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Review article: breath testing for human liver function assessment

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SUMMARY

Carbon-labelled breath tests were proposed as tools for the evaluation of human liver function 30 years ago, but have never become part of clinical routine. One reason for this is the complex role of the liver in metabolic regulation, making it difficult to provide essential information for the management of patients with liver disease with a single test and to satisfy the hepatology community. As a result, a battery of breath tests have been developed. Depending on the test compound administered, different metabolic pathways (microsomal, cytosolic, mitochondrial) can be examined. Most available data come from microsomal function tests, whilst information about cytosolic and mitochondrial liver function is more limited. However, breath tests have shown promise in some studies, in particular to predict the outcome of patients with chronic liver disease or to monitor hepatic function after treatment. Whilst we await new substrates that can be used to measure liver function in a more valid manner, and large prospective studies to assess the usefulness of available test compounds, the aim of this review is to describe how far we have come in this controversial and unresolved issue.

INTRODUCTION

Function tests in hepatology should provide accurate information about diagnosis, severity estimation, prognosis assessment and therapy evaluation in patients with liver disease.[1] Unfortunately, the 'ideal' test does not exist and the search for the best test has been ongoing for years. The main reason is probably the huge number of functions accomplished by the liver, which render hepatic function assessment a very complex challenge. Carbohydrate, protein and lipid metabolism, the synthesis, storage and secretion of new products into the blood and bile, and xenobiotic detoxification are, in fact, only some of the important major functions of the liver, thus explaining the difficulties and also probably the impossibility of obtaining a single test to assess these different metabolic pathways.

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Conventional static biochemical liver tests, such as transaminase, bilirubin, alkaline phosphatase and albumin plasma levels or prothrombin time, provide information as a mixture of injury and function, but none may be regarded as a reliable marker either to quantify functional hepatic reserve or to reflect life-threatening complications of acute and chronic liver diseases. To improve these shortcomings, several dynamic tests have been proposed over recent decades. Although each of these tests, exploring a specific function, can provide useful information about the functional hepatic mass, they have not been widely used in clinical practice.[2] As a result, the Child–Turcotte and Pugh scoring system,[3, 4] which was developed several years ago as a concerted evaluation of clinical criteria and laboratory data, currently is considered to be the most important tool for the staging of liver disease by the degree of impaired hepatic function.

The general principles of liver dynamic tests are founded on the administration of a given exogenous substance, which is mostly metabolized or eliminated by

the liver, and on the subsequent measurement of the substance concentration in plasma or metabolite formation.[5] These tests allow the determination, at a given time, of the liver's capacity to metabolize or eliminate the exogenous substance, thus reflecting the concept of 'hepatic functional mass'. The basic principle for most of these tests is the hepatic clearance (C, i.e. the test substance is primarily removed by the liver), which is related to the hepatic perfusion (Q) and the extraction ratio [E, i.e. the ratio of the difference between the inflow (c_i) and outflow (c_o) substance concentrations and the inflow substance concentration]. Therefore $C = Q \times E$, where $E = (c_i - c_o)/c_i$.

By means of these equations, it is possible to distinguish between tests providing information on liver blood flow ('flow-limited' test compounds: hepatic extraction ratio above 0.7) and hepatic metabolic capacity ('enzyme-limited' test compounds: hepatic extraction ratio below 0.3).[6]

Because of the large number of criteria which need to be fulfilled to render a given substrate 'ideal' for assessing human hepatic function (Table 1), the advent of a single dynamic test able to provide reliable information about chronic liver diseases as a whole seems unlikely.[7] However, long-term assessment of a given hepatic metabolic activity by means of a dynamic liver test could be useful to define the severity at the time of diagnosis, to check the progression or regression of liver disease as a result of intervention, or to obtain prognostic information not accessible through conventional clinical, biological and histological data.[8]

Various dynamic tests have been proposed, including dye clearance tests,[9, 10] galactose,[11] sorbitol,[12] antipyrine[13] and caffeine[14] clearance tests, urea synthesis[15] and the monoethylglycinexylidide test.[16] In addition, a battery of breath tests, utilizing carbon-labelled compounds (indicated by asterisk in text) (^{14}C or the stable isotope ^{13}C), have been investigated to examine the integrity or derangement of different liver metabolic pathways.[17]

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 (digestive and absorptive processes, presence of bacteria, activity of organ-specific organelles, etc.).[18, 19] In the past, these tests were generally performed with radioactive ^{14}C-labelled substrates, measuring the radioactivity by means of a liquid scintillation counter.[20] However, 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).[21] Results of *C-breath tests may be expressed in different ways, the manner of presentation being chosen somewhat arbitrarily and therefore varying between investigators.[22, 23] The percentage of administered dose of *C recovered per hour (% *C dose/h, the shape of the curve reflecting the dynamics of the studied process) and the cumulative percentage of administered dose of *C recovered over time (% *C cumulative dose, the shape of the curve providing information about the global studied process) are the most convenient methods of presentation.[21–23]

With regard to the liver metabolic function, the appearance of $^*CO_2$ in breath after *C-substrate administration means that the administered substance has

Table 1. Main criteria of an 'ideal' substrate for the assessment of human hepatic function

1	Rapid and consistent absorption if administered orally
2	Elimination solely dependent on hepatic metabolism
3	Water solubility and simple pharmacokinetics
4	Metabolism independent of liver blood flow (i.e. low hepatic extraction) and protein binding
5	Well-known metabolic pathway (enzymes involved, rate-limiting step)
6	Safe, with no significant pharmacological effects in both healthy subjects and patients
7	Minimal interaction with extra-hepatic pathologies, environmental (e.g. diet, nutritional state, medications) and genetic factors
8	Fairly rapid metabolite appearance (short sampling time) in blood, breath, saliva or urine
9	Easy and cheap to prepare, perform and analyse (to allow widespread use)

undergone liver oxidation, thus reflecting the function investigated (microsomal, cytosolic, mitochondrial).

Several specific breath tests have been introduced for the non-invasive assessment of human hepatic function (Table 2):[24] in particular, *C-labelled aminopyrine,[25] phenacetin,[26] methacetin,[27] caffeine,[28] diazepam[29] and erythromycin[30] breath tests have been proposed for the assay of different cytochrome P450 enzymatic systems of liver microsomes, whereas *C-labelled phenylalanine[31] and galactose[32] breath tests look promising for the assessment of different liver cytosolic pathways. Finally, breath tests utilizing substrates producing $*CO_2$ during liver mitochondrial metabolism, such as α-ketoisocaproic acid[33] and the amino acid methionine,[34] have been proposed for the assessment of hepatic mitochondrial function *in vivo*.

MICROSOMAL LIVER TESTS

Aminopyrine breath test

Historically, the aminopyrine breath test (ABT) was the first breath test proposed for the assessment of patients with liver disease.[35] At present, ABT is one of the most frequently utilized tests for probing hepatic microsomal P450 enzyme activity and investigating hepatocellular function.[36]

After administration, *C-labelled dimethylaminoantipyrine undergoes two-step *N*-demethylation through the cytochrome P450 mono-oxygenase system of liver microsomes, yielding formaldehyde and aminoantipyrine. The formaldehyde, generated by hepatic *N*-demethylation of dimethylaminoantipyrine and monomethylaminoantipyrine, is then oxidized to bicarbonate, which may either be exhaled as CO_2 in breath (about 30%) or equilibrated with the central bicarbonate pool.[37] Despite the multi-step metabolism, *N*-demethylation of aminopyrine has been documented as the rate-limiting step of a process that occurs almost exclusively in the liver,[38] and it can be assumed that ABT gives a global assessment of the P450-dependent mono-oxygenase system.[36] Moreover, because of the low hepatic extraction ratio ($E = 0.2$), aminopyrine metabolism is mostly dependent on hepatic metabolic capacity ('functional hepatic mass') rather than on portal blood flow.[6]

The clinical utility of ABT lies in its capacity to reflect hepatic residual functional microsomal mass, thus providing useful information for establishing the prognosis or for predicting the response to therapy of certain types of liver disease. It must not be considered a screening test for establishing a diagnosis, because similar impaired ABT results could be expressed by any type of liver disease.[24]

As far as chronic hepatitis is concerned, several studies have shown that ABT values are significantly reduced in patients with chronic active hepatitis, compared to healthy controls[39–41] and to patients with chronic persistent hepatitis.[23] In particular, Monroe *et al.* prospectively evaluated patients with chronic hepatitis by histology, serum bile acids, standard liver function tests and ABT.[42] They reported that 30 of 35 patients with chronic active hepatitis and bridging or cirrhosis had ABT values (% cumulative dose over 2 h) of less than 5.7%, whereas values higher than 5.7% identified 21 of 25 patients with mild chronic active hepatitis or chronic persistent hepatitis (Figure 1). Recently, Herold *et al.* have shown that, in a well-characterized cohort of 367 patients with chronic hepatitis B or C, ABT was significantly correlated with liver histology (inflammation and grading of fibrosis) and Child–Pugh score.[43] Similarly, Giannini *et al.* have reported that, in a group of patients with chronic hepatitis C or Child A cirrhosis,

Table 2. *C-labelled breath tests for human liver function assessment

Probe substrate	Hepatic function	Enzyme studied
Aminopyrine	Microsomal	P450s (CYP1A2?, 2C9?, 3A4?)
Phenacetin	Microsomal	CYP1A2 (CYP2E1)
Methacetin	Microsomal	CYP1A2
Caffeine	Microsomal	CYP1A2 (CYP2E1, 3A3, 2B6)
Diazepam	Microsomal	CYP2C19 (CYP3A)
Erythromycin	Microsomal	CYP3A (CYP3A4, A5, A7?)
Galactose	Cytosolic	Galactokinase
Phenylalanine	Cytosolic	Hydroxylase
α-Ketoisocaproic acid	Mitochondrial	Branched-chain α-ketoacid dehydrogenase complex
Methionine	Mitochondrial	Krebs cycle enzymes (?)

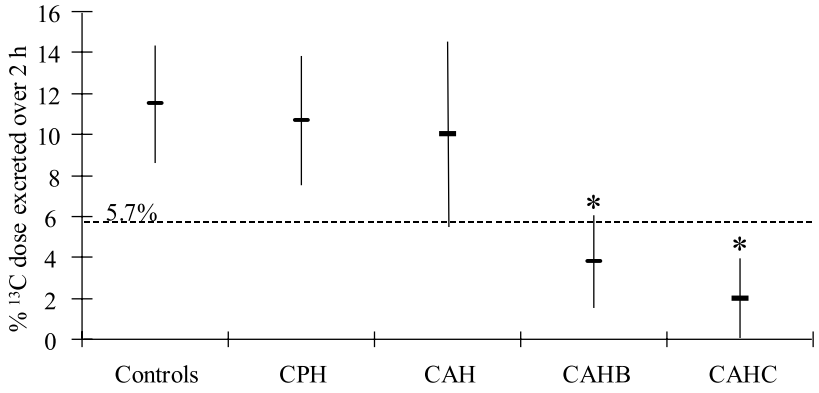

Figure 1. The aminopyrine breath test (ABT) reflects the histological severity in chronic hepatitis. ABT values (expressed as the percentage of the ^{13}C dose of aminopyrine administered recovered over 2 h; mean ± s.d.; horizontal and vertical bars, respectively) in normal subjects, patients with chronic persistent hepatitis (CPH), patients with chronic active hepatitis (CAH), patients with chronic active hepatitis with bridging (CAHB) and patients with chronic active hepatitis with cirrhosis (CAHC). *$P < 0.05$ vs. controls or CPH or CAH. Moreover, 85.7% of patients with CAHB or CAHC showed ABT results lower than 5.7%, whereas 84% of patients with CPH or CAH showed ABT results higher than 5.7% ($P < 0.001$); this value (indicated by the broken line) has a sensitivity of 0.86 and a specificity of 0.84 for the detection of severe liver disease.[42]

^{13}C-ABT values (% dose/h at 30 min) were able to discriminate between the study groups.[44] ABT also significantly correlated with the degree of fibrosis and necro-inflammatory activity. A significant correlation between ABT and portal vein velocity in a group of patients with chronic hepatitis C and mild fibrosis has also been documented.[45] These results suggest that ABT may have a complementary role to liver histology in the staging and monitoring of the evolution of disease. Only prospective studies, however, will indicate whether ABT can provide further information in patients with chronic hepatitis, which is superior to that obtained by standard laboratory tests or prognostic scores.

ABT has been used extensively in cirrhotic patients as a method of assessment of the residual functional hepatic microsomal mass and to predict the prognosis. In particular, Hepner and Vesell reported that ABT was significantly decreased in cirrhotic patients with respect to controls, with a significant correlation with the plasma aminopyrine clearance rate, serum albumin and retention of bromosulphalein.[35] They also found a strong correlation between extremely low ABT results and poor early outcome. A correlation between ABT scores and the severity of disease, prothrombin time,[46] galactose elimination capacity, Child–Pugh classification and hepatic volume[47] in cirrhotic patients has also been reported. Amongst others, Herold *et al.* have recently shown that, in a group of 86 patients with chronic hepatitis C-related cirrhosis, ABT values were significantly correlated with the Child–Pugh classification and were significantly different between the three Child–Pugh grades.[48] The prognostic value of ABT compared to conventional scores in cirrhotic patients has been investigated in several studies.[49–51] The concomitant use of ABT, however, did not add any information to the prognostic accuracy of the Child–Pugh classification. On the contrary, after following 125 cirrhotic patients for up to 4 years, Merkel *et al.* found that ABT was a strong predictor of survival and that, when combined with the Child–Pugh clinical and biochemical data, it improved the prognostic accuracy of prediction of death from liver failure based on the Child–Pugh classification.[52] The same authors reported that serial measurements of ABT and the Child–Pugh score proved to be of significant value in updating the prognosis of patients with advanced cirrhosis.[53] Finally, a study aimed at investigating the urinary sodium balance in comparison to quantitative methods of liver function in a group of 75 cirrhotic patients showed that ABT was the only independent predictor of urinary sodium excretion, and therefore the best parameter to relate liver function to renal impairment.[54] These results suggest that ABT has a good diagnostic sensitivity in cirrhotic patients, but the importance of its prognostic information with respect to conventional prognostic parameters (i.e. Child–Pugh score) remains controversial.

ABT has also proved to be useful in the diagnostic work-up of alcoholic liver disease. ABT was more reliable than standard liver function tests in identifying

the presence of alcoholic cirrhosis,[55–57] and in predicting short-term survival of patients with alcoholic cirrhosis.[58] ABT has also been used to monitor alcohol abstinence.[55, 59] In particular, in a retrospective study of a cohort of 32 patients with alcoholic cirrhosis followed up for 42 months, Lotterer *et al.* showed that ABT was the best surrogate marker to define the severity, progression or regression of disease as a result of intervention (Figure 2).[60] ABT best reflected the differences between the two groups (abstinent and non-abstinent), whereas the time course of various laboratory tests and the Child–Pugh score did not.

The clinical utility of ABT in cholestatic diseases is limited, because aminopyrine is eliminated mostly by hepatic metabolism and does not undergo enterohepatic circulation: its elimination is consequently expected to be unaffected by cholestasis. Hepner and Vesell found that aminopyrine metabolism was normal in most cases of benign obstructive cholestasis and abnormal in a few patients with acute cholestasis caused by gallstone obstruction, drugs or late primary biliary cirrhosis.[35] ABT values appeared to be higher in patients with early primary biliary cirrhosis than in patients with chronic active hepatitis.[41] On the contrary, low ABT values were found in advanced primary biliary cirrhosis where hepatocellular failure was present.[39] Recently, in a 2-year prospective study, Herold *et al.* have reported that ABT did not predict the prognosis of patients with primary biliary cirrhosis.[61] According to these considerations, ABT has been proposed as a screening test in patients with hyperbilirubinaemia in order to distinguish between cholestasis and hepatocellular disease.[62]

The only and rather provocative report about the diagnostic utility of ABT in the detection of hepatic neoplasms showed that, in a group of 153 patients with a variety of previously documented malignant tumours, ABT established the presence of hepatic malignancy in 83% of patients and excluded it in 74%.[63]

The usefulness of ABT for surgeons has been documented in some studies. Gill *et al.* reported that ABT (2-h percentage cumulative dose < 2.3) was a predictor of death in cirrhotic patients undergoing elective or emergency surgery.[64] ABT also proved to be better than the Child–Pugh score in predicting prognosis. Horsmans *et al.*, using ABT before and after surgical portocaval shunting in cirrhotic patients, showed that pre-operative ABT values were significantly higher in patients surviving 1 year than in those who died within the same period.[65] The authors therefore proposed ABT as an additional pre-operative prognostic test for a better selection of patients for shunt surgery. ABT has also been used in a recent pre-operative risk analysis of patients with oesophageal cancer, thus contributing to a composite pre-operative risk score of individual organ dysfunction.[66] Finally, ABT appears to better predict acute allograft rejection than other laboratory tests when performed after orthotopic liver transplantation (Figure 3).[67] Mion *et al.* used ABT to monitor liver graft recovery in the early post-orthotopic liver transplantation period.[68] They reported a progressive increase of ABT values after 48 h up to 7–10 days, whereas ABT

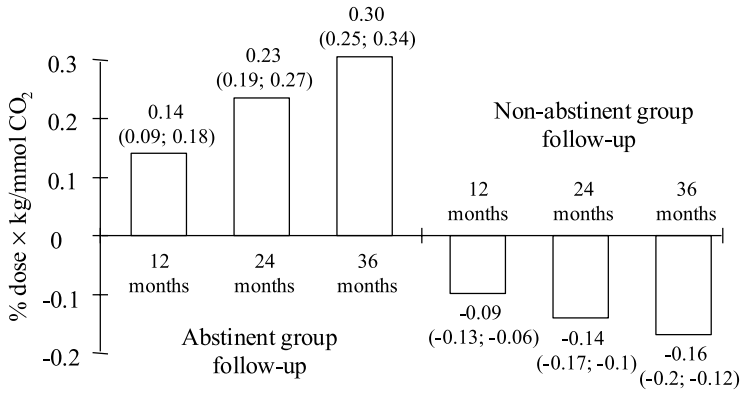

Figure 2. Retrospective analysis of the time course of aminopyrine breath test (ABT) changes in a cohort of 32 alcoholic cirrhotic patients followed up for 12–42 months. ABT results (expressed as the percentage of the administered dose over 30 min: % dose × kg/mmol CO_2) significantly improved in abstinent alcoholic cirrhotic patients ($n = 15$) and deteriorated in non-abstinent patients ($n = 17$) during the follow-up period (repeated-measurement ANOVA: $P < 0.01$). Data are expressed as the mean (95% CI) of the absolute differences (i.e. differences between the test results at the indicated time and the test results at time zero).[60]

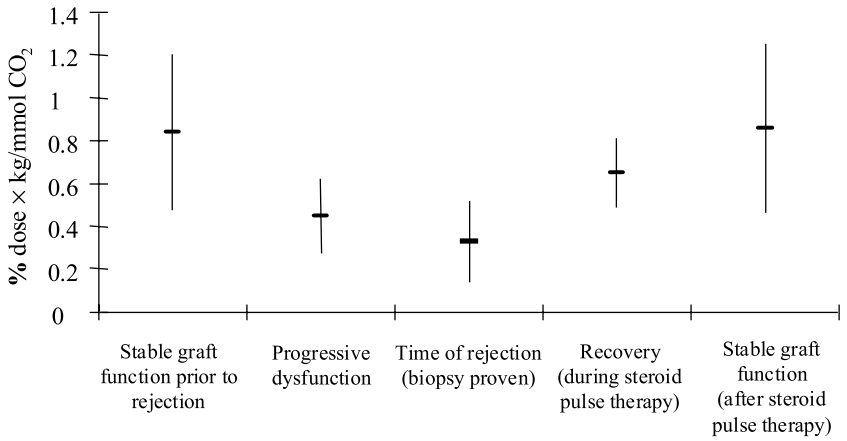

Figure 3. Aminopyrine breath test (ABT) course over 30 days of post-operative observation in 10 patients with acute liver allograft rejection. ABT values (mean ± s.d.; horizontal and vertical bars, respectively) are expressed as the percentage of the administered dose over 30 min (% dose × kg/mmol CO_2). ABT results showed a significant decrease (mean, 65%; $P < 0.05$) in all patients, whereas typical clinical signs of rejection (e.g. increasing liver cell enzymes, decrease in bile production, fever) were observed in less than 50% of patients. Moreover, the decline in ABT appeared 1–2 days before clinical symptoms in 40% of patients.[67]

values decreased when liver graft dysfunction occurred. Potential confounding factors (e.g. drug interactions, nutritional status, basal metabolic rate, sepsis, blood transfusions, gastrointestinal motility) in the interpretation of the ABT results in these patients, however, were not evaluated.

Finally, ABT has been proposed to assess the severity and to predict the evolution of acetaminophen-related liver injury. In particular, Saunders *et al.* reported that ABT correctly identified patients who developed severe hepatic injury or died after paracetamol poisoning.[69]

In spite of these extensive studies, some limitations in the use of ABT as a marker of hepatic function and reserve must be recognized. Cytochrome P450 is induced or inhibited by many endogenous/exogenous factors that may render ABT results difficult to interpret. Factors potentially affecting the interpretation of aminopyrine and other microsomal function tests include the age-related changes in liver *N*-demethylase activity, the concomitant presence of chronic diseases or the administration of *N*-demethylase enzyme modulators. Maturational changes in *N*-demethylase activity have been documented in infants, in whom demethylation of aminopyrine was found to be positively correlated with age,[70, 71] and was significantly greater in males than females.[72] Conversely, ABT showed a progressive decrease with advancing age.[73] Although sex differences in aminopyrine metabolism have not been found in adult humans,[74] exogenous female sex hormones have been shown to decrease aminopyrine *N*-demethylation.[75, 76] The role of nutritional status is not clear, but malnutrition seems to decrease the metabolism of aminopyrine.[77] Moreover, congestive heart failure or chronic renal failure have been shown to decrease aminopyrine *N*-demethylation.[78, 79] Drug interference and other environmental factors are probably the most important confounders in the interpretation of ABT results. In particular, aminopyrine *N*-demethylase activity has been found to be induced after phenobarbital,[80] glutethimide,[22] diphenylhydantoin,[29] steroids[39] and spironolactone[81] administration. On the other hand, aminopyrine *N*-demethylation is depressed after cimetidine,[82] disulfiram,[25] allopurinol,[83] albendazole,[84] cytostatic drugs,[85] interferon[86] and influenza vaccination[87] administration and after long-term exposure to pesticides.[88] Cigarette smoking has also been reported to increase ABT values,[89] whereas aminopyrine *N*-demethylation is depressed after acute ethanol intake[76] and increased during chronic ethanol consumption.[59] Other factors may influence ABT results by modifying endogenous CO_2 production (increased with fever, physical exertion, meal intake and hyperthyroidism; lowered with sleep, hypothermia and hypothyroidism).[22, 36]

All of the above-mentioned potential confounding factors should be kept in mind when normal ABT values are found in patients with clinical and laboratory signs of liver dysfunction, or when abnormal ABT results are found in patients without liver disease.

Finally, some concern persists about the safety of aminopyrine because of the occurrence of agranulocytosis

in up to 1 in 10 000 individuals with chronic administration of pharmacological doses,[90] although this complication has never been reported following the small single doses required for the breath test.[35, 91]

In conclusion, ABT is one of the most frequently utilized tests to assess functional liver microsomal mass. ABT may have a complementary role to liver histology in grading chronic hepatitis. It is a reliable method for predicting the occurrence of cirrhosis and could be proposed, together with other conventional tests (e.g. biochemical liver tests, ultrasound), to stage liver disease when liver biopsy is not diagnostic or not performed. ABT seems to be a sensitive survival predictor in patients with liver disease, adding, however, little information to the Child–Pugh score. It also shows a prognostic value in patients undergoing hepatic or shunt surgery. Finally, ABT is useful in assessing liver function after treatment, showing potential application in the longitudinal monitoring of liver transplantation. However, ABT has never been the subject of an organized clinical trial and has never been fully used in clinical practice. This could be related in part to bias in the design of the studies (e.g. the lack of well-defined groups of patients in the studies, different expression of results) which may have generated data that are difficult to interpret. At the same time, several recognized factors that interfere with aminopyrine metabolism may have discouraged clinical investigators from employing ABT to define the severity of liver disease at the start of a trial and the progression or regression as a result of intervention.

Phenacetin breath test

Information regarding the usefulness and possible clinical implications of the phenacetin breath test (PBT) (and other microsomal, cytosolic and mitochondrial liver tests) as a hepatic function test is more limited than for ABT.

Phenacetin [*N*-(4-ethoxyphenyl)acetamide] undergoes *O*-de-ethylation through the hepatic mixed function oxidase system, in particular cytochrome P450 1A2. A minor metabolic pathway involves cytochrome P450 2E1,[92] the resulting acetaldehyde or ethanol being oxidized via the tricarboxylic acid cycle to CO_2. Moreover, unlike aminopyrine, phenacetin is a high extraction drug ($E > 0.8$),[6] and undergoes an extensive first-pass clearance that reflects its hepatic extraction from portal blood.[93]

Because of the potential for aminopyrine-induced agranulocytosis, PBT was proposed as an alternative to ABT for the evaluation of hepatic function in humans. Breen *et al.* assessed the reliability of ^{14}C-PBT in healthy subjects, hospitalized patients without liver disease and cirrhotic patients without ascites or peripheral oedema.[26] In spite of the high hepatic extraction ratio of phenacetin, results were similar with both intravenous and oral administration, and a good correlation between the peak rate of $^{14}CO_2$ excretion and the clearance of phenacetin was also observed. Moreover, the $^{14}CO_2$ excretion profile was depressed in cirrhotic patients with respect to controls and patients free from liver disease. The authors therefore proposed PBT as a simpler and more rapid tool for the evaluation of hepatic drug metabolism than other available breath tests, although the potential delay of phenacetin metabolism by intra- or extra-hepatic shunting in cirrhotic patients was not assessed. Schoeller *et al.* subsequently compared PBT with ABT in healthy controls and patients with liver disease, reporting a high correlation ($r = 0.77$) between the results of both breath tests only in 'liver' patients, whereas a poor correlation ($r = 0.21$) was shown in healthy subjects.[94] Moreover, in spite of increasing the phenacetin dose administered, the saturation of phenacetin de-ethylation was not obtained and an increase in PBT values was not observed in healthy subjects after induction with rifampin. These observations led the authors to suppose that phenacetin de-ethylation was not the rate-limiting process in hepatic metabolism. Finally, in a recent study, Kajiwara *et al.* proposed the association of ^{13}C-PBT (using 1-^{13}C-ethoxy-phenacetin) and a urine test (using ^{13}C-nuclear magnetic resonance spectroscopy) as a valuable tool for hepatic function assessment in liver patients.[95]

Apart from these investigations, PBT has not undergone further extensive study, and the effect of well-known inducers of phenacetin metabolism, such as cigarette smoking[96] and dietary factors (e.g. eating charcoal-broiled meat or some vegetables), on breath test results has never been assessed. Based on these limited data, clinical application of PBT is not currently justified.

Methacetin breath test

Methacetin [*N*-(4-methoxyphenyl)acetamide], a derivative of phenacetin, undergoes *O*-demethylation

through the hepatic mixed function oxidase system to acetaminophen and CO_2, the latter being exhaled in breath.[97] Like phenacetin, methacetin is a high extraction drug ($E > 0.8$),[6] undergoing extensive first-pass clearance. Methacetin was suggested as an alternative to aminopyrine, because of its rapid metabolism in normal subjects and the lack of toxicity in small doses.[97] A less pronounced induction of methacetin than aminopyrine metabolism by cigarette smoking and anticonvulsant drugs has also been reported.[89]

The need for a test able to estimate metabolic liver capacity and applicable to all patients without any radiation hazard led Krumbiegel *et al.* to assess the reliability of the ^{13}C- with respect to the ^{14}C-methacetin breath test (MBT) in healthy subjects and patients with liver cirrhosis.[98] In both study groups, ^{14}C and ^{13}C-MBT curves were nearly congruent and a good discrimination between healthy volunteers and patients was observed. In order to determine the efficacy of ^{13}C-MBT, Fahl *et al.* assessed different doses of labelled methacetin in normal subjects, in whom a linear increase of $^{13}CO_2$ excretion rates without saturation was observed;[99] they also reported a significant correlation between MBT values, total serum bile acids and histology in patients with liver disease.[100] Matsumoto *et al.* studied patients with histologically confirmed chronic hepatitis, liver cirrhosis (compensated, advanced, with hepatocellular carcinoma) or late primary biliary cirrhosis, and showed that ^{13}C-MBT values were decreased and delayed according to the severity of liver damage.[27] The ^{13}C recovery over 30 min was regarded as the best parameter for comparison between the groups: no significant differences were observed between healthy controls and those with chronic persistent hepatitis, but MBT values were significantly lower in patients with chronic active hepatitis or compensated cirrhosis. Significantly lower values were observed for patients with advanced cirrhosis or hepatocellular carcinoma in comparison with the former groups. No information on the potential influence of intra- or extra-hepatic shunting or environmental factors (e.g. smoking, eating charcoal-broiled meat), which may influence methacetin metabolism,[97] was reported.

Klatt *et al.* subsequently pointed out that MBT values were able to discriminate between cirrhotic and non-cirrhotic subjects (sensitivity, 93.5%; specificity, 95%).[101] MBT values were also significantly lower in Child C than in Child A/B patients, and the correlation between the Child–Pugh score and MBT appeared to be significant ($r = 0.67$) and better than that of the monoethylglycinexylidide test ($r = 0.39$) and indocyanine green clearance ($r = 0.43$). As in the above study, Pfaffenbach *et al.* tested the ^{13}C enrichment in the breath of cirrhotic patients and normal volunteers by means of isotope-selective non-dispersive infrared spectrometry.[102] Both the ^{13}C-MBT maximal percentage rate and cumulative rate over 30 min up to 3 h were significantly lower in cirrhotic patients than in controls. Moreover, significant differences were found among cirrhotic subjects, depending on their Child–Pugh score. Both studies[101, 102] highlighted the potential advantages of ^{13}C-MBT (safe, rapid and easy to perform) and the cost-effectiveness of breath ^{13}C enrichment measurement by isotope-selective non-dispersive infrared spectrometry, the reliability of which is being further validated in larger studies.[103]

Finally, a recent study by Lara Baruque *et al.*, in which ^{13}C-MBT was used to investigate the hepatic functional capacity in healthy controls and patients with chronic hepatitis and/or Child A–C cirrhosis, showed that ^{13}C-MBT (% dose/h at 10 min as the best result) discriminated between all the different groups, with the highest regression coefficients for healthy controls vs. chronic hepatitis and for Child B vs. Child C cirrhosis.[104]

Based on the data produced so far, MBT could have the potential to become a reliable test to probe liver function. The test seems to discriminate well between different stages of liver cirrhosis, with a good correlation with the Child-Pugh score, and could become an additional tool for predicting the occurrence and monitoring the progression of chronic liver disease. However, methacetin has a high hepatic extraction, and the effects of altered blood flow on its pharmacokinetics need to be studied before MBT can be rationally applied to larger prospective studies.

Caffeine breath test

Caffeine (3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione) undergoes *N*-demethylation through the hepatic mixed function oxidase system, mainly cytochrome P450 1A2 (other minor metabolic pathways involve cytochromes 2E1, 3A3, 2B6).[6] Caffeine is a low extraction drug ($E < 0.3$), and thus its elimination is primarily dependent on the hepatic metabolic capacity ('liver functioning mass').[6] Caffeine undergoes complete absorption and is metabolized almost entirely by the liver,[84] through which three major dimethyl

metabolites are produced: paraxanthine (80% of products of demethylation in humans), theobromine and theophylline.[105]

As caffeine shares many of the characteristics of an 'ideal' liver test substrate (Table 1), Arnaud *et al.* assessed the feasibility of a breath test in healthy subjects using oral 1,3,7-methyl-^{13}C-caffeine:[28] the rapid (15 min after administration) and significant (maximum during the first hour after administration) ^{13}C enrichment in the expired breath, and the amount of ^{13}C recovery over 24 h (21–26%), led to further interest in the caffeine breath test (CBT) as a liver function test. CBT was thus subsequently evaluated in cirrhotic patients,[106] showing a slower rise and a marked decrease in the ^{14}C exhalation curve compared to healthy volunteers. Further investigations were carried out to identify the optimal CBT conditions, to explore which labelled methyl group best reflected caffeine *N*-demethylation and to determine whether labelled CO_2 excretion was increased by cigarette smoking (as the caffeine metabolic clearance rate). Kotake *et al.* demonstrated that, in healthy volunteers, the absolute rate of trimethyl-labelled CO_2 exhibited dose-dependent kinetics, the labelled CO_2 output was two-fold greater in smokers than in non-smokers and the 2-h cumulative labelled CO_2 excretion was the best CBT parameter (significant correlation with the oral caffeine metabolic clearance rate: $r = 0.90$) to assess the effect of smoking on caffeine *N*-demethylation.[107] Moreover, monomethyl-labelled CBTs showed that 3-methyl-C-labelled caffeine was the most suitable to explore *N*-demethylation caffeine pathways.

The reliability of CBT in yielding quantitative information on liver function was then evaluated by Renner *et al.* by testing patients with chronic liver disease and healthy subjects.[108] After the injection of 3-methyl-^{14}C-caffeine and unlabelled compound, liver patients showed a significant decrease in CBT results with respect to healthy controls. Moreover, CBT values showed a parallel decrease with caffeine plasma clearance ($r = 0.83$) and a significant correlation with the bromosulphalein plasma disappearance rate ($r = 0.83$). Apart from a few other investigations, in which the effect of the hepatitis B surface antigen carrier state on the cytochrome P450 system was explored,[109] CBT has not undergone further extensive studies to assess the severity of liver disease, and its prognostic capacity in comparison with commonly used liver function parameters has never been demonstrated. The reliability of this compound for the evaluation of patients with liver disease is, in fact, limited by several factors that may induce or inhibit its metabolizing enzymes.[24, 36]

However, as caffeine metabolism reflects hepatic cytochrome P450 1A2 activity, CBT has been adopted as a specific probe to explore the effect of xenobiotic and exogenous compounds on the specific P450 metabolizing activity *in vivo*.[110–117] Moreover, because of its safety, CBT is useful for studying the paediatric population.[118–123]

Diazepam breath test

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one), a low extraction drug ($E < 0.3$), mainly undergoes *N*-demethylation through the hepatic mixed function oxidase system, in particular cytochrome P450 2C19.[6]

In 1977, Hepner *et al.* studied the hepatic drug metabolism of diazepam, antipyrine, aminopyrine and indocyanine green in patients receiving anticonvulsants and those with various types of hepatobiliary disease.[29] In particular, diazepam metabolism was assessed by a breath test (DBT) after the intravenous administration of ^{14}C-labelled compound. They observed a significant increase in $^{14}CO_2$ (24-h value was the best discriminator between study groups) in the breath of patients taking anticonvulsants compared to controls, and a significant decrease in those with liver disease, except patients with cholestasis. DBT showed a good correlation with the diazepam plasma half-life ($r = -0.65$, $P < 0.001$) and diazepam metabolic clearance rate ($r = 0.54$, $P < 0.001$); a weak, but significant, correlation with the 24-h recovery of ^{14}C in urine ($r = 0.42$, $P < 0.01$) was also observed. However, the DBT values were significantly correlated with other drug elimination tests (antipyrine clearance, $r = 0.75$, $P < 0.001$; ABT values, $r = 0.79$, $P < 0.001$; indocyanine green elimination, $r = 0.48$, $P < 0.01$) only when all patient groups were combined. Sonnenberg *et al.* studied diazepam and aminopyrine demethylation in women taking oral contraceptive steroids by means of ^{14}C-DBT and ^{14}C-ABT.[124] They reported that, with ^{14}C-DBT, the short-term measurement of $^{14}CO_2$ in the breath of women taking oral contraceptive steroids did not differ from that of controls, whereas ABT showed a half-life of $^{14}CO_2$ in breath that was significantly prolonged in women taking oral contraceptive steroids (thus partially

reflecting different P450 enzyme specificities for the two drugs). Moreover, during long-term $^{14}CO_2$ assessment, a bi-exponential decline of $^{14}CO_2$ in breath with a marked circadian rhythm was observed for both DBT and ABT (thus partially reflecting the circadian rhythm of liver demethylase activity).

Because of the genetic polymorphism of the CYP2C19 gene,[6] diazepam was subsequently considered as an unsuitable substrate for general metabolic liver function assessment.

Erythromycin breath test

Erythromycin undergoes *N*-demethylation through cytochrome P450 3A (CYP3A4, CYP3A5, probably CYP3A7), and the carbon atom in the cleaved methyl group is rapidly converted to exhaled CO_2.[6] CYP3A is one of the major cytochrome P450 subfamilies in humans, and is the most abundant cytochrome P450 present in adult human liver, accounting for up to 30% of the total cytochrome proteins.[125] The CYP3A subfamily in humans is composed of at least CYP3A4 (the predominant isoform), CYP3A5 (present in 30% of adult livers, with a catalytic activity and substrate spectrum similar to the 3A4 isoform), CYP3A7 and CYP3A43.[126] CYP3A is one of the most important CYP subfamilies involved in the gastrointestinal and hepatic metabolism of several drugs (immunosuppressants, calcium channel blockers, antihistamines, sedatives, macrolides, steroids, synthetic oestrogens, human immunodeficiency virus protease inhibitors, cytotoxic drugs, etc.) and in the bioactivation of some xenobiotics into potential carcinogens or toxins.[127] It has also been shown that the catalytic activity of CYP3A may vary considerably (up to 10–20-fold) in humans,[125] this heterogeneity accounting in part for inter-individual differences in both the CYP3A drug substrate dose requirement and response to some exogenous toxins.

The erythromycin breath test (ERMBT) was therefore proposed as a non-invasive means to measure CYP3A activity in humans. In particular, Watkins *et al.* intravenously administered ^{14}C-*N*-methyl-erythromycin in a group of patients with normal liver and kidney functions, and observed a six-fold inter-individual variation in breath CO_2 production unrelated to medications, smoking or age.[30] A significant increase or decrease in ERMBT values was reported after treatment with CYP3A inducers (dexamethasone and rifampicin) or inhibitors (triacetyloleandomycin), respectively. The same group subsequently found a significant correlation between ERMBT results and hepatic CYP3A levels ($r^2 = 0.56$) in patients with severe liver disease before transplantation.[128] However, neither ERMBT nor CYP3A hepatic levels reflected the severity of liver disease, as they were unrelated to standard liver function tests. Because of the presence of large inter-individual variations in ERMBT values and the independence of the severity of liver dysfunction, the reliability of ERMBT as a test of general liver function is considered to be unlikely. Conversely, its usefulness as a specific assay of *in vivo* CYP3A activity has been emphasized extensively, but with some criticism.[129]

As CYP3A4 is the main enzyme responsible for cyclosporin A metabolism, ERMBT has been used to predict cyclosporin blood levels,[130] and has been demonstrated to detect potentially dangerous low levels of this cytochrome isoform.[131] The importance of CYP3A enzyme activity in influencing the dose of both cyclosporin and FK 506 given after liver transplantation was also investigated by Cakaloglu *et al.*, who performed ERMBT in 37 stable orthotopic liver graft recipients 1 year after surgery.[132] Blood cyclosporin and FK 506 levels were significantly related to the ratio: dose cyclosporin or FK 506/ERMBT results; this confirms that the appropriate dosage of these immunosuppressants may be obtained by means of ERMBT. Finally, Schmidt *et al.* have recently shown a significant correlation between ERMBT and the hepatic CYP3A protein level and catalytic activity in liver transplant recipients in the early post-operative phase.[133] Whether early ERMBT can be clinically useful as a predictor of cyclosporin or FK 506 pharmacokinetics in liver transplantation needs further investigation.

In addition to clinical transplantation, ERMBT has also been applied to quantify CYP3A down-regulation after interferon treatment,[134] and to predict docetaxel clearance for dosage optimization of this chemotherapeutic agent.[135]

In conclusion, current data indicate that ERMBT has gained acceptance for the guidance of individual drug dosages of immunosuppressants after transplantation,[136] and of various anticancer drugs.[137] However, the need to administer a ^{14}C-labelled compound and the intravenous administration, with the consequent bypass of intestinal CYP3A enzymes (a significant source of first-pass metabolism for orally administered CYP3A substrate[138]), are limitations that hamper the spread of ERMBT in clinical practice.

CYTOSOLIC LIVER TESTS

Galactose breath test

In humans, galactose is mainly metabolized in the cytosol of hepatocytes, the rate-limiting step being the initial phosphorylation by the enzyme galactokinase.[139] The high extraction ratio ($E > 0.8$)[6] of galactose by the sinusoidal membrane of hepatocytes implies a blood flow-dependent liver metabolism when galactose is given at low doses. Therefore, the administration of large doses of this carbohydrate is necessary to saturate its metabolic pathway and to obtain information on liver function mass rather than hepatic blood flow.[140]

For some 40 years, the galactose elimination capacity (GEC) test has been performed as a quantitative liver function test,[11] showing a significant correlation with the severity of chronic liver disease,[141, 142] and a usefulness for the prognosis of liver cirrhosis.[143–145]

The galactose breath test (GBT) was developed to overcome some limitations of the GEC test, such as intravenous galactose injection and serial blood sampling. Shreeve *et al.* studied the feasibility of GBT in healthy subjects and patients with alcoholic cirrhosis after oral administration of D-galactose (10 g/m^2) labelled with ^{14}C or ^{13}C.[32] The mean rate of oxidation of *C-galactose to *CO_2 was significantly lower in cirrhotic patients than in healthy controls. Moreover, a definite correlation between GBT values and serum albumin was observed in the cirrhotic group ($r = 0.81$). A further study was performed by Caspary and Shaffer using an oral galactose load (40 g) labelled with ^{14}C in healthy controls and patients with histologically proven chronic active hepatitis, alcoholic or post-necrotic cirrhosis.[146] The GEC test, ABT and standard liver tests were also performed. However, GBT was less accurate than the GEC test and ABT in identifying patients with liver disease. These results, which conflict with those of the previous study, led to the conclusion that GBT could be used to quantify hepatic function during the follow-up of patients with chronic liver disease, but was less accurate than the GEC test for the diagnosis of liver disease. Following some modifications (e.g. intravenous injection of 0.5 g/kg body weight of ^{14}C-galactose), GBT subsequently discriminated well between controls and patients with chronic liver disease ($P < 0.001$), also showing a significant correlation with the GEC test ($r = 0.87$) and bromosulphalein clearance ($r = 0.92$).[147] These conflicting data may possibly be explained by differences between the groups of patients studied, the complexity of galactose metabolism to CO_2 or the influence of extra-hepatic factors (e.g. ethanol ingestion, diabetes with hyperglycaemia).[1, 24]

Recent interest in the usefulness of GBT has been triggered by the results obtained by Mion and Rousseau. In a first report, they investigated patients with chronic liver disease (histologically proven chronic hepatitis and Child A–C cirrhosis) with both GBT and the GEC test, which showed a significant correlation with the degree of fibrosis in liver biopsy specimens.[148] A further study aimed to determine whether GBT was altered early in the course of chronic hepatitis C.[149] GBT (intravenous injection or oral administration of 495 mg/kg body weight of unlabelled galactose, together with 5 mg/kg body weight of 1-^{13}C-galactose) was performed in 50 chronic hepatitis C patients and 10 healthy controls, and the results were compared with the GEC test, histological data (METAVIR)[150] and standard biochemical data. No differences were observed between the two routes of administration. GBT was significantly decreased in patients vs. controls (% ^{13}C dose/h at 60 min, $P < 0.0001$) and showed a significant inverse correlation with the fibrosis score ($P < 0.0001$), but no correlation with the activity score. Finally, GBT results showed a weak correlation with both the GEC test and some of the standard biochemical liver function tests. On the basis of these data, GBT looks promising for the correct identification of mild chronic liver disease and for the improvement of the initial staging and follow-up of patients with chronic hepatitis C. Further prospective studies should clarify whether GBT (a non-invasive, but expensive test) correlates better than the GEC test (an invasive, but cheaper test) with the progression of liver fibrosis over time.

Phenylalanine breath test

The essential aromatic amino acid L-phenylalanine is mostly metabolized by the liver.[151] In particular, phenylalanine is hydroxylated to tyrosine and converted by tyrosine aminotransferase into hydroxyphenylpyruvic acid. Hydroxyphenylpyruvate is further converted, through dioxygenation, to homogentisic acid, finally producing CO_2. These reactions occur inside the hepatocyte cytoplasm, and may thus reflect hepatic cytosolic activity.

Liver disease is associated with decreased metabolism of the aromatic amino acids phenylalanine and tyrosine, leading to a rise in their plasma concentrations,[151–155]

and an 80% decrease of phenylalanine hydroxylase activity in liver biopsy specimens of cirrhotic patients has also been reported.[156]

Based on these considerations, and because of the previous evidence that the rate of hepatic phenylalanine metabolism can be calculated quantitatively from the appearance of $^{13}CO_2$ in breath, using L-1-^{13}C-phenylalanine as a tracer, Burke *et al.* studied the feasibility of the ^{13}C-phenylalanine breath test (PHBT) in patients with end-stage liver disease.[31] After the ingestion of ^{13}C-phenylalanine, significant differences were observed in PHBT recovery over 1 h between healthy volunteers and cirrhotic patients and between Child A and Child B/C patients; PHBT values also showed a significant correlation with conventional liver function tests (e.g. albumin plasma levels, $r = 0.54$) and Child–Pugh score ($r = 0.51$).

In addition to this study, a few others have since been performed on the usefulness of PHBT for liver function assessment. It has been reported that a dose of 100 mg of L-1-^{13}C-phenylalanine gives the best results for the rapid evaluation of liver function,[157] with a good correlation with conventional liver blood tests.[158] Another study, evaluating ^{13}C-PHBT in advanced cirrhotic patients and healthy controls by both isotope ratio mass spectrometry and non-dispersive infrared spectrometry, indicated the superiority of mass spectrometry breath analysis.[159] In confirmation of the reliability of PHBT for quantifying the functional hepatic reserve in a short period of time, a study by Lara Baruque *et al.*, in which ^{13}C-PHBT was used to investigate different groups of patients with liver disease, showed that the percentage ^{13}C dose per hour at 30 min was the best value to discriminate between controls and chronic hepatitis and between Child B and Child C cirrhosis.[104] Finally, in a recent report, Kobayashi *et al.* demonstrated that, in groups of patients with progressive stages of liver disease, PHBT values significantly correlated with the plasma retention rate of indocyanine green ($r = -0.7$, $P < 0.0001$), the Child–Pugh score ($P < 0.0001$) and standard liver blood tests ($P < 0.01$).[160]

In conclusion, PHBT is a rapid test potentially able to discriminate between healthy subjects and patients with liver disease and between progressive stages of liver cirrhosis, with a good correlation with the Child–Pugh score. However, the information produced so far is rather limited and further optimization (e.g. discrepancies between intravenous and oral administration of the tracer[161]) is needed to justify the use of PHBT in future prospective studies.

MITOCHONDRIAL LIVER TESTS

α-Ketoisocaproic acid breath test

The impairment of hepatic mitochondrial function has been proven in a broad spectrum of liver conditions, having a genetic or an 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).[162, 163] Therefore, the 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,[164] metabolism of benzoic acid,[165] hepatic nitrogen clearance determination[166] and ^{31}P-nuclear magnetic resonance spectroscopy[167] are some examples.

Notable exceptions could be breath tests using substrates producing CO_2 during mitochondrial metabolism. α-Ketoisocaproic acid (KICA, a branched-chain α-ketoacid) may undergo two different metabolic pathways: oxidative decarboxylation through a branched-chain α-ketoacid dehydrogenase complex located exclusively in mitochondria, or conversion via transamination into the corresponding branched-chain amino acid leucine.[168]

In humans, a large portion of the branched-chain α-ketoacid dehydrogenase complex is present in extra-hepatic mitochondria (e.g. muscle), but most is phosphorylated and should be inactive.[169] Therefore, Lauterburg *et al.* evaluated human mitochondrial function by administering 2-keto-1-^{14}C-isocaproic acid, together with a leucine load (to inhibit the KICA transamination pathway), in patients with alcoholic and non-alcoholic liver disease and healthy controls.[33] Interestingly, 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 h was observed in alcoholic patients, despite normal conventional and quantitative liver function tests, with respect to patients with non-alcoholic liver disease and controls ($P < 0.01$).

Similar results were reported by the same group, this time using the ^{13}C-KICA breath test.[170] Because, in alcoholic patients, KICA decarboxylation was again impaired in spite of normal quantitative liver function tests, the authors suggested that the KICA breath test does not simply reflect a loss of functional hepatic mass, but probably a specific ethanol-induced marked decrease in the activity of the branched-chain α-ketoacid dehydrogenase complex. On the basis of these considerations, the ^{13}C-KICA breath test has also been used for the diagnostic work-up of hepatic steatosis, and has been proven to be helpful in distinguishing alcoholic steatosis from non-alcoholic steatosis of the liver.[171] However, the usefulness of the KICA breath test as a marker of excessive ethanol consumption has been rejected recently. Bendtsen *et al.* reported no difference in ^{13}C-KICA breath test values between male alcoholic patients and healthy male subjects, nor between patients with alcoholic hepatitis or steatosis and controls.[172] Moreover, significantly higher total amounts of exhaled $^{13}CO_2$ were found in healthy females with respect to healthy males ($P < 0.01$). These conflicting data may be partly explained by the complex regulation of the activity of the branched-chain α-ketoacid dehydrogenase:[173] the phosphorylation state of the enzyme complex, availability of coenzyme A, intra-mitochondrial calcium levels and hormonal status are other variables that might be influenced by the chronic intake of ethanol. Therefore, the value of the KICA breath test for the assessment of long-term excessive ethanol consumption deserves further study.

Nevertheless, the ^{13}C-KICA breath test appears to be useful for the assessment of subtoxic and reversible mitochondrial dysfunction, such as that caused by socially consumed amounts of ethanol and therapeutic doses of acetylsalicylic acid.[174] In healthy subjects, ^{13}C-KICA breath test values were significantly lower ($P < 0.01$) after the acute administration of ethanol [the metabolism of which induces a decrease in the ratio of the oxidized to reduced forms of nicotinamide adenine dinucleotide (NAD^+/NADH)[175]], whereas, after the ingestion of acetylsalicylic acid (the metabolism of which induces an increase in the NAD^+/NADH ratio[176]), they appeared significantly higher with respect to normal conditions (Figure 4). Because of its ability to detect subtoxic effects of xenobiotics on mitochondria, the KICA breath test has also been used to assess the effect of lamivudine[177] and FK 506[178] on the mitochondrial function of patients with chronic hepatitis B and those who have undergone orthotopic liver transplantation, respectively.

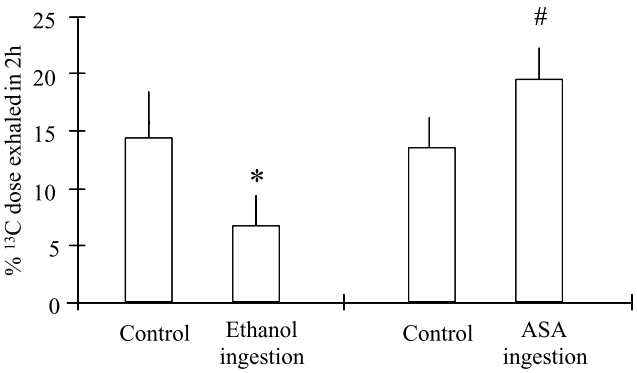

Figure 4. Effect of xenobiotics on hepatic mitochondrial function assessed using the ^{13}C-α-ketoisocaproic acid (^{13}C-KICA) breath test. In healthy subjects, the ingestion of ethanol (0.5 g/kg body weight) resulted in a significant decrease in the KICA breath test values with respect to the control period (*$P < 0.001$). Conversely, after acetylsalicylic acid (ASA) administration (30 mg/kg body weight), the KICA breath test results significantly increased (#$P < 0.01$ vs. control period). Data are expressed as the mean ± s.e. of the percentage of the administered dose exhaled in 2 h.[174]

Although the KICA breath test was proposed as a marker of liver mitochondrial function in chronic alcoholics more than 10 years ago,[33] it has never gained general acceptance. Its use as a marker of xenobiotic-induced impairment of human liver mitochondrial function appears promising, although a comparison with other liver mitochondrial function tests has never been performed.

Methionine breath test

The essential amino acid methionine is mostly metabolized by the liver,[179] because the highest specific activity of methionine adenosyltransferase in mammals occurs in hepatic tissue.[180] However, methionine metabolism is very complex, as this amino acid has several important functions.[181, 182] The main operating reaction in methionine metabolism is the formation of the high- energy sulphonium compound *S*-adenosyl-L-methionine.[180] Further metabolism can subsequently occur through the transmethylation–trans-sulphuration pathway or the polyamine pathway.[183] Therefore, depending on the labelled carbon ($^{14/13}$C) unit of the methionine molecule administered, different metabolic pathways could be explored through a breath test. Hepatic mitochondrial

function could be assessed using the 3- or 4-carbon-labelled methionine and following the fate of the amino acid through 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. Alternatively, the 1-carbon-labelled methionine could give an estimate of mitochondrial α-ketobutyrate decarboxylase activity.[182] Finally, the oxidative capacity of liver mitochondria could be tested through the administration of the methyl-*C-labelled methionine, 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.[184]

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 to assess hepatic mitochondrial function in 20 healthy subjects before and after ethanol-induced oxidative stress.[34] Methionine breath test values were significantly decreased after ethanol intake; however, the low percentage of ^{13}C recovered over the test period (at 180 min: 7.81 ± 0.66% in normal conditions and 4.25 ± 0.14% after ethanol ingestion) 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. 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.*, using a breath test with 1-^{13}C-methionine to monitor mitochondrial functional changes in a patient with biopsy-proven, acute, valproate-associated microvesicular steatosis.[185]

Few data are available (only as abstract reports) on the use of the methionine breath test in patients with liver diseases. In particular, the methionine breath test has been evaluated in patients with various chronic liver diseases, showing interesting results in terms of the ability to distinguish between various groups of liver disease patients,[186, 187] and the capacity to assess xenobiotic-induced reversible modulation of hepatic mitochondria in healthy subjects.[186]

In conclusion, further investigation of the methionine breath test is needed to evaluate its usefulness for liver mitochondrial function assessment. In particular, the identification of which methionine-labelled carbon unit best reflects mitochondrial metabolism and the comparison of methionine breath test values with other liver mitochondrial function tests should be clearly addressed.

CONCLUSIONS

The availability of breath tests for the assessment of liver function in humans goes back nearly 30 years when ABT was proposed by Hepner and Vesell.[25] Since then, several 'liver function' breath tests have been developed, assessing the specific microsomal,[26–30] cytoplasmic[31, 32] or mitochondrial[33, 34] metabolism of hepatic substrates. However, they have not become common practice amongst hepatologists and, although their use appears to be conceptually logical and rather simple, they are still performed only in a few medical centres. Furthermore, in spite of the marked advances in hepatology research (e.g. genetic, diagnostic, medical and surgical therapy), and the need for an accurate measurement of hepatic function to monitor the progression of liver disease or to predict the long-term prognosis, risk of surgical interventions and optimal timing of liver transplantation, a breath test able to replace the Child–Pugh score[3, 4] and other 'static' conventional liver tests has not yet been accepted. This indicates a general dissatisfaction and uncertainty amongst the scientific community in these other 'dynamic' function tests.

However, the usefulness of breath tests as hepatic function tests in the clinical setting of patients with liver disease has been documented by several studies. In particular, with regard to their use for the grading of hepatitis and/or the diagnosis of cirrhosis, aminopyrine,[23, 42–44, 48] methacetin,[27, 101–104] galactose,[149] phenylalanine[31, 104, 160] and ketoisocaproic acid[171] breath tests have shown some interesting results. Moreover, ABT has been proven to be useful as a survival predictor in patients with chronic liver disease,[52, 53] although it adds little information to the Child–Pugh score.[49–51] Similarly, ABT has shown prognostic value in patients undergoing surgery or shunt procedures,[64–66] and in patients with paracetamol poisoning[69] or acute alcoholic liver disease.[57] Finally, the most interesting data have been obtained from studies in which breath tests have been used to monitor liver function or determine immunosuppressive drug dosage after treatment, giving, in some cases, more information than other biochemical, clinical or dynamic liver function parameters. In particular, ABT is the best

method to relate hepatic function to renal impairment in cirrhotic patients,[54] to reflect differences between abstinent and non-abstinent patients with alcoholic cirrhosis,[60] and to predict acute allograft rejection after orthotopic liver transplantation.[65] Similarly, ERMBT has been proven to be extremely useful in predicting ciclosporin and FK 506 blood levels after orthotopic liver transplantation.[130, 132, 133] Therefore, as the most favourable information suggests that the main application of breath tests in hepatology is in defining the prognosis and following the treatment response of patients with liver disease, large, well-designed, prospective, longitudinal studies (e.g. defined sets of patients, uniformity of test conditions, substrate dose and analytical procedures, combination of various breath tests, comparison with traditional static and dynamic parameters, definition of end-points) are needed to assess their usefulness as hepatic function tests. The results of such studies will help to clarify Bircher's long-standing, but yet unsolved, question, 'To score or to measure?',[188] and possibly to obtain official approval for the commercial development of these tests.[189]

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