

## CLINICAL INVESTIGATION OF LIVER MITOCHONDRIAL FUNCTION: A ROLE FOR <sup>13</sup>C-STABLE ISOTOPE BREATH TESTS?

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### Abstract

*Assessing in vivo mitochondrial function in patients with liver diseases helps the study of specific hepatic metabolic functions and the development of more rational diagnostic, prognostic and therapeutic approaches. Specific breath tests use substrates (e.g. alpha-keto-isocaproic acid and methionine) marked with the natural stable isotope (<sup>13</sup>C). Following oral ingestion, substrates, are decarboxylated by liver mitochondria and time-dependent concentrations of the derivative <sup>13</sup>C-CO<sub>2</sub> can be measure in breath samples by mass spectrometry. Long and medium chain fatty acids that are metabolized through the Krebs cycle and benzoic acid which undergoes glycine conjugation may also reflect the function of mitochondria. We discuss the utility of this novel diagnostic approach, i.e. stable isotope breath tests to assess mitochondrial function for potential applications in clinical practice.*

**Keywords:** Breath test, Chronic liver disease, Mitochondria.

**Abbreviations:** BT, breath test; KICA, alpha-ketoisocaproic acid; MeBT, <sup>13</sup>C-Methionine breath test; mtDNA, mitochondrial DNA; NASH, nonalcoholic steatohepatitis.

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### Introduction

Mitochondria are heavily involved in the metabolism of carbohydrates, proteins, lipids and xenobiotics and are the major cellular source of energy. Dysfunction of mitochondria contribute to the onset and progression of chronic liver diseases [1]. Mitochondrial dysfunction is thought to play key role in the progression of fatty liver to steatohepatitis and eventually cirrhosis, as well as in drug-induced liver injury following a number of therapeutic agents [2]. Assessment of hepatic mitochondrial function might provide more insight into the pathogenesis of liver diseases and might provide clinically relevant information regarding effects of drug on mitochondria and regarding diagnosis and prognosis of acute and chronic liver diseases.

### Dynamic tests of mitochondrial function – breath tests

Dynamic tests of mitochondrial function imply that known quantities of labeled exogenous substrates that can easily be traced are administered and their metabolism is quantitatively and qualitatively followed. If a given administered substrate is exclusively metabolized by hepatic mitochondria, metabolites generation will reflect mitochondrial function [3]. The hepatic clearance of a variety of exogenous substances can be used to study specific metabolic pathways, to predict drug metabolism, to estimate the prognosis of liver disease and to assess the outcome of treatment [4]. If the hepatic metabolism of a test compound results in the formation of carbon dioxide CO<sub>2</sub> and the appropriate C atom is labeled, the exhalation of labeled CO<sub>2</sub> (which is measurable in mass spectrometry), reflects the hepatic clearance of the original

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labeled compound [5]. Breath tests have several advantages which include: the use of naturally occurring  $^{13}\text{C}$  and safe substrates, easy procedures which can be repeated several times in the same patient and in critically ill patients and end-stage liver diseases, possibility to follow-up different liver diseases, and to be adapted also in infants and pregnant women. Potential pitfalls, however, include long duration of the test (at least 2 hrs), need for patient's supervision by MDs, costs of substrates, and use of expensive equipment (i.e. mass spectrometry). Labelled alpha-ketoisocaproic acid (KICA), methionine and octanoic acid have been utilized as substrates in clinical research following more or less extensive validation in animal models, and, albeit the results clearly discriminate between different patients populations, none of them has been currently approved for clinical use. The decarboxylation of KICA is a specific function of mitochondria [3]. In the KICA BT the major competing pathway for the elimination of KICA, the transamination to leucine, must be suppressed by the concomitant administration of leucine. The utility of this test has been shown in experimental models [3], isolated mitochondria [6], and in healthy subjects [7]. Another substrate employs  $^{13}\text{C}$ -Methionine (MeBT): transmethylation of methionine results in the removal of the labeled methyl group if [methyl- $^{13}\text{C}$ ]-methionine is used as substrate. Either L-(1- $^{13}\text{C}$ ) methionine or [methyl- $^{13}\text{C}$ ]-methionine are used. **Acute ethanol consumption** impaired the decarboxylation of  $^{13}\text{C}$ -methionine in healthy volunteers [8]. In a case of valproic acid intoxication with microvesicular steatosis of the liver, a consequence of mitochondrial toxicity, the MeBT was impaired and recovered in parallel to the liver damage, suggesting that the test indeed reflects mitochondrial function in patients [9]. An additional substrate is octanoate, a medium chain fatty acid that enters mitochondria independently of the carnitine transport system. Within mitochondria, octanoic acid undergoes  $\beta$ -oxidation generating acetyl coenzyme A which enters the Krebs cycle and is oxidized to  $\text{CO}_2$  unless it is utilized for the synthesis of other energy-rich compounds. The decarboxylation of octanoate was decreased in rats developing thioacetamide-induced acute hepatitis and liver cirrhosis but not in cholestatic liver injury following bile duct ligation [10]. Interpretation of results is not straightforward, and problems do exist. Potential limitations for the interpretation of breath test results include: the presence of confounding variables (e.g. exercise), the typology of gastric emptying kinetics, the hepatic first pass metabolism of the administered substrates, the presence of competing pathways of elimination and metabolism of compounds. Furthermore, one should take into account additional factors like the presence of mitochondrial metabolism occurring in organs other than the liver, possible dilution of exogenous labeled methionine in a larger pool of unlabelled methionine, and endogenous production of unlabelled  $\text{CO}_2$  which can vary substantially from subject to subject.

### **Assessment of drug effects on mitochondrial function by $^{13}\text{C}$ -breath tests**

Drugs may impair mitochondrial function, and cumulative mitochondrial damage could provoke adverse drug effects [11]. For example, mitochondrial ATP production in animals is reduced by tacrolimus, an immunosuppressive drug. In humans, tacrolimus inhibits the decarboxylation of KICA, the resting energy expenditure and the respiratory rate in a dose-dependent manner, suggesting inhibition of mitochondrial respiration [12]. **Nucleoside analogues are partly incorporated into mitochondrial DNA (mtDNA) with adverse effects suggesting mitochondrial dysfunction.** Nucleoside analogues, because of their similarities with nucleosides, hind the replication process by inhibition of mitochondrial  $\gamma$ -DNA-polymerase. **Whether anti-retroviral therapy in HIV-infected subjects impairs mitochondrial function is controversial, so far.** In one study antiretroviral-treated HIV-positive patients undergoing MeBT had decreased decarboxylation of methionine compared with healthy controls and untreated subjects, especially in those patients with hyperlactatemia [13]. Other breath test studies in HIV patients reported a relationship between decreased decarboxylation of methionine and hypertriglyceridemia, hepatic steatosis, macrocytosis and age, rather than to drug therapy [14,15]. Indeed, HIV infection may also directly impair mitochondrial function: in a study [16], breath test was better in asymptomatic patients treated on antiretroviral drugs but declined in those treated patients developing lipodystrophy. Apparent divergent findings in HIV-infected patients might depend on the different combination of drugs and their variable effects on mitochondrial function. For example, lamivudine, a antiretroviral agent (reverse transcriptase inhibitor - Nucleoside) is not incorporated into mtDNA and does not inhibit mitochondrial  $\gamma$ -DNA-polymerase. In a study in patients with chronic hepatitis B, lamivudine had no effect on the KICA BT [17]. Hepatitis viral infection itself, however, may impair mitochondrial function: **mitochondrial alterations have been documented in hepatitis C infected cells [18].** Hepatitis C patients exhibit a markedly decreased MeBT which further impaired during treatment with pegylated interferon and ribavirin and recovered when treatment ceased, reaching values above the baseline MeBT in some of the patients with sustained virological response [19].

### **Assessment of liver function by $^{13}\text{C}$ -breath tests**

Several studies have highlighted the use of BT as specific and non-invasive tool to assess mitochondrial function as a marker of liver injury in patients with non-alcoholic fatty liver and alcoholic liver disease. Abnormalities of mitochondria occur early in animals and humans when exposed to excessive and prolonged amounts of ethanol [20]. The demethylation of aminopyrine (catalyzed by cytochrome P-450 in the endoplasmatic

reticulum) and the clearance of galactose (another extra-mitochondrial cytosolic process) were not impaired early in alcoholic patients.

By contrast, single doses of moderate amounts of ethanol decreased KICA decarboxylation [7], and KICA decarboxylation was decreased in alcoholic patients compared to patients with non-alcoholic liver disease and controls [21,22]. These data, although not always confirmed [23], indicate that KICA does reflect the mitochondrial function. KICA BT could also discriminate between patients with non-alcoholic liver steatosis of and those with alcoholic steatosis [24], although the extent of fat accumulation in the liver was not further investigated in this study. Indeed, the extent of steatosis may influence BT performance, since decarboxylation of methionine was decreased in patients with **biopsy-proven severe steatosis** involving over 40% of hepatocytes [9]. In a recent study from our group, KICA decarboxylation was impaired in patients with advanced nonalcoholic steatohepatitis (NASH) but not in those with simple steatosis detected by liver histology [25]. The impaired metabolism of KICA was inversely related to the stage of fibrosis. Obese control subjects had normal KICA decarboxylation, but obesity in those patients with NASH independently contributed to the further decrease in the KICA BT. Thus, obesity *per se* does not affect the KICA BT [26], although it has been suggested that adaptive modifications in mitochondrial function may occur in response to caloric restriction [26]. In patients with different stages of NASH the oxidation of octanoate was unchanged [27] or even increased [28]. The metabolism of octanoate was also unchanged in patients with early stage or advanced cirrhosis with and without porto-systemic shunt [29]. Subtle differences in the metabolic pathways of different substrates or extra-hepatic mitochondrial oxidation of octanoate might explain such discrepancies. Another field of application of breath tests is liver cirrhosis and hepatocellular carcinoma (HCC). We have recently shown that liver mitochondrial function, as assessed by KICA BT, was decreased in cirrhotic patients with HCC suggesting a possible tumor-induced suppressant effect [30]. Radiofrequency ablation but not transarterial chemoembolization appears to spare residual (microsomal) liver mass, but induces such a transient stunning effect on mitochondrial function. Improved mitochondrial function after 1 and 6 mo from radiofrequency ablation may represent an additional parameter of treatment efficacy. Therefore, breath test assessing liver function may have potential applications in HCC management, and might predict prognosis after a therapeutic challenge. Indeed, mitochondria isolated from HCC exhibit a defective ATP synthesis, indicating mitochondrial dysfunction [31]. Searching for a correlation between mitochondrial function (by breath tests) and markers of liver mitochondrial biogenesis in patients with HCC before/after invasive procedures might provide useful additional information of potential prognostic value.

### Perspectives

Many variables other than mitochondrial function are potentially able to influence the results of breath tests in patients with liver diseases. So far, marked steatosis and advanced NASH independent of ethanol consumption will result in a decreased decarboxylation of KICA and methionine. Mitochondrial dysfunction has been proposed to be involved in all pathogenic steps leading to fatty liver and alcoholic and non-alcoholic steatohepatitis [32]. The results of the breath tests, although consistent with the hypothesis that mitochondrial dysfunction plays a key role in the progression of liver damage, do not allow conclusive clinical decisions. Further studies are warranted also for the potential implication of easily performed breath test to select outpatients requiring consultation.

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