheap to prepare, perform and analyse. By means, it is clear that a single dynamic liver test could not be enough to provide reliable informations about chronic liver disease[2].

A large battery of dynamic tests have been proposed, including dye clearance tests, galactose, sorbitol, antipyrine and caffeine clearance tests, urea synthesis and the monoethylglycinexylidide test[9,11-17].

These "quantitative" tests, allowing prediction of death and assess disease severity, although in appearance effective to explore liver function are time-consuming, complex, expensive, invasive and, sometimes, associated with the occurrence of side effects. In addition, during severe chronic liver disease, abnormalities in portal circulation and hepatic blood flow, changes in renal function may influence the reliability of such tests.

The introduction of breath testing in hepatology, utilizing carbon-labelled compounds (^{14}C or the stable isotope ^{13}C), have been provided a new and potentially important chance in the study of liver dynamic properties[18].

Breath tests using carbon-labelled compounds share the principle that a subject is administered a given test compound in which the common ^{12}C atom of a functional group has been replaced by the radioactive ^{14}C or stable ^{13}C isotope. The functional group is then enzymatically cleaved and undergoes further metabolic processes up to labelled CO_2 production. Finally, labelled CO_2, after mixing with the bicarbonate central body pool, is then expired. Depending on the location of the speed-determining enzyme (rate-limiting step) of the metabolic process, information may be obtained with regard to different physiological and pathological metabolic pathways (than it is possible to explore microsomal, mitochondrial and cytosolic function of liver cells)[19]. In the past, these tests were generally performed with radioactive ^{14}C-labelled substrates but, the potential radiation hazards, especially for pregnant women and children, shifted interest to the development of non-radioactive, stable, ^{13}C-labelled substrates, the ^{13}C enrichment of expired carbon dioxide being analysed by means of isotope ratio mass spectrometry (the reference technique)[20-22].

With regard to the liver metabolic function, the appearance of $*CO_2$ in breath after *C-substrate administration means that the administered substance has undergone liver oxidation, thus reflecting the investigated function (microsomal, cytosolic, mitochondrial).

Several specific breath tests have been introduced for the non-invasive assessment of human hepatic function (Table I): in particular, ^{13}C-labelled aminopyrine, phenacetin, methacetin, caffeine, diazepam and erythromycin breath tests have been proposed

Table I. ^{13}C-Substrates used in hepatolgy.

Substrate	*Liver function explored*
α-ketoisocaproic acid	Mitochondrial
Methionine	Mitochondrial
Aminopyrine	Microsomal
Phenacetin	Microsomal
Methacetin	Microsomal
Caffeine	Microsomal
Diazepam	Microsomal
Erythromycin	Microsomal
Galactose	Cytosolic
Phenylalanine	Cytosolic

for the assay of different cytochrome P450 enzymatic systems of liver microsomes[23-28], whereas ^{13}C-labelled phenylalanine and galactose breath tests look promising for the assessment of different liver cytosolic pathways[29,30]. Finally, breath tests utilizing substrates producing $*CO_2$ during liver mitochondrial metabolism, such as α-ketoisocaproic acid and the amino acid methionine, have been proposed for the assessment of hepatic mitochondrial function *in vivo*[31-34].

Breath tests for liver function evaluation are disarmingly simple, safe, cost effective tools, which can provide useful informations on liver disease severity and patients prognosis. In addiction, the fact that they do not require repeated blood sampling represent a further advantage. However, more studies are needed to identify the best substrate for the investigation of hepatic metabolic pathways, and to evaluate the usefulness of breath test in increasing accuracy of conventional tests in the staging and prognosis of liver disease.

This review aims to summarize relevant currently available data about the study of liver mitochondrial function as assessed by breath test.

Test for Evaluation of Liver Mitochondrial Function

Hepatic mitochondrial function, essential for liver cells live, result impaired in a wide range of liver diseases, either of genetic or acquired origin (e.g., Reye's syndrome, acute fatty liver of pregnancy, alcoholic liver disease, liver injury from xenobiotics, liver cirrhosis, primary non-function after liver transplantation)[35,36]. Studies conducted on human beings and animal models with chronic liver cirrhosis revealed a variety of alterations of mitochondrial structure and function in comparison with controls.

Respect to different aetiology of chronic liver disease, oxidative metabolism of various substrates as far as recovery of the hepatic energy state after a metabolic insult, is impaired as a result of the disfunction of the electron transport chain and/or ATP synthesis. Hepatic mitochondria of patients with alcoholic liver disease show, in fact, morphological and functional alterations (as pleomorphism, megamitochondria, crystalloid inclusions and reduction of cristae)[37,38].

Sudies performed in animal models of alcoholic injury showed a decreased respiratory capacity and impaired oxidative phosphorylation of liver mitochondria after ethanol administration[39].

Moreover, mitochondria are premature targets of toxicity from a number of xenobiotics. The adverse effects of several clinically useful compounds, such as acetylsalicylic acid (ASA), amiodarone, valproate, amineptine, mexiletine and fialuridine, are thought to be due to drug-induced deranged mitochondrial function[40,41].

However, the functional consequences of the structural mitochondrial dysfunctions are still object of intense study, as far as the possibilities to probe hepatic mitochondrial function reserve without complex and often invasive methods. Availability of simple, non-invasive tests for the quantification of the hepatic mitochondrial function *in vivo* could be extremely useful for prognostic evaluation and therapeutic choices of patients with acute or chronic liver disease. In spite of this, methods available for the assessment of hepatic mitochondrial function *in vivo* are mostly invasive and/or complex: the acetoacetate/β-hydroxybutyrate ratio in arterial blood, metabolism of benzoic acid, hepatic nitrogen clearance determination and ^{31}P-nuclear magnetic resonance spectroscopy are some examples[42-45] (Table II).

A valid alternative to these complex tools is represented by ^{13}C-breath test utilizing given exogenous substances mainly metabolised by hepatic mitochondria. Among the best studied substrates producing CO_2 during mitochondrial metabolism we can include α-ketoisocaproic acid and methionine.

Table II. Methods available for the assessment of hepatic mitochondrial function.

Methods for the study of hepatic mitochondrial function
Acetoacetate/β-hydroxybutyrate ratio in arterial blood
Metabolism of benzoic acid
Hepatic nitrogen clearance determination
^{31}P-nuclear magnetic resonance spectroscopy
Breath test

Ketoisocaproic Acid (KICA) Breath Test

The KICA-breath test assesses the decarboxylation of KICA which occurs almost exclusively in hepatic mitochondria by following the exhalation of labelled CO_2 after the administration of labelled KICA[46].

Ketoisocaproic acid (a branched-chain α-ketoacid) undergoes two main enzymatic pathways: oxidative decarboxylation by a branched-chain α-ketoacid dehydrogenase complex located exclusively in mitochondria, or conversion via transamination into the corresponding branched-chain amino acid leucine[47].

The human branched chain 2-keto acid dehydrogenase complex is located not only in liver, but also in the pancreas, kidney, brain, skeletal muscle and adipose tissue. In particular, muscle contains large amounts of branched-chain 2-oxoacid dehydrogenase complexes but most of the enzymatic complex is phosphorylated and hence inactivate in extra-hepatic mitochondria. The enzymatic activity is dependent on the mitochondrial integrity and is regulated by several agents, such as redox status of cells, availability of CoA, or endocrin factors as insulin[48,49].

The administration of carbon labelled ketoisocaproic acid concomitant with a leucine load, necessary to inhibit KICA transamination pathway and thus increasing the rate of the decarboxylated KICA, allows the evaluation of hepatic mitochondrial function in humans. Fasting patients, at rest for 30 min prior to and during the test, receive 1 mg/kg 2-keto(1-^{13}C) isocaproic acid (sodium 1-^{13}C ketoisocaproic acid, 99 atom % enriched) together with 20 mg/kg L-leucine dissolved in 200 ml of orange juice[50].

The capacity of KICA-breath test to discriminate sub-toxic and reversible mitochondrial dysfunction, previously demonstrated in rats utilizing ^{14}C-α-ketoisocaproic[46], has been evaluated in healthy subjects with the study of acute effects of socially consumed amounts of ethanol and acetylsalicyc acid on mitochondrial function.

Lauterburg et al. measured the decarboxylation of KICA in healthy volunteers following ingestion of 0.5 g/kg of ethanol or 30 mg/kg of acetylsalicylic acid, respectively. Although the ^{13}C enrichment of circulating KICA and leucine were similar in the presence or absence of ethanol, the decarboxylation of KICA was significantly lower ($p < 0.01$) at each time point in the presence of ethanol (the metabolism of which induces a decrease in the ratio of the oxidized to reduced forms of nicotinamide adenine dinucleotide NAD^+/NADH). On the contrary, the ingestion of acetylsalicylic acid (the metabolism of which induces an increase in the NAD^+/NADH ratio) significantly increased the decarboxylation of KICA[51].

The ability of KICA breath test to detect sub-toxic effects of xenobiotics on mitochondria, has also been tested to assess the effect of lamivudine, a nucleoside analogue which acts as active inhibitor of hepatitis B viral replication, on mitochondrial function of patients affected by chronic hepatitis B. In this study normal KICA breath test values concorded with the absence of signs of toxicity assessed by routine histological evaluation and by electron-microscopic studies of mitochondria in liver biopsy specimens[52].

Gabe et al. evaluated the effect of tacrolimus (FK506), a potent immunosuppressive drug that decreases mitochondrial adenosine triphosphate production and increases intestinal permeability in animals, in patients undergoing orthotopic liver trasplantation by using a combined absorption-permeability-mitochondrial function test (5 g lactulose, 1 g L-rhamnose, 0.5 g D-xylose, 0.2 g 3-O-methyl-D-glucose, 1 mg/kg ^{13}C-KICA, and 20 mg/kg L-leucine). They observed that tacrolimus inhibited the decarboxylation of KICA and that the resting energy expenditure in proportion to drug exposure (area under the concentration–time curve over 2 or 5 hours) is consistent with an acute inhibition of mitochondrial energy production[53].

The possibility to investigate alterations of mitochondrial function with a simple and non-invasive test, able to evidence metabolic changes related to xenobiotics led to the opportunity to use KICA breath test in the work up of alcoholic liver disease.

Lauterburg et al. investigated mitochondrial dysfunction assessed by KICA-breath test in patients with alcoholic liver disease in comparison with non-alcoholic liver disease and healthy controls. Mitochondrial function was evaluated by measuring the exhalation of $^{14}CO_2$ after administration of 2-keto-1-^{14}C-

isocaproic acid. Interestingly, alcoholic patients showed a significant decrease in both the $^{14}CO_2$ peak exhalation and the fraction of the administered dose exhaled as $^{14}CO_2$ after 1 hour, despite normal conventional and quantitative liver function tests. Similar results were obtained when they used ^{13}C-KICA breath test instead of ^{14}C-KICA[31,32]. Moreover, in alcoholic patients followed during the time, the impaired decarboxylation of KICA was partially reversible upon astinence. The authors suggested that, since patients with non alcoholic-liver disease had normal values at KICA breath test, the impairment of KICA decarboxylation in alcoholics could not be attributed to the loss of functional hepatic mass, but to the ethanol-induced decrease in the activity of the branched-chain a-ketoacid dehydrogenase complex. In fact, even when they compared patients with similar severity of liver disease, as assessed by galactose elimination rate and aminopyrine breath test, patients with non-alcoholic liver disease showed a significantly higher peak exhalation of $^{13}CO_2$ and fraction of the dose decarboxylated in two hours than alcoholics.

KICA breath test has been proven to be helpful in the characterization of hepatic steatosis, distinguishing between alcoholic and non-alcoholic fatty liver disease. Mion et al. studied mitochondrial function, based on ^{13}C-KICA breath test in patients with alcoholic and non-alcoholic steatosis compared with healthy volunteers. Only patients with alcohol related steatosis showed a significant reduction (42%) of ^{13}C-KICA decarboxylation[54].

However, in contrast with these data, successively Bendtsen *et al.* showed the uselessness of KICA-decarboxylation as marker of ethanol abuse. They compared the ^{13}C-KICA breath test values of patients affected by severe alcohol dependence with healthy volunteers: no differences were found between alcoholic patients and healthy subjects, nor between patients with alcoholic hepatitis or steatosis and controls. Moreover, healthy women had a higher percentage exhalation of $^{13}CO_2$ than both healthy males and alcoholic males[55].

The complex regulation of the activity of the branched-chain α-ketoacid dehydrogenase explains at least partially these conflicting results: ethanol might influence other determinants of KICA decarboxylation as the phosphorylation state of the enzyme complex, the availability of coenzyme A, intramitochondrial calcium levels and hormonal status[56]. Moreover, although functional and structural alterations of mitochondria during ethanol abuse have been described only in liver, the main site of KICA decarboxylated, extrahepatic contributions to the impaired KICA metabolism in alcoholic patients cannot be underrate.

Although KICA decarboxylation results impaired in chronic alcholics independently from decreased microsomial or cytosolic function, the possibility to use KICA breath test as a marker of liver mitochondrial function in chronic alcoholics has never gained general acceptance. Contrasting data may be related to the complex metabolic activity of the branched-chain α-ketoacid dehydrogenase, remarkablly influenzed by a wide number of factors. Moreover, monitoring of xenobiotic-induced toxic effects on mitochondrial activity by KICA breath test appears promising, but needs further studies to understand if and when interindividual variability and environmental factors may influence the results.

Methionine breath test

Methionine is an essential amino acid mostly metabolised via two major pathways by the liver, since most other tissues lack one or more of the enzymes involved in these complex processes. In physiological conditions methionine is converted via the trans-sulfuration pathway into homocysteine (a high-energy sulphonium compound *S*-adenosyl-L-methionine) by methionine adensyltransferase. The alternative pathway consists in the transamination to α-keto-β-methiolbutyric acid, but it does not occur under normal metabolic conditions. Therefore, methionine breath test could explore different metabolic pathways, and thus hepatic mitochondrial function, in relation to the labelled carbon ($^{14/13}$C) unit of the methionine molecule administered. Using 3- or 4-carbon labelled methionine it is possible to follow the amino acid metabolism through the the trans-sulphuration pathway, in which

the carbon chain is released as α-ketobutyrate and further metabolized to CO_2 via the tricarboxylic acid cycle. Instead, if 1-carbon-labelled methionine is administered, the excess of ^{13}C recovered in the breath shoul indicate the activity of liver mitochondrial α-ketobutyrate decarboxylase, assuming that this enzyme is the rate-limiting step in the substrate oxidation to CO_2[57,58].

Moreover, the administration of the methyl-*C-labelled methionine could estimate the oxidative capacity of liver mitochondria, because it has been shown that the most important mechanism for removing the excess of methionine methyl groups is via sarcosine production and further mitochondrial oxidation[59].

On the basis of these considerations and because of the cheaper availability of the methyl-^{13}C-labelled oral tracer with respect to other ^{13}C-labelled methionine molecules, our group investigated the feasibility of a breath test with methyl-^{13}C-methionine (2 mg/kg body wheight) to assess hepatic mitochondrial function in healthy subjects before and after ethanol-induced oxidative stress. Methionine breath test values were significantly decreased after ethanol intake; however, the low percentage of ^{13}C recovered over the test period and the inter-individual variations pointed to an incomplete recovery of tracer in breath and to a major flux of methionine labile methyl groups towards different metabolic pathways[60].

The potential usefulness of the methionine breath test for the detection of toxic effects of xenobiotics on liver mitochondria has also been reported by Spahr et al. They used a breath test with 1-^{13}C-methionine to monitor mitochondrial functional changes in a patient with biopsy-proven, acute, valproate-associated microvesicular steatosis: the initially abnormal breath test improved togheter with the recovery of liver failure suggesting that ^{13}C-methionine breath test may provide a noninvasive monitoring of hepatic mitochondrial function *in vivo*[61]. The same authors used a breath test with 1-^{13}C-methionine to explore mitochondrial oxidation in patients with pure non-alcohl related severe macrovescicular steatosis and in patients with cirrhosis. Exhalation of $^{13}CO_2$ was reduced both in patients with steatosis and in patients with cirrhosis of mixed etiologies; moreover in the latter group dose/h correlated to aminopyrine breath test value and was inversely correlated to Child-Pugh score[34].

Recently, our group evaluated the relationship between graft viability, mainly related to the energy production of hepatic mitochondria and to the drug metabolism capability at the microsomal level, and the time course of aminopyrine and methionine breath test in the early phase following liver transplant. In all patients with successful OLTx the cumulative percentage of the dose of ^{13}C progressively increased after transplantation, reaching values not significantly different from controls (methionine at day 5). In the two patients primary non function occurred: in these patients the cumulative percentage of the ^{13}C dose did not increase after the ortotopic transplant and the result of both breath tests indicated that it always remained significantly lower than those observed in successfully transplantated patients at all time points. As a result, a combination of breath tests exploring both mitochondrial and microsomal pathways, impaired because of the metabolic debt accumulated during the graft preservation and implantation phases, could be useful in the early phase after OLTx to estimate the graft outcome[62]. Today, only few data document the uselfull of ^{13}C-methionine breath test in hepatology than further investigation is needed in order to confirm the possible predictive value of MBT in clinical setting. Therefore, complex metabolic pathways of this amino acid need to be studied with the aim to identify which of the different ^{13}C-labeled methionines best reflects mitochondrial metabolism in basal conditions and in liver disease.

New Substrates

Preliminary studies have suggested the possibility to assess mitochondrial β-oxidation by means of sodium octanoate [$CH_3(CH_2)_6CO_2Na$], a medium chain fatty acid. This new substate has physical and chemical features that make it a good alternative to classical mitochondrial substrates. First of all, it is completely absorbed from the intestinal lumen; it is also able to enter liver mitochondria independently from the carni-

tine transport system and is metabolized through β-oxidation, producing acetyl coenzyme A (CoA) that is finally metebolized leading to the production of CO_2.

In two recent studies ^{13}C-sodium octanoate breath test was used to assess acute oxidative stress in healthy subjects and in patients with NASH[63,64]. In both cases ^{13}C-octanoate breath test was able to assess mitochondrial β-oxidation. In the second study this breath test confirmed the enhanced mitochondrial β-oxidation found in *in vitro* studies[65].

Conclusion

Several "liver function" breath tests have been developed in the last three decades, assessing the specific microsomal, cytoplasmic or mitochondrial metabolism of hepatic substrates. The widespread of spetroscopic methods for measuring $^{13}CO_2$ means that liver function testing by a breath test procedure can be performed in the course of an hour, in an incredible safe, easy and potentially accurate way. Furthermore interesting data have been obtained from studies in which breath tests have been used to monitor the progression of liver disease, to predict the long-term prognosis, efficacy of therapy, risk of surgical interventions and optimal timing of liver transplantation and sub-toxical effects of xenobiotics.

In particular, KICA and methionine breath tests appear promising in the work up of the several diseases that may impair the complex and sensitive oxidative metabolic pathways of mitochondria as it has been showed in several studies.

However, today a breath test that measures hepatic function better than the conventional liver tests and the Child-Pugh classification has not been described. A general dissatisfaction and uncertainty amongst hepatologists, together with the lack of commercial sponsorship, explain the restricted use of these tools to still few medical centre.

Therefore, further well-conducted studies are needed to define the real usefulness of hepatic function assessed by breath test in comparison with standard methods, as to obtain a general approval that allows a rationale use of them in clinical setting.

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