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

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Abstract. – **Conventional liver tests can be used to estimate a mixture of injury and function but none of these 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 overcome this limit, many dynamic tests have been developed in order to evaluate the "hepatic functional mass". Among these tests we can include breath tests with ^{13}C-labeled substrates undergoing different metabolic pathways. As concerning the evaluation of microsomal function, two main categories of breath tests have been developed based on the limiting step in the different substrates metabolism. The first group include aminopyrine, caffeine and diazepam, all substrates with a metabolism independent from hepatic blood flow and dependent almost exclusively from the enzymatic activity of different cytochromes P450. The other group is composed of substrates with flow dependent metabolism like methacetin, phenacetin, erythromycin.**

The aim of this review is to describe the clinical applications of microsomal liver breath tests in different hepatic diseases.

Key Words:

Microsomal function, Liver, Breath test.

P450 Cytochrome and Microsomal Function

The main part of drug metabolizing-enzymes are located on the smooth endoplasmic reticulum which costitute the microsomal system. These enzymes are similar to mitochondrial cytochromes and, since they are members of heme proteins family are able to bind molecular oxigen when the iron is in the reduced state. The most important class of such enzymes is cytocrome P450, whose name is related to the absorption properties[1]. In the reduced state it binds carbon monoxide in a complex with an absorption peak at 450 nm[2]. Altough it is generally accepted that the mammalian P450 genes come from a common precursor gene (supergene family), they costitute a very heterogeneous group of proteins with differences in structure, regulation and chromosomal location. All these enzymes are involved in microsomal oxidation. In this process molecular oxygen binds to heme iron in the reduced state. The reduction is mediated by NADPH cytocrome P450 reductase. Drugs bind to this cytocrome in a region next to heme iron; during this process iron is oxidized and one atom of molecular oxygen is inserted in the drug while the other one contributes to the water formation[3-4] (Figure 1).

Conventionally it is possible to classify P450 proteins on the basis of the amino acid sequence homology. Collecting proteins that share more than 40% amino acid structure, we know at least 10 families involved not only in drug metabolism, but also in normal cellular activities such as steroid hormones and prostaglandin biosynthesis. It is universally accepted that the enzymes of cytochrome P450 involved in drug metabolism are part of the gene families I, II, III.

The cytochrome P450 I includes 2 genes but only P450 IA2 is expressed in human liver and is able to metabolize phenacetin with an oxidative O-demethylation and caffeine with an oxidative N-demethilation.

In P450 II family we can include several subfamilies. Among these P450 IIC and IID seem to be the most important ones, implicated in mephenytoin and debrisoquin metabolism.

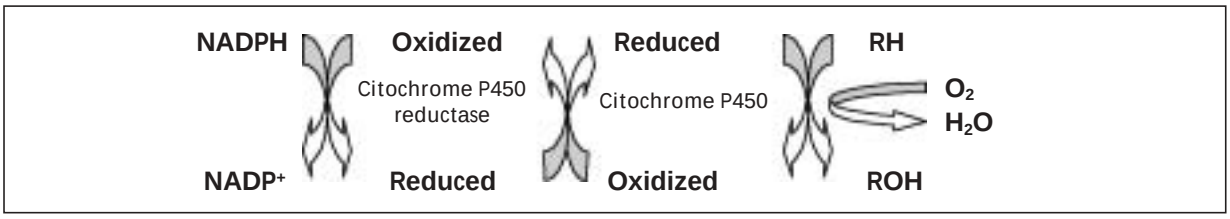

Figure 1.

Finally, we should consider P450IIIA, one of exponent of the cytochrome P450 III family that is the most important drug metabolizing enzyme, representing the 25% of total cythocromal proteins. This cytochrome can be induced by macrolides, benzodiazepins and glucocorticoides and is able to metabolize estrogens (hydrossilation), erythromicin (oxidative N-demethilation), and cyclosporine (hydrossilation and oxidative N-demethilation).

While different cytochromes are involved in aminopyrine metabolism, the specific cytochrome reponsible for methacetin metabolism are yet unknown[2-5-6-7].

Tests Used in the Evaluation of Microsomal Function

The liver is involved in many vital functions such as protein synthesis, bile excretion, xenobiotic detoxification, nutrients metabolism. In many liver diseases these functions can be decreased. In clinical practice, liver function is evaluated by some biochemical markers such as aminotransferase, bilirubin, alkaline phosphatase and albumin plasma levels or prothrombin time. Even if these conventional static biochemical liver tests can be useful to value a mixture of injury and function, 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. Although the Child-Pugh Score, obtained by laboratory and clinical parameters, is commonly used to estimate disease severity and patient prognosis, it is not able to estimate the "hepatic functional mass"[7]. To overcome this limit, a lot of dynamic tests have been developed[8]. The general principles of liver dynamic tests are founded on the administration of a exogenous substance, whose metabolic "rate limiting step" is controlled by hepatic enzymes. The hepatic clearance of xenobiotics depends on both hepatic perfusion (Q) and extraction ratio (E), where the latter is the ratio of the difference between the inflow and outflow substrate concentrations and inflow substrate concentration. Based on the extraction ratio, some tests can provide information about liver blood flow (when the extraction ratio is above 0.7) or about hepatic metabolic capacity (when the extraction ratio is below 0.3). The presence of a metabolite or the substance concentration in plasma or in breath samples can reflect a specific hepatic function which depends on the rate limitig step. In particular, as regards detoxification function, microsomal activity can represent a good index of functionality. Moreover P450 function is decreased in chronic liver disease because of the down-regulation of gene expression by interferons, tumor necrosis factor and other cytokines. It has been reported that in animal models it is related to hepatic fibrosis with capillarization of hepatic sinusoids. In the last decades many microsomal function tests have been defined: antypirine clearance, monoethylglycinexylide test and caffeine clerance but also non invasive tests such as ^{13}C-breath tests (Table I)[9].

Antypirine clearance, measured in the blood or saliva 24 hr after oral administration, is able to predict fibrosis evolution. It has been demostrated that it is more sensitive than serum albumin and other conventional liver tests to predict the disease progression in patients with chronic hepatitis B and autoimmune hepatitis and changes in liver histology in patients with chronic hepatitis C. Antypirine clearance is not dependent on hepatic blood flow and it is alterated in patients with diabetes or dysthyroidism[9]. Monoethylglycinexylide

Table I. Tests used to assess microsomal liver function.

MICROSOMAL PATHWAY			
Invasive tests		Not-invasive tests	
Enzyme-limited	*Flow-limited*	*Enzyme-limited*	*Flow-limited*
Antipyrine cl	Lidocaine/MEGX	Aminopyrine	Methacetin
Caffeine cl		Caffeine diazepam	Phenacetin

No. of patients: placebo-treated, 147 (infertile, 19; hirsutism, 22); inositol-treated, 136 (infertile, 23; hirsutism, 13). P values are NS. CIs, Confidence interval (95%).

(MEGX) test is also able to evaluate hepatic functional mass. It is based on intravenous administration of lidocaina that is metabolized by hepatic cytochrome through oxidative N-demethylation leading to the production of MEGX, measured in plasma samples. Even if this test presents a good correlation with liver biochemical tests, it does not seem to be reliable indicator of liver histology. Moreover, it can be affected by hepatic shunt because of its high hepatic extraction ratio and is associated with the occurence of side effects such as hypotension[10]. Finally, caffeine clearance is assessed by blood sampling over 24 hr after oral administration. Caffeine is characterized by low extraction ratio and its clearance correlates with other quantitative assays[11].

Even if it has been shown that such invasive quantitative tests can have some utility to assess disease severity and to predict mortality, they have not a large application in clinical practice. Intravenous administration and repeated blood sampling, the risk of side effects, pharmacological interferences, inter and intra assay variability represent important shortcomings. Furthermore, the presence of alterations in fluid homeostasis, splanchnic and systemic hemodynamics and renal function can make them unreliable. To improve these drawbacks, non invasive breath test, using carbon-labelled compounds, have been proposed. These tests are based on the replacement of one ^{12}C atom of the substrate with the radioactive ^{14}C or stable ^{13}C isotope, that is, finally, estimated in breath samples. The aim of this review is to describe clinical applications of liver breath tests that assess microsomal pathway. These tests can be divided into two main groups: enzyme-limited tests such as aminopyrine and caffeine and flow-limited tests which include methacetin, phenacetin, erythromycin and diazepam[7].

Microsomal Liver Breath Test

Enzyme-Limited Breath Test

Aminopyrine Breath Test

Aminopyrine breath test (ABT) was the first breath test proposed for the evaluation of liver function in patients with liver disease[12] and still now is one of the most frequently utilized test for assessing cytochrome P450 metabolism[13]. After oral administration, *C-labelled dimethylaminoantipyrine is completely absorbed from the gut and, after distribution in the total body water, it is metabolized in the liver[2]. Subsequently, it undergoes two-step *N*-demethylation through the cytochrome P450 mono-oxygenase system of liver microsomes, leading to the formation of formaldehyde and aminoantipyrine. The formaldehyde is then oxidized to bicarbonate, which may either be exhaled as CO_2 in breath (about 30%) or equilibrated with the central bicarbonate pool[2]. Since *N*-demethylation of aminopyrine has been documented as the rate-limiting step of a process that occurs almost exclusively in the liver[13], it is possible to assume that ABT reflects the activity of P450-dependent mono-oxygenase system[14]. In addiction, considering the low hepatic extraction rate (E = 0.2), aminopyrine metabolism is related to 'functional hepatic mass' and does not reflect changes or shunting of liver blood flow[15].

ABT reflects hepatic residual functional microsomal mass, thus providing useful information in clinical practice for the evaluation and staging of hepatic disorders including cirrhosis, chronic and acute hepatitis from different aetiologies. However, this test should not be considered a screening test for establishing a diagnosis because different types of liver diseases can present the same ABT results[16].

Since its initial appearance in clinical practice, the ABT has been extensively used in cirrhotic patients as a method for the assessment of the residual functional hepatic mass and for prognosis prediction. In particular, Hepner and Vesell showed that patients with cirrhosis had a lower $*CO_2$ breath excretion after ABT than controls and ABT results correlated well with the plasma aminopyrine clearance rate, serum albumin and bromosulphalein retention score[12]. The same authors described a strong correlation between extremely low ABT results and a bad early outcome. Several other studies have shown that in cirrhotic patients ABT scores correlate quite well with severity of disease, prothrombin time[17], galactose elimination capacity, Child-Pugh classification and hepatic volume[18-22]. In these studies, however, the concomitant use of ABT did not add any information to the prognostic accuracy of the Child-Pugh classification. On the contrary, Merkel et al. found that ABT was an effective predictor of survival and that it improved the prognostic accuracy of Child-Pugh classification[13,24]. It has, also, been demonstrated that ABT is the only independent predictor of urinary sodium excretion and, therefore, the best parameter to relate liver function to renal impairment[25]. In a recent study, Herold et al. used microsomal enzymes induction as a novel method to evaluate functional hepatic reserve and to predict prognosis in patients with cirrhosis. In particular, they examined changes in quantitative tests of liver function after treatment with phenobarbital, a potent cytochrome P450 inducing agent. These authors observed that microsomal liver function assessed by ABT was significantly increased in a subgroup of patients with liver cirrhosis, whereas galactose elimination capacity (cytosolic liver function), sorbitol clearance (liver plasma flow) and indocyanine green clearance (liver perfusion) remained unchanged[26]. In conclusion, all these results suggest that ABT has a good diagnostic sensitivity in cirrhotic patients, but is still controversial the importance of its prognostic information with respect to conventional prognostic indexes.

As concerning chronic hepatitis, several studies have shown that ABT values are significantly reduced in patients with chronic active hepatitis, compared to healthy controls and to patients with chronic persistent hepatitis[27-28-29]. Among these, Monroe et al. performed ABT in patients with chronic hepatitis and compared its results with histology, serum bile acids and standard liver function tests[30] . They showed that a percentage of cumulative dose over 2 hours lower than 5.7% correctly identified 86% of patients (30 of 35) with chronic active hepatitis and bridging or cirrhosis. A value higher than 5.7% identified 84% (21 of 25) of patients with chronic persistent hepatitis or chronic active hepatitis.

Similar results were obtained from Herold et al.[31]. In addiction, Giannini et al. have reported that, in a group of patients with chronic hepatitis C or Child A cirrhosis, ^{13}C-ABT values (% dose/h at 30 min) were able to discriminate between the study groups[32]. ABT also significantly correlated with the degree of fibrosis and necro-inflammatory activity and with portal vein velocity[33]. These results suggest that ABT may have a complementary role to liver histology in the staging and monitoring of the evolution of disease. To date, however, is still not possible to assess whether ABT can provide further information in patients with chronic hepatitis with respect to that obtained by standard laboratory tests or prognostic scores.

ABT was also assessed in patients with of alcoholic liver disease. In these subjects, ABT was more reliable than standard liver function tests in identifying the presence of alcoholic cirrhosis[34-36], in defining severity and progression of the disease and in predicting short-term survival[37-39].

The clinical utility of ABT in cholestatic diseases is limited. Since aminopyrine is eliminated almost entirely by hepatic metabolism and does not undergo enterohepatic circulation, its elimination is expected to be unaffected by cholestasis. In a study performed by Hepner and Vesell aminopyrine metabolism was found to be normal in most cases of benign obstructive cholestasis and abnormal in

few patients with acute cholestasis caused by gallstone obstruction, drugs or late primary biliary cirrhosis[12]. Furthermore, ABT values resulted higher in patients with early primary biliary cirrhosis than in patients with chronic active hepatitis[29]. Finally, low ABT values were found in advanced primary biliary cirrhosis associated with hepatocellular failure. Based on these results, ABT could be used as a screening test in patients with hyperbilirubinaemia in order to distinguish between cholestasis and hepatocellular disease[40-41].

As concerned the usefulness of ABT in patients with liver neoplasms, the results of the only study performed are inconclusive[42].

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[43]. 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[44]. 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 pre-operative risk analysis of patients with oesophageal cancer, thus contributing to a composite pre-operative risk score of individual organ dysfunction[45].

In recent years breath tests have been proposed as a non-invasive method for monitoring hepatic function in patients waiting for orthotopic liver transplantation and during follow-up after this procedure. In particular, Heidecke showed that daily ABT measurements after orthotopic liver transplantation is a better predictor of acute allograft rejection than other laboratory tests performed[46]. Mion et al. used ABT to monitor liver graft recovery in the early post-orthotopic liver transplantation and reported a progressive increase of ABT values after 48 h up to 7-10 days in normal patients compared to a reduction of ABT values that occurred in patients with liver graft dysfunction[47]. Recently, Di Campli et al. have shown that ABT test is useful in the early phases after orthotopic liver transplantation to predict primary non function of the transplanted liver.

Finally, ABT has been proposed to assess the severity and to predict the evolution of liver injury in drugs mediated hepatotoxicity[48].

In spite of these extensive studies, some limitations in the use of ABT as a marker of hepatic function and reserve must be recognized. First of all we should remember that cytochrome P450 activity could be induced or inhibited by many endogenous/exogenous factors that may influence ABT results. Among the potential confounding factors in interpreting the ABT we can include the age-[49,50] and sex-related[51] changes in liver *N*-demethylase activity, the concomitant presence of chronic diseases or the administration of *N*-demethylase enzyme modulators. ABT results are greatly influnced by age since they showed a progressive decrease with advancing age[52]. If we consider sex differences, it is possible to hypothize the role of exogenous female sex hormones in decreasing aminopyrine *N*-demethylation[54-55] even if sex differences in aminopyrine metabolism have not been found in adult humans[53]. As concerning the influence of nutritional status, malnutrition seems to decrease the metabolism of aminopyrine[56]. Equally, congestive heart failure or chronic renal failure have been shown to decrease aminopyrine *N*-demethylation[57-58]. However, drug interference and other environmental factors are likely the most important confounders in the interpretation of ABT results. In particular, aminopyrine *N*-demethylase activity has been found to be induced after treatment with phenobarbital[59] glutethimide[60], diphenylhydantoin[61], steroids[27] and spironolactone[62]administration. On the other hand, aminopyrine *N*-demethylation is depressed after cimetidine[63], disulfiram, allopurinol[64], albendazole[65], cytostatic drugs[66], interferon[67]and influenza vaccination[68]administration and after long-term exposure to pesticides[69]. Cigarette smoking has also been reported to increase ABT values[70], whereas aminopyrine *N*-demethylation is depressed after acute ethanol intake and increased during chronic ethanol consumption[38]. Moreover, several factors such as fever, physical exertion, meal intake and hyperthyroidism could affect the ABT results by increasing the endogenous CO_2 production. Similarly other conditions like sleep, hypothermia and hypothyroidism are able to influence aminopyrine metabolism by decreasing the endogenous CO_2 production[60]. In conclusion, all of the above described potential confounding factors should be considered when normal ABT values are

found in patients with clinical and laboratory signs of liver dysfunction and, on the other hand, when abnormal ABT results are found in patients without liver disease.

Finally, as concerning the toxicity of aminopyrine. No side effects has been reported in literature with the low single doses required for the breath test analysis[72], although the occurrence of agranulocytosis has been described with the chronic pharmacological administration[71]

In conclusion, we can assess that ABT is one of the most widely used tests to assess functional liver microsomal mass. There are several reasons that justify its use: (1) ABT may have a complementary role to liver histology in grading chronic hepatitis; (2) 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; (3) seems to be a sensitive survival predictor in patients with liver disease; (4) shows a prognostic value in patients undergoing hepatic or shunt surgery; (5) is useful in assessing liver function after treatment, showing potential application in the longitudinal monitoring of liver transplantation. On the other hand there are still some reasons that limit it use in the clinical practice: ABT has never been tested in organized clinical trial. 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 and the follow up of patients after treatment.

Caffeine Breath Test

Caffeine is a common substance, widespread in human nutrition. Its pharmacokinetics is well-know: completely absorbed in intestinal tract, it undergoes N-demethylation through cytochrome P450, in particular IA2 subtype[7] , with production of paraxanthine (its most important metabolite), theobromine and theophylline[73]. It is a good substrate to provide information on hepatic metabolic capacity, because of its low extraction ratio (below 0,3)[74]. Although it has a low plasma binding, it is safe and inexpensive. Caffeine seems to have all the characteristics of an ideal liver test substrate. Initial researches studied its metabolism through serum and salivary samples. Although they had yielded promising results, there were many shortcomings to introduce them in clinical practice. Plasma caffeine clearance required repeated blood sampling while salivary concentration could be affected by pH level or by flow rate[75-76]. ^{13}C-caffeine breath test allows to overcome the drawbacks of traditional tests. Many researches explored which labeled methyl group best reflected caffeine N-demethylation, concluding that 3-methyl-C-labelled caffeine was the best to estimate hepatic caffeine metabolism[77]. Further investigations were carried out to clarify the effect of smoking on caffeine metabolism. Although it is well-established that cigarette smoking is able to induce the activity of P450 IA1, multivariate analysis assesses that only smoking and disease state were independent predictors of caffeine breath test[78]. Sex and age differences have not been found[78-79].

To date, caffeine breath test has been well-studied to explore the effect of xenobiotics on P450 IA1 activity. The degree of induction of hepatic microsomal activity can represent a good indicator of hepatic functional reserve. A recent study has shown that the inducibility of microsomal function, assessed by caffeine, may differentiate cirrhotic patients with maintained and compromised liver reserve[80]. As well as aminopyrine, caffeine metabolism increases less in cirrhotic smokers comparated with smoker controls[78]. There is lacking in studies to ascertain whether caffeine breath is a hepatic metabolizing good predictor of disease. In 1984, Renner et al evaluated breath samples after intravenous administration of radioactively ^{14}C-caffeine in patients with chronic liver disease and healthy subjects. It seemed to correlate with plasma caffeine clearance and to indicate varying degrees of liver disease. Nevertheless comparison with traditional liver parameters had not been performed[81]. Recently, Park et al. clarified many aspects of caffeine breath test. Using the stable isotopic label ^{13}C administrated *per os* (2 mg/kg), they demonstrated that ^{13}C-caffeine breath test is a valid

indicator of plasma caffeine clearance and correlates with varying degrees of liver impairment. There is a good correlation with serum albumin and platelet count and a weak but significant correlation with international normalized ratio and transaminase levels. One study has shown a close inversely association between caffeine breath test and Child-Pugh Score and demonstrated the ability of this test to predict cirrhosis state in patients with normal biochemical parameters[78]. Nevertheless, its prognostic potential deserves further investigation. In addition, a very interesting application could be about the evaluation of the P450 IA1 cytochrome ability to activate carcinogenic arylamines. Estimating specifically P450 IA1 cytochrome activity, it is possible to characterize arylamine N-oxidation phenotype and to predict the susceptibility to arylamine-induced cancer. Since tobacco increases this specific cytochrome activity, the carcinogenic effect of smoking could be explained[82].

In conclusion, ^{13}C-caffeine breath test is a reliable "enzyme-limited" test, able to provide information on hepatic metabolic capacity and to distinguish between cirrhotic, hepatopatic patients and controls. It could be introduced as a complementary test in the assessment of liver disease, in addition of traditional biochemical, histologic and imaging data. Because of its safety, it is useful for studying hepatic disorders in children and in pregnant women. Its low cost or its availability must be underlined. Finally, although it has been generally accepted that 2 hour cumulative exhalation of labelled CO_2 represents the best parameter of caffeine clearance, a recent study has demonstated that a single hour measure can be enough and simplifies the test[78].

Diazepam Breath Test

Diazepam, such as other benzodiazepins, is metabolised by cytochrome P450 and, in particular, by 2C19 subtype. It undergoes N-demethiyation with production of desmethyldiazepam, which is subsequently conjugated in phase II reactions. Finally, the soluble metabolite is excreted with urine. It has been demonstrated that diazepam breath test (DBT) has a good correlation with the diazepam plasma half-life or diazepam metabolic clearance rate and a significant but weak correlation with the 24hr recovery of ^{14}C in urine. Comparing DBT and ABT, aminopyrine has a half-life of $^{14}CO_2$ longer than diazepam in women taking oral contraceptive steroids: this is the proof of different P450 subtype involved in the metabolism of the two drugs[83]. Many researchers have studied the application of diazepam breath test in clinical practice. Hepner et al compared diazepam metabolism in patients receiving anticonvulsants and patients with hepatobiliary diseases. The value of $^{14}CO_2$ was increased in the first group, decreased in the latter but not in patients with cholestasis[84].

Although diazepam has a low extraction ratio and it could be a good mass index, it has been considered an unsuitable substrate to assess hepatic function because of the genetic polymorphism of the P450 2C19 gene and therefore the inter-assay variability[85].

Flow-Limited Breath Tests

Methacetin Breath Test

Methacetin [*N*-(4-methoxyphenyl)acetamide], a derivative of phenacetin, is metabolized through *O*-demethylation by the hepatic function oxidase system leading to the final production of acetaminophen and CO_2[86]. Methacetin has been proposed as an alternative to aminopyrine, because of its rapid metabolism in normal subjects and the lack of toxicity in small doses. Moreover, it has been showed a less pronounced induction of methacetin than aminopyrine metabolism by cigarette smoking and anticonvulsant drugs[87].

In order to develop a test able to estimate metabolic liver capacity and, at the same time, applicable to all patients without any radiation hazard, Krumbiegel et al. evaluated the reliability of the ^{13}C- with respect to the ^{14}C-methacetin breath test (MBT) in healthy subjects and patients with liver cirrhosis[88]. In both study groups, ^{14}C and ^{13}C-MBT curves were nearly overlapping and a good discrimination between healthy volunteers and patients was observed.

The efficacy of ^{13}C-MBT was evaluated in a further study by Fahl et al. using different doses of labelled methacetin in healthy subjects[89]. The same authors reported a significant correlation between MBT values, total serum bile acids and histology in patients with liver disease[90].

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[91]. In this study no significant differences were founded between healthy controls and patients 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.

Recently it has been reported that the MBT is influenced by both age and blood oxygenation . In particular, the results of a study performed by Ciccocioppo et al showed that the MBT values are influenced by age in healthy subjects and inversely related to the intra-hepatic resistence index, assessed by Doppler pulsed wave analysis. Zipprich et al demonstrated that oxigen supplementation increased the liver oxidation capability in cirrhotic patients in different Child-Pugh classes but is not clear if MBT alterations after oxygen supplementation are related to metabolic factors (e.g., interference with the bicarbonate central body pool and increased excretion of CO_2) or liver microsomal activity[92-93].

In a successive study performed by Klatt et al, MBT values were used to discriminate between cirrhotic and non-cirrhotic subjects with a sensitivity of 93.5% and a specificity, 95%[94] and correlated with Child-Pugh score better than monoethylglycinexylidide test and indocyanine green clearance. In a similar study Pfaffenbach et al found that the ^{13}C-MBT maximal percentage rate and the cumulative rate over 30 min up to 3 h were significantly lower in cirrhotic patients than in controls and among the former were able to discriminate different Child-Pugh categories[95].

Further studies have supported these results. In particular a study performed by Lara Baruque et al, showed the abilty of MBT to discriminate among healthy controls, patients with chronic hepatitis and patients with Child A-C cirrhosis[97].

MBT was also used to monitor hepatic function and to define prognosis of patients with advanced liver cirrhosis listed for orthotopic liver transplantation The same authors demonstrated that MBT is a potentially useful tool for assessing the recovery of hepatic function immediately after graft reperfusion and in the first weeks after liver transplantation[98].

Based on these data, we can assess that MBT is a promising test to probe liver function. It is able 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 and for following cirrhotic patients awaiting or undergoing orthotopic liver transplantation . The only limit of methacetin is the high hepatic extraction, so that becomes very important to study the effects of altered blood flow on its pharmacokinetics before MBT can be rationally applied in larger prospective studies.

Phenacetin Breath Test

Phenacetin is mainly metabolised by cytochrome P450 IA2 with O-de-ethylation. Subsequently acetaldehyde or ethanol are oxidized via the tricarboxilic acid cicle to CO_2, detectable in breath samples[7]. Nevertheless, this pathway could not be the rate limiting step of phenacetin metabolism, since there is neither an increase in CO_2 excretion after induction with rifampin or a saturation of enzyme increasing the dose. Because of its high extraction rate, it undergoes an extensive first pass clearance and it is affected by hepatic shunt. Nevertheless, there are not differences between intravenous or oral administration and it has been demonstrated that the peak of $^{14}CO_2$ excretion correlates with the plasmatic clearance. Breen et al. found that phenacetin metabolism was decreased in patients with liver disease ,but the effect of hepatic shunt in cirrhotic patients was not evaluated[99]. Schoeller et al compared phenacetin and aminopyrine breath test and demonstrated a good correlation only in hepatopatic patients but not in controls[100]. Controversial results have been reported about the use of phenacetin breath test and the reliability of this compound is limited by several factors that may induce its metabolising enzymes such us smoking or dietary factors. Recently, Kajiwara et al tried to increase the specificity and sensitivity with the association of ^{13}C-

breath test (using 1-^{13}C-ethoxy-phenacetin) and urine test (using ^{13}C-nuclear magnetic resonance spectroscopy)[101]. Since more investigations are required to assess its role in clinical practice, at the moment ^{13}C-phenacetin breath test application does not seem reliable.

Erythromycin Breath Test

Erytromycin is metabolised by cytochrome P450IIIA, that is the most important drug metabolizing enzyme, representing the 25% of total cythocromal proteins. It is also involved in the metabolism of many drugs, such as steroids, immunosuppressants, sedatives, and the bioactivation carcinogenic xenobiotics[85]. Few studies have been carried out to assess the role of erytromycin breath test as a test of general liver function, in fact it does seem neither correlated with biochemical test or indicative of varying degrees of liver disease[102]. On the other hands, it represents the gold standard to quantify, *in vivo*, the activity of cytochrome P450IIIA[103]. Most drugs can follow two metabolic pathway: phase I reactions in cytochrome P450 families (IIIA subfamily is the most representative) and interrelations with the P-glycoprotein (PGP), able to put xenobiotics out of the cells. Both the proteins are located in hepatocyte and in enterocyte. In particular , PGP is situated at the apical membrane of the cells in intestinal tract and in pericalicular domain in hepatocyte[104]. It may be useful to quantify the activity of both proteins to predict drug interactions or dose adjustment. Many methods have been proposed. *In vitro,* it is possible to measure P450 IIIA4 enzymatic activity directly on liver biopsy or cell cultures, while *in vivo* ^{14}C- breath test after intravenous administration of eritromycin represents the gold standard. Nevertheless the latter method does not allow to receive information about drug metabolism in intestinal tract. To overcome such shortcomings, Lemahieu et al tried to dose eritromycin in urine and breath samples after oral and intravenous administration. With mathematical analysis it was possible to distinguish between P450 and PGP activity in the liver and in intestinal tract, predicting different enzymatic phenotypes. A great varability has been described and it could explain, at least in part, interindividual differences in kinetics and dosing requirements of drugs. However a good application of eritromycin breath test could be the therapeutic monitoring of tacrolimus or cyclosporine, metabolised specificately by P450 IIIA4[105]. Recently, Schmidt et al have demonstrated that eritromycin breath test can be a reliable predictor of tacrolimus nephrotoxicity, severe graft dysfunction or graft loss liver transplant recipients. In addition, eritromycin breath test has been used to assess P450 IIIA4 down-regulation after interferon treatment. In conclusion, although erythromicin breath test is a useful tool to predict drug interactions or to adjust the therapeutic dose of immunosuppressants, many drawbacks limit its diffusion in clinical practice, in particular the utilization of radioactive ^{14}Carbon and the intravenous administration. If the utilization of the stable ^{14}Carbon may be possible, the oral administration does not seem reliable because of intestinal metabolism[106].

Conclusion

Since the initial use of liver breath test by Hepner and Vesell occurred nearly 30 years ago, several 'liver function' breath tests have been developed assessing the specific hepatic metabolism of specific substrates. However, at time they are still performed only in a few medical centres, although their use appears to be conceptually logical and rather simple. This indicates a general dissatisfaction and uncertainty amongst the scientific community in these other 'dynamic' function tests.

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, as concerning the assessment of hapatic microsomal function, aminopyrine and methacetin have shown some interesting results. To date ABT has been proven to be useful tool to predict survival in patients with chronic liver disease or acute alcoholic liver disease and in patients undergoing surgery or shunt procedures. Finally, they have been used to monitor liver function or to determine immunosuppressive drug dosage, giving, in some cases, more information than other biochemical, clinical or dynamic liver function parameters.

For example, ERMBT has been proven to be extremely useful in predicting ciclosporin and FK 506 blood levels after orthotopic liver transplantation.

We can conclude that, although microsomal breath tests are useful for defining the prognosis and following the treatment response of patients with liver disease, large, well-designed, prospective, longitudinal studies are needed to assess their clinical usefulness to evaluate liver function.

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