Specific ^{13}C functional pathways as diagnostic targets in gastroenterology breath-tests: tricks for a correct interpretation

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Abstract. Breath tests are non-invasive, non-radioactive, safe, simple and effective tests able to determine significant metabolic alterations due to specific diseases or lack of specific enzymes. Carbon isotope ^{13}C, the stable-non radioactive isotope of carbon, is the most used substrate in breath testing, in which ^{13}C/^{12}C ratio is measured and expressed as a delta value, a differences between readings and a fixed standard. ^{13}C/^{12}C ratio is measured with isotope ratio mass spectrometry or non-dispersive isotope-selective infrared spectrometer and generally there is a good agreement between these techniques in the isotope ratio estimation. ^{13}C/^{12}C ratio can be expressed as static measurement (like delta over baseline in urea breath test) or as dynamic measurement as percent dose recovery, but more dosages are necessary. ^{13}C Breath-tests are involved in many fields of interest within gastroenterology, such as detection of Helicobacter pylori infection, study of gastric emptying, assessment of liver and exocrine pancreatic functions, determination of orocaecal transit time, evaluation of absorption and to a lesser extend detection of bacterial overgrowth. The use of every single test in a clinical setting is vary depending on accuracy and substrate costs.

This review is meant to present ^{13}C the meaning of ^{13}C/^{12}C ratio and static and dynamic measure and, finally, the instruments dedicated to its use in gastroenterology.

A brief presentation of ^{13}C breath tests in gastroenterology is also provided.

Keywords:

^{13}C/^{12}C ratio, C13 hysotope, Mass spectroscopy, Infrared spectroscopy breath test.

Introduction

Breath-tests are based on the collection of breathed air samples at certain time intervals, starting from the administration of a specific substrate. Several substrates may be used for the purpose, although the most important in clinical practice remain H_2 and ^{13}C.

Carbon isotope breath test have been developed three to four decades ago for testing specific patterns in pancreatic and hepatic metabolism. The first breath test introduced in clinical practice used medium-chain triglyceride (MCT) trioctanoate-1-^{14}C$_4$ for studying steatorrhea. In normal subjects, MCTs passively diffuse from the GI tract to the portal system without requirement for modification and are metabolized by liver with production of ^{14}C-carbon dioxide that is eliminated by lungs. In subjects with steatorrhea there was lower production of ^{14}C-carbon dioxide[1].

^{14}C breath tests were good models for studying metabolic pathways and develop correct mathematical algorithm, but this isotope cannot be used routinely in clinical practice due to its radioactivity, with decaying with a half-life of about 5,730 years. More recently, ^{13}C was introduced in gastroenterology and suddenly became more utilized until substituting almost completely ^{14}C.

Although since the beginning the importance of ^{13}C utilization in functional pathways in gastroenterology was clear, the relevance of ^{13}C breath testing was consolidated when ^{13}C urea breath test was utilized for diagnosing *Helicobacter pylori* (*H. pylori*) infection.

^{13}C isotope in human and in external environment

Carbon is the most diffused element in living organisms, plants and animals. It is a non-metallic chemical element, tetravalent, making four electrons available to form covalent chemical bonds. In the human body, carbon is the second most abundant element by mass (about 18,5%) after oxygen,

but the main element found in organic compound: in sugars, chitins, alcohols and fats it is conjugated with oxygen and hydrogen, in proteins it is conjugated with nitrogen and sulfur while in nucleic acids (DNA and RNA) with phosphorus.

Carbon exists in nature in few isotopic forms; the most widespread forms are ^{12}C, ^{13}C and ^{14}C isotopes. While ^{14}C is instable radioactive isotope, with a half time decay of 5730 years, only ^{12}C and ^{13}C are stable forms. Most of the carbon is ^{12}C (98.98%), with 1.11% being ^{13}C. These isotopes are unevenly diffused among the different compounds deriving, for instance from different regions or part of the world, and this isotopic distribution can reveal information about chemical, physical and metabolic processes during carbon transformation. Several data demonstrated that ^{13}C is diffused in a higher quantity in carbonates and plants (corn and cane sugar), while it is absent in fossil fuels. In the human body the pool of ^{13}C is approximately 2 g/kg body weight and up to 25 mg/kg are additionally ingested every day.

Meaning of $^{13}C/^{12}C$ iso-type ratio and its application in breath testing

Because the ^{13}C and ^{12}C are stable isotopes, the information inherent in the $^{13}C/^{12}C$ ratio is invariant as long as carbon is not lost, but its variation is the consequence of isotope effects expressed during the formation and destruction of chemical bonds involving carbon atoms, that are affected by atomic mass. $^{13}C/^{12}C$ ratio indicates the proportions of the two different isotopes in a specific compound or in a specific metabolic flow, and this difference is expressed with a Delta, a value given in parts per thousand or per million. Delta value does not express absolute isotope abundance but the difference between sample readings and one of the widely used natural abundance standards which are considered delta = zero. The common reference for delta ^{13}C was obtained from *Belemnitella Americana*, a genus of belemnite from the late cretaceous of Europe and North America, that has the higher $^{13}C/^{12}C$ ratio than nearly all other natural carbon-based substances. For convenience it is assigned a delta ^{13}C value of zero, giving other samples negative delta values.

^{13}C breath-tests are based on a ^{13}C-labelled substrate, specifically designed for a gateway enzyme in a specific metabolic pathway. The turnover of the substrate can be measured by controlling the unidirectional decomposition to ^{13}C-labelled carbon dioxide, that can be found in the exhalation. The timing and the quantity of breathed ^{13}C distribution is helpful for discrimination of pathological conditions, because they may reflect the alteration of a specific metabolic pathway; the time production and the quantity of ^{13}C labeled carbon dioxide in exhalation is determined by the cleavage of the substrate in GI lumen, the transport processes and the enzymatic degradation. Alterations in each of these points can modify the concentration of ^{13}C carbon dioxide in the exhalation and alter the usual ^{13}C-^{12}C ratio. For example, in Urea Breath test, the difference between DOB at baseline and DOB following external ^{13}C urea administration, is considered positive in case of differences among the two values of 2.5 or 3.5 or 5, depending on the protocol utilized.

Tracer could be detected and expressed as static or dynamic variable. In the first case it's considered simply the delta over baseline, like in urea breath test. In the second case $^{13}C/^{12}C$ can be expressed as recovery rate per hour or percent dose recovery and more than 2 dosages are necessary and special algorithms are used.

Moreover, in order to get accurate results, measurement or estimation of basal ^{13}C value is recommended. This value is usually approximated to 300 mmol CO_2/m^2 bsa/h and is potentially influenced by any condition able to alter endogenous CO_2 production or excretion. Hence, breath-test results may be affected by several conditions including fever, physical exercise, respiratory diseases, food ingestion and dys-thyroidisms. Moreover, ^{13}C recovery is never complete as a substantial amount of tracer is retained in the carbon pool of the body. Therefore, ^{13}C breath test analysis is a semi-quantitative diagnostic tool.

$^{13}C/^{12}C$ ratio detection and available technologies

Breath samples are analyzed by high-resolution mass spectrometers, which are able to measure the slight mass difference of one neutron between ^{13}C-labelled carbon dioxide and the naturally most common carbon dioxide with the carbon isotope ^{12}C, on the base of different light absorbance between the two isotopes. The precision of high resolution mass spectrometers is very high, so very low isotopic quantity can be detected, thus, permitting the use of small samples of exhaled air; however, mass spectrometers are highly expensive, they have relatively long analysis time and they require well trained personnel for their use.

Core elements for mass spectroscopy are the ion source, the magnet chamber and the detector.

**Table I.** Main clinical indication for ¹³C breath tests and their description.

Indication	Substrate	Substrate costs	Static/ Dynamic	Clinical importance	Diagnostic alternative
Helicobacter pylori infection	¹³C-urea	Low	Static	High	Fecal antigen test, histology, rapid urease test, culture, serum antibodies
Gastric emptying – solid phases	¹³C-octanoate ¹³C-spirulina	Low High	Dynamic	High	Scintigraphy
Gastric emptying – liquid phases	¹³C-acetate ¹³C-glycine	Low Low	Dynamic	Moderate	Scintigraphy
Liver function	¹³C-aminopyrine ¹³C-methacetin ¹³C-galactose ¹³C-phenacetin ¹³C-phenylalanine ¹³C-erythromycine ¹³C-caffein ¹³C-methionine ¹³C-ketoiso-caproic acid ¹³C-octanoic acid	Moderate Low High Moderate Moderate High High High Moderate Low	Dynamic Static/Dynamic Dynamic Dynamic Dynamic Static Dynamic Static Dynamic Dynamic	Moderate	Clinical scores, MEGX-test, Galactose elimination capacity, Indocyanine green retention test
Exocrine pancreatic function	¹³C-mixed triglycerides ¹³C-triolein ¹³C-trioctanoin ¹³C-tripalmitin ¹³C-hiolein ¹³C-cholesterol-octanoate ¹³C-protein ¹³C-starch	Moderate High Moderate High High High High Low	Dynamic Dynamic Dynamic Dynamic Dynamic Dynamic Dynamic Dynamic	Low	Secretin pancreozymin test, faecal fat analysis, faecal elastase
Absorption	¹³C-palmitic acid ¹³C-olein ¹³C-protein	High High High	Dynamic Dynamic Dynamic	Very low	Faecal fat
Orocoecal transit time	¹³C-lactosoureide	High	Dynamic	Low	Lactulose and inuline H₂ breath test, scintigraphy
Carbohydrate assimilation	¹³C-lactose ¹³C-fructose ¹³C-glucose ¹³C-starch ¹³C-saccharose	High Moderate Moderate Low High		Very low	H₂ breath tests, measurement of enzyme activity in the duodenal biopsy
Bacterial overgrowth	¹³C-glycocholate ¹³C-xylose	High High	Low		Glucose H₂ breath test, microbiological culture from jejunal aspirate

The first element is the ion source, which is the chamber where gases become ionized and then pushed into the magnet chamber. Magnet chamber, which is kept with vacuum, is the place where ions are separated in order to be able to reach the sensor according to each ion weight mass.

Usually, measures from a mass spectroscopy display standard deviation of only 0.1-0.2‰ of delta values.

Although the capability of mass spectrometry in measuring the difference of one neutron between ¹²C and ¹³C, the introduction of infrared technologies (non-dispersive isotope selected infrared spectrometers, NDIRS), based on the use of photo-acoustic detectors, made this goal cheaper with adequate accuracy.

The ability of photo-acoustic detectors, in fact, is that of being able to measure variation in
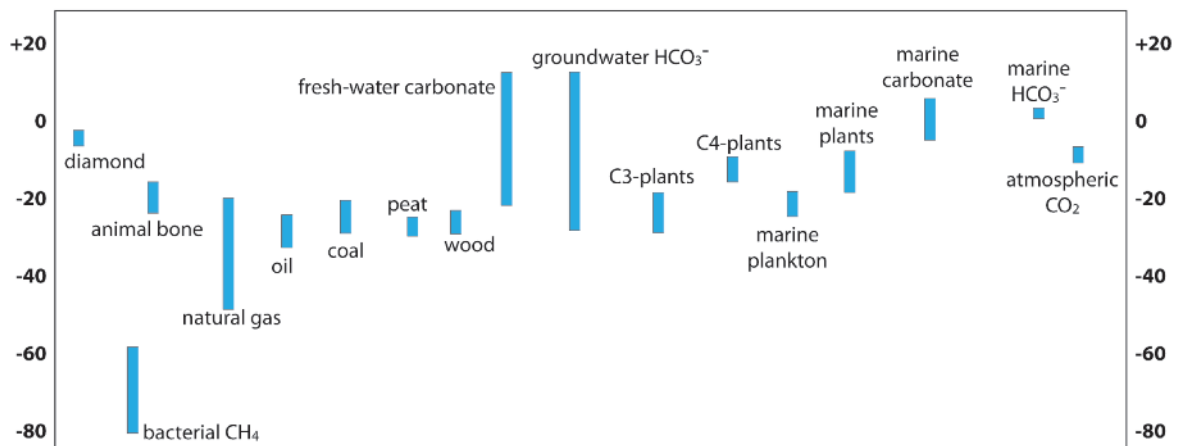
$$Delta\ (‰) = 1000 * (^{13}C/^{12}C_{sample} - \ ^{13}C/^{12}C_{PDB})\ /\ ^{13}C/^{12}C_{PDB}$$

Figure 1. Schematic representation of Delta from different products from soil, plants, food or air.

the absorbance of the light through different combination of circuits using standard gases. The measure is, therefore, not depending directly on mass weight like for mass spectroscopy. It is easy to understand that in this case standard deviation for measures coming from infrared based spectrometer is usually 0.2-0.3‰ of delta values.

Operating and handling of NDIRS is easy, even for non-experienced users. This enables not only the performance, but also the diffusion of ^{13}C-breath tests in primary care settings. Generally, there is a good agreement between mass spectroscopy and infrared-based technology, for ^{13}C/^{12}C ratio estimate, particularly for Urea breath test[2,3]. For dynamic tests, however, and when a major precision of the parameter is required, the real validity of infrared instrument needs to be checked according to protocol of validation of the given test.

Clinical applications in gastroenterology

[13]C Breath-tests are involved in many fields of interest within gastroenterology, such as detection of *H. pylori* infection, study of gastric emptying, assessment of liver and exocrine pancreatic functions, determination of orocaecal transit time, evaluation of absorption and to a lesser extend detection of bacterial overgrowth.

The use of a single test in a clinical setting varies depending on accuracy and substrate costs.

Among the breath tests utilizing a single difference measurement of ^{13}C/^{12}C ratio, there is the urea breath test for diagnosing *H. pylori*, the first cause of peptic ulcer disease and dyspepsia. The ^{13}C-urea

breath test is probably the best known, best standardised and most widely used breath test; the non-invasiveness of this breath test results in a high acceptance in patients. The easy execution and the safety of this test make it also applicable for primary testing in adult, young and paediatric patients. Moreover, multiple studies and a meta-analysis including more than 3500 patients demonstrated excellent sensitivity (95%) and specificity (>95%) compared to histology and faecal test; so ^{13}C urea breath test remains a cornerstone in diagnosis and control of *H. pylori* infection[4,5]. The ^{13}C urea breath test is based on the action of bacterial enzyme urease: in subjects affected by Helicobacter infection ^{13}C-urea is hydrolyzed by the bacterial urease activity to ammonia and ^{13}C-labelled carbon dioxide, that is eliminated by lungs, therefore an increase of [13]CO_2 in breath appearing 30 min after drinking a test solution containing 75-100 or 125 mg ^{13}C-urea reliably detects *H. pylori* infection. Since the first description by Graham et al[6] who performed ^{13}C-Urea breath test with a test meal containing 350 mg urea, doses have been gradually reduced until the standard of 100 mg or 75 mg or less.

Urea breath tests is the only available test using a well defined metabolic pathway determined by the presence of urease, a key enzyme utilized by Helicobacter to survive in the stomach.

Other breath tests, as they are more related to digestion or metabolic functions of the host rather than a single chemical reaction, require a longer observation period as they are more influenced by time and concomitant factors. That is the reason while "dynamic" measures have been adapted to synthetize complex phenomena.

^{13}C-octanoate and ^{13}C-acetate are the most used substrate in breath testing, respectively used to label solid and liquid components of test meals and they represent the main trace element for gastric emptying. ^{13}C-octanoic acid is mixed into egg yolk and baked and when this compound has passed the pylorus together with the ingested food, the ^{13}C middle chain fatty acid is rapidly absorbed in duodenum and jejunum and is oxidised to ^{13}C-labelled carbon dioxide, eliminated by lungs. Main clinical indications for gastric emptying breath test are functional dyspepsia and autonomic diabetic neuropathy. Simultaneous labelling of solid and liquid phases, though could be done in nuclear medicine, is possible in breath-testing only with the addition of ^{14}C, whose clinical use is obsolete because of its long half-time decay[7].

^{13}C breath tests can be used to study liver function: they are non invasive tests that quantities the residual liver function in patients with chronic hepatopaties, from minimal stages up to liver cirrhosis. Several substrates have been proposed but the most used in clinical practice are methacetin and aminopyrine[8,9].

Most hepatic breath tests measure the microsomal dealcylation of ^{13}C-labelled substrates and the cytochrome P450 dependent enzymatic system. The cleaved methyl group is oxidized to formic acid, which enters the Carbon-pool, and is finally exhaled as carbon dioxide. Moreover, the measure of ^{13}C labeled carbon dioxide is proportional to liver enzymatic system integrity.

Another application of ^{13}C breath testing involves the study of pancreatic activity. The secretin pancreozymin test is the most accurate of the pancreatic exocrine function tests, but it is invasive as it requires duodenal intubation and hormonal stimulation of the pancreas, while fecal analysis of fat, fecal elastase or chymptrypsine are more practicable, but less sensitive to detect early stages of pancreatic exocrine insufficiency. ^{13}C breath test with ^{13}C labeled triglycerides, cholesterol and proteins can be used as indirect and non invasive tests for evaluate pancreatic exotic function. These valid substrates of pancreatic enzymes are metabolized in the duodenum under physiological conditions, they are easily absorbed by mucosal wall and metabolized in the liver cells with production of ^{13}C labelled carbon dioxide, excreted by lungs. Low ^{13}C/^{12}C ratio in expired air indicate a not completed digestion of marked substrates due to impaired pancreatic secretion. The triglycerides are ^{13}C-labelled at the carboxy group of the fatty acids. Only in pres-ence of valid intra-luminal lipolysis, the free fatty acids or monoglycerides can be absorbed and oxidized to produce ^{13}C carbon dioxide.

Sensitivity and specificity of the mixed triglyceride breath test compared to the stimulated lipase output in the pancreocymin test is 89%, respectively 81%[10-12]. ^{13}C-labelled starch is a good substrate for amylase. But the ^{13}C-starch breath test is not very sensitive because can be influenced by other pathologies characterized by alteration of amylase; while a ^{13}C-protein breath test based on chicken egg white has been described for testing the activity of trypsine[13-15].

If breath tests are valid methods for evaluate pancreatic function, the costs of the substrates, the high time expenditure and the lack of standardization limit the utilization and the diffusion in clinical settings.

^{13}C breath tests can be used also in the measure of oro-caecal transit time. For example ^{13}C lactose has proven to be a valid marker to evaluate oro-caecal transit time: it resists the action of intestinal enzymes and it is metabolized by colonic microflora in the cecum. The valuation of time from ingestion of the ^{13}C labeled substrate to the first increase in breath ^{13}C/^{12}C ratio indicates the oro-caecal transit time.

Conclusions

Carbon isotope ^{13}C is a stable non-radioactive isotope of carbon naturally present in nature. The study of ^{13}C/^{12}C ratio is able to detect differences in compounds with different origin. In human biology ^{13}C/^{12}C ratio could be studied for several applications, although the one that resulted in the greatest success was for the detection of *Helicobacter pylori* infection.

Among ^{13}C breath tests, urea breath test is depending mostly by one metabolic pathway and for this reason it presents the highest sensibility and specificity. Other breath tests, as they are influenced by many physiological functions like digestion and adsorption, need more measurements and display different degree of specificity and sensibility.

Finally most of C13 products rather then urea breath tests, are still missing a clear classification of their status: they are not registered as drugs and not like food supplements.

^{13}C breath tests are interesting tools in gastroenterology and probably, besides urea breath allow test, they require a major interest in order to let them answering questions still un-answered in clinical practice.

Conflict of interest

The Authors declare that they have no conflict of interests.

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