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Stable Isotope Techniques for the Assessment of Host and Microbiota Response During Gastrointestinal Dysfunction

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ABSTRACT

The International Atomic Energy Agency convened a technical meeting on environmental enteric dysfunction (EED) in Vienna (October 28–30, 2015; https://nucleus.iaea.org/HHW/Nutrition/EED_Technical_Meeting/index.html) to bring together international experts in the fields of EED, nutrition, and stable isotope technologies. Advances in stable isotope–labeling techniques open up new possibilities to improve our understanding of gastrointestinal dysfunction and the role of the microbiota in host health. In the context of EED, little is known about the role gut dysfunction may play in macro- and micronutrient bioavailability and requirements and what the consequences may be for nutritional status and linear growth. Stable isotope labeling techniques have been used to assess intestinal mucosal injury and barrier function, carbohydrate digestion and fermentation, protein-derived amino acid bioavailability and requirements, micronutrient bioavailability and to track microbe–microbe and microbe–host interactions at the single cell level. The noninvasive nature of stable isotope technologies potentially allow for low-hazard, field-deployable tests of gut dysfunction that are applicable across all age groups. The purpose of this review is to assess the state-of-the-art use of stable isotope technologies and to provide a perspective on where these technologies can be exploited to further our understanding of gut dysfunction in EED.

Key Words: environmental enteric dysfunction, gut dysfunction, gut microbiota, malnutrition, stable isotopes

(*JPGN* 2017;64: 8–14)

Environmental enteric dysfunction (EED) is thought to be a major contributing cause of the failure of nutritional interventions, be they based on micronutrient, macronutrient, and combined supplementation with behavioral change to normalize postnatal linear growth in populations living in poverty (conceptualized in Fig. 1 adapted from (1)). EED, among other factors, is the result of multiple and continuous infections even in the absence of diarrhea or well-defined clinical illness. It is posited to lead to local intestinal inflammation, altered intestinal permeability with subsequent bacterial translocation and immune activation. Functional aberrations

What Is Known

- Linear growth retardation (stunting) currently affects 165 million children worldwide.
- Nutritional interventions have had only moderate effects on linear growth.
- Environmental enteric dysfunction is thought to be a major contributing cause of the failure of nutritional interventions.

What Is New

- There is a lack of functional biomarkers to assess gut dysfunction in environmental enteric dysfunction. Biomarkers of nutrient digestion and absorption capacity, gut permeability, and gut and systemic inflammation may allow a greater understanding of the role of environmental enteric dysfunction in nutritional interventions. Stable isotope techniques have the potential to assess multiple aspects of gut dysfunction in environmental enteric dysfunction.

in EED may include a reduced ability to digest macronutrients, reduced capacity to absorb essential nutrients, and disturbances in micronutrient requirements. The role of the small intestinal microbiota is unclear but clearly is an important factor in the development and pathogenesis of EED (2). In EED, the state of chronic immune activation compromises intestinal function and host metabolism in a way that derails normal prescribed linear growth patterns in affected populations.

Received April 22, 2016; accepted August 2, 2016.

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Work at the Stable Isotope Biochemistry Laboratory, SUERC is funded in part by BBSRC (BB/L004259/1, BB/L025418/1). A.L. is supported by the Vienna Science and Technology Fund (WWTF, project LS12-001) and the Austrian Science Fund (FWF, project I 2320-B22). The IAEA convened a Technical Meeting on EED in Vienna in which all the authors participated.

The authors report no conflicts of interest.

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DOI: 10.1097/MPG.0000000000001373

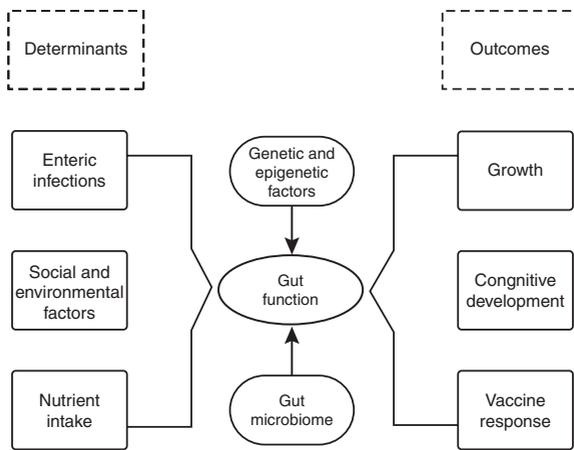


FIGURE 1. Repeated enteric infections coupled with altered mucosal immune responses and macronutrient/micronutrient absorption and availability are major determinants of gut dysfunction observed in environmental enteric dysfunction (EED). The role of the gut microbiome requires further investigation. Together, these may be important drivers of the outcomes observed in EED such as growth faltering, reduced vaccine response, and impairments in cognitive development.

Stunting, defined as linear growth (height-for-age) 2 standard deviations below the population median growth curve, currently affects 25.7% of the world's children; the total number of affected individuals being 165 million (3). Despite this burden, controlled interventions including the provision of food in food insecure settings, and the provision of education regarding feeding practices have had only moderate effects on linear growth (4). EED is thought to be one of the major causes of the failure of nutritional interventions in children at risk from stunting (5). The validation of this theory is however currently limited by a lack of reliable assays to measure some of these key parameters that are thought to be deranged in EED.

Identification of better biomarkers and delineation of the key biological pathways may also guide better-targeted interventions. Although markers of systemic inflammation are plentiful and well described (6), markers of intestinal permeability, nutrient absorption, enterocyte metabolic capacity, intestinal inflammation, and intestinal regeneration and repair are extremely limited. This lack of functional measurements severely limits our understanding of the intestinal processes that control nutrient bioavailability and inflammation and our ability to focus on effective interventions. Here, we review and discuss the potential of available stable isotope techniques as non-/less-invasive and safe tools for advanced diagnosis and research of gastrointestinal dysfunction.

PRINCIPLE OF USING STABLE ISOTOPES IN GASTROENTEROLOGY

The basic principle of all stable isotope techniques in gastroenterology is to administer a stable isotope-labeled compound to the body (ie, orally, rectally, or intravenously) in “trace” amounts to minimally disturb normal physiology, and to subsequently track the fate of the compound or its catabolic products in breath, tissue, feces, urine, and/or blood (7). The route of administration and type of isotope label and compound essentially determine which gastrointestinal function is assessed. Stable isotope techniques are often used to determine general epithelial function such as absorption and permeability. They, however, can also rather precisely measure

specific metabolic features in the intestinal tract such as uptake of a defined micronutrient or a defined physiological property of intestinal microbiota members metabolizing the administered compound. The attraction of stable isotope-based technologies in EED is their potential for noninvasive diagnosis in field research settings to assess a wide assortment of different components of gut physiology and function. By measuring the magnitude of deviation from normal physiology in different epidemiologic contexts, the underlying nature and extent of pathology that underscores gut dysfunction in EED could be elucidated facilitating the development of effective diagnostic tests and interventions.

BREATH TESTS AND SMALL INTESTINAL FUNCTION

Breath tests are characterized by their noninvasive technology and as such are ideal for both diagnostic and longitudinal studies. The test performance characteristics are particularly well-suited for studying children as testing is painless and noninvasive (8). The most robust, safe, sensitive, and specific stable isotope breath test devised is the ^{13}C urea breath test for the diagnosis and monitoring of *Helicobacter pylori* (Hp). It has repeatedly been shown to have >95% sensitivity and specificity (9). There are some intrinsic advantages that favor this. First, when Hp is present it is usually the dominant microorganism in the low pH environment of the stomach and may affect linear growth (10). By thriving in the mucosa of the stomach, Hp is also close to the site of oral tracer administration, leading to rapid delivery of the tracer bolus to the site of infection. As the tracer target moves more distal in the intestine, complex delivery formulation may be needed to allow for site-directed tracer delivery, and there may be variations in transit time that reduce sensitivity and specificity of the test.

Therefore, the measurement of functional characteristics in gastrointestinal tract regions distal to the stomach presents greater challenges with respect to targeting functional characteristics, epithelial damage, and barrier integrity and specifically targeting pathogens (11,12). Several stable isotope breath tests for assessing gastric emptying have been designed and validated against scintigraphic methods (13,14). These tests have also been combined with nonabsorbable carbohydrates, such as lactulose, using H_2 measurements in breath to determine orocecal transit time of a particular meal (15). This in turn gives an indication and allows calculation of small intestine residency time. Less attention has been given to interrogating the patency of the small intestinal mucosa. A notable exception is the assessment of lactase deficiency or lactose malabsorption and intolerance (16). This has traditionally been detected using a H_2 breath test or with a combined $\text{H}_2/^{13}\text{CO}_2$ breath test (17).

Only a small percentage of individuals exhibit a genetically determined sucrase deficiency, much less than the global 70% to 90% prevalence for lactase deficiency (18). This raises the possibility of using a ^{13}C sucrose breath test (SBT) as a noninvasive reporter for detecting small intestinal villus dysfunction and damage, by targeting sucrase activity as the surrogate for quantifying the maturity of the absorptive capacity of the epithelium (19,20). A villus atrophy induced by chemotherapy is a common occurrence, but ethical practice precludes endoscopy to assess the severity and to thus monitor any ameliorating interventions. The SBT has been reported in animal models and in childhood cancer chemotherapy and appears to act as a surrogate marker for small intestinal villus dysfunction and damage (21–23).

Other settings where significant small intestinal damage occurs include rotavirus infection, *Giardia lamblia* infestation, celiac disease, and the endemic spectrum of EED. In many of these diseases and disorders, the degree of absorptive impairment is

not known nor easily measurable; thus, its contribution to health and linear growth in childhood is not fully understood. Preliminary studies in children with acute diarrhea have shown lowered SBT response that improves as disease resolution occurs (24). In infants with EED, resolution of the small intestinal impairment in the same time frame, however, did not occur. Correspondingly, nor did the severity of diarrhea in these children (25).

Multiple isotope labels, targeted delivery, and additional stable isotope end-points, including breath $^{13}\text{CO}_2$, will potentially move this strategy forward for understanding gut dysfunction in EED.

STABLE ISOTOPES, MACRONUTRIENT BIOAVAILABILITY, AND HOST RESPONSE

Classical stable isotope–dilution techniques, which use steady-state tracer infusion protocols, have become an essential tool for determining metabolite flux in humans (26). They, however, only focus on a limited number of targeted metabolites and are

unsuitable for investigations in young children because they involve extended tracer infusions and serial blood and/or tissue sampling.

Intrinsic labeling of macronutrients for human feeding studies allows for an integrative assessment of nutrient bioavailability. The photosynthetic pathways of carbon sequestration in plants (C3 vs C4) provide natural isotopic carbon-13 (^{13}C) enrichment in C4 plant material that is sufficient for measurement by isotope ratio mass spectrometry (27). C4 plant-derived carbohydrates have been allowed assessment of liver glycogen oxidation (28), starch digestion, and exogenous glucose production in adults (29,30) and children (31). This approach is however limited by the low signal to background ^{13}C abundance. Studies using isotope labeling (enrichment) techniques have largely overcome these challenges. This experimental approach is exemplarily outlined for stable isotope–labeled, plant-derived dietary compounds in Figure 2. Isotope labeling has been used to describe quantitative exogenous glucose production and insulin sensitivity from native plant-derived starches (32–34). Beyond starch, ^{13}C labeling has been used to determine quantitatively short chain fatty acids (SCFA) production

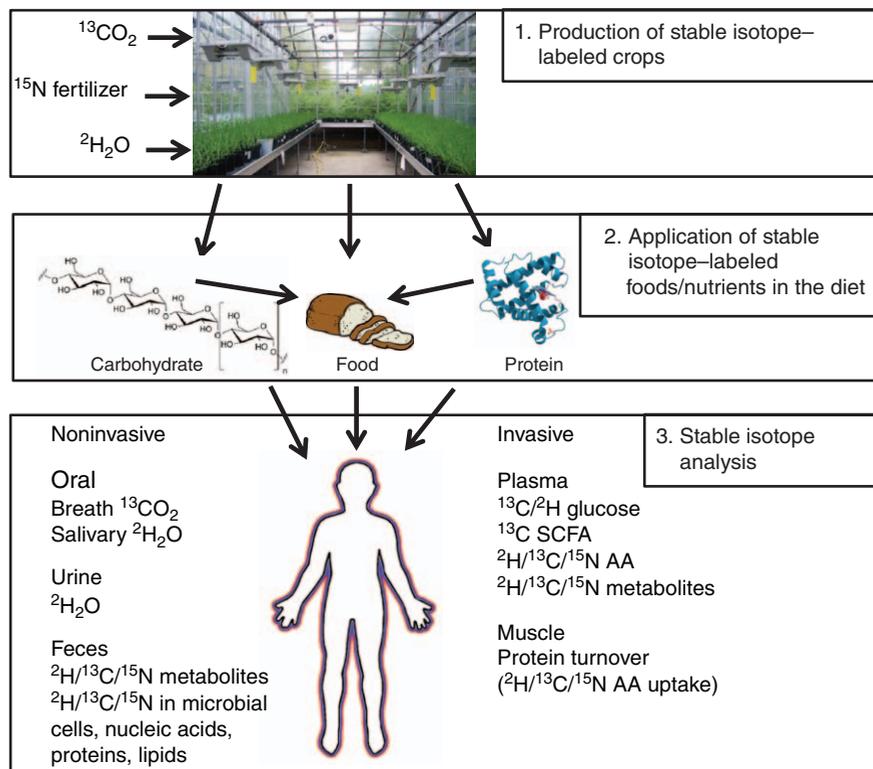


FIGURE 2. The 3 domains of a human stable isotope–labeling experiment using labeled macronutrients. Stable isotope labeling of macronutrients from plant sources has been achieved by both $^{13}\text{CO}_2$ enrichment of the plant atmosphere, use of ^{15}N (nitrate, ammonium) fertilizers or $^2\text{H}_2\text{O}$ labeling of plant feed water. Deuterium labeling lends itself to both low and highly enriched plant products, whereas carbon labeling is mostly (but not exclusively) restricted to low-enriched products. The labeling strategy has consequences for the technologies required to measure isotopic enrichment in target metabolites. Highly enriched metabolites (>1 atom percent excess) are amenable to “organic” mass spectrometry and NMR. Low-enriched metabolites are largely restricted to isotope ratio mass spectrometry approaches. Once a labeling protocol has been undertaken, the plant can be used whole, or fractionated into macronutrients and incorporated into foods (the food matrix remaining unlabeled but target macronutrients labeled within the food). Once ingested, the analysis required depends on the question being asked. If rate and extent of oxidation are the only requirements (as in ^{13}C breath tests), collection of expired breath $^{13}\text{CO}_2$ over time is sufficient. If molecular information is required about metabolites from digestion and absorption of a labeled macronutrient, then compound-specific isotope analysis is necessary, often through serial blood sampling to gather time-course information. Such information may also be available through urine metabolite excretion. In order to derive true molecular flux information, a second tracer administered (orally or intravenously) in a known quantity may sometimes be required to account for the unknown fraction of labeled metabolites entering a biological pool from macronutrient digestion and absorption. Finally fecal microbiota isotope sequestration yields information on nutrient uptake and microbial activity specific to the labeled substrate.

from inulin *in vivo* (35), and the extent of carbohydrate oxidation from β -glucan-enriched barley (36). These tools uniquely allow a full understanding of macronutrient bioavailability, requirements, and metabolism and potentially provide new mechanistic insights into how carbohydrate requirements and metabolism are altered in EED.

Of considerable interest in EED is the bioavailability of, and nutritional requirements for, protein-derived amino acids. Energy, protein, and usable protein availability are negatively associated with stunting (37). Recent evidence has demonstrated reduced serum essential amino acids in stunted children (38). What role EED plays in altering protein digestion and amino acid absorption and use is unclear at the present time. The limitations of current techniques to assess protein quality and amino acid requirements have been extensively reviewed elsewhere (39,40). ^{15}N -labeled proteins have been used to determine net postprandial protein use (41,42), by measuring of the kinetics of dietary N appearance in ileal contents, plasma proteins, plasma free amino acids, body and urinary urea, and urinary ammonia in a 13-compartment, 21-parameter model (43). The complexity of the technique means it is unlikely to be widely adopted for routine applications (39). In children, $^{13}\text{CO}_2$ production has been used as an index of bioavailability from oxidation of protein-bound indicator ^{13}C -labeled amino acids whereby the extent of amino acid oxidation is inversely related to amino acid uptake through protein synthesis (44). Amino acid infusion studies using animals to intrinsically label-specific amino acids in milk and meat protein have shown a dose-response of *de novo* muscle protein synthesis to whey protein in human feeding studies (45), and that minced beef increases postprandial protein retention compared with beef steak (46). A combined dual stable isotope study using ^{15}N -labeled Spirulina protein and ^2H -phenylalanine has demonstrated that protein digestibility is severely compromised in patients with cystic fibrosis (47). Of significant potential is intrinsic labeling of cereal, legume, and animal-derived protein with ^2H , in combination with ^{13}C -labeled amino acids from a (predigested) crystalline amino acid mixture having the same composition as the intact protein to assess amino acid bioavailability (48,49). In EED, this approach has the potential to assess amino acid bioavailability and requirements, taking into account directly the disturbances in digestion and absorption. Finally, intrinsic labeling of amino acids in humans using $^2\text{H}_2\text{O}$ has been used to determine the role of diet and disease on whole-body and tissue-specific protein synthesis (50–52).

Isotope studies on lipid use related to gut dysfunction have largely been focused on the efficiency of fat digestion in the intestine. The use of ^{13}C -labeled triglyceride combined with $^{13}\text{CO}_2$ appearance in breath has been used to assess pancreatic exocrine insufficiency (53), efficacy of pancreatic enzyme supplementation in disease (54), the effects of gastrectomy on fat digestion (55), and to determine how oral fat exposure, enteral lipid metabolism impacts upon postprandial lipemia (56).

The power of stable isotopes to trace macronutrient assimilation is unparalleled because they can be used in physiologically relevant settings. The challenge is to develop methodologies that are minimally invasive while yielding maximum information on how EED affects the digestion, absorption, and the use of the major macronutrients.

MICRONUTRIENT ABSORPTION AND BIOAVAILABILITY

Zinc and iron are 2 micronutrients of particular importance for investigations of etiology and therapy for EED. Deficiencies of each nutrient are common, and disrupted absorption and homeostasis are considered to be likely. Stable isotope studies have been widely applied to examine bioavailability of each of these nutrients from local diets, fortified and bio-fortified foods, and from supplements in vulnerable populations. Great potential exists for detailed

studies in children with EED to better characterize the extent and impact of the condition on micronutrient use.

Zinc

Three stable isotopes of zinc (^{70}Zn , ^{67}Zn , and ^{68}Zn) have low enough natural abundance to be useful as tracers of zinc homeostasis (57). The most commonly applied approach to measurement of zinc absorption is the “dual isotope tracer ratio” method, which involves oral administration of 1 isotope and intravenous administration of another isotope. Measurement of the ratio of the isotope enrichment in plasma or urine several days later provides a determination of the fractional absorption of zinc; multiplication of this times the amount of ingested zinc yields the amount of absorbed zinc. Oral isotope is ideally administered with all meals of the day to yield total daily absorbed zinc; administration in postabsorptive state with a standardized dose can provide estimate of absorption “capacity.” Excretion of endogenously secreted zinc in the intestine, a critical aspect of zinc homeostasis, can also be estimated by the “isotope dilution” method, by determination of the ratio of the amount of an intravenously administered isotope in feces to that in urine during the same time period (58). Because the endogenous fecal zinc appears to be more reflective of host zinc status than the absorption efficiency, simplified approaches to its determination are being explored (eg, using partial instead of complete fecal collections). The size of the exchangeable Zn pool, a putative index of zinc status, can also be estimated by measurement of urine enrichment of an intravenously administered stable isotope. All of these approaches to zinc homeostasis have been safely applied in pregnant women, infants, and young children. Although administration and collection procedures require meticulous care and attention to detail, they have been successfully applied in many austere environments (59).

As appreciation of the complexity of EED has been increasingly recognized, it also seems likely that micronutrient requirements are higher, as a result of the impaired absorption and/or increased losses. Several approaches using zinc stable isotopes are relevant: studies comparing absorption in children with and without EED; measuring absorption from a range of doses to identify that which is high enough absorption to meet “pathophysiologic requirements”; and studies to document endogenous intestinal losses in EED. As a potential biomarker of zinc status and response to zinc interventions, exchangeable Zn pool is expensive, but it could usefully be measured in subsets of subjects participating in intervention trials to evaluate response to intervention and to link to functional outcomes.

Iron

The criterion standard for measuring iron absorption and bioavailability from foods or supplements, both in healthy individuals and those with malabsorption, is the iron stable-isotope technique based on erythrocyte incorporation of the stable isotope labels 14 days after intake (60). Three stable isotopes of iron are commonly used (^{54}Fe , ^{57}Fe , and ^{58}Fe). Labeled iron compounds (eg, ferrous sulfate, ferrous fumarate, NaFeEDTA) can be prepared from isotopically enriched elemental iron. The labels are added to test meals or supplements that are fed to fasted human subjects (61). The labels mix with the total nonheme iron pool from the meal or supplement in the stomach and are mainly absorbed in the upper duodenum (62). Either within- or between-subject designs can be used. Fourteen days after the administration of the test meals, on the basis of the shift in the iron isotope ratios in the blood samples measured by mass spectrometry and the amount of iron circulating

in the body, the amounts of the labeled iron compounds in the blood and thereby fractional absorption of the iron is calculated (63). Because these techniques involve no radioactivity, they can be used in infants and young children with no health risk (60,64).

Stable iron isotopes could be used to quantify, with high precision, iron absorption and bioavailability from diets, lipid-based nutritional supplements, and/or micronutrient powders in children with EED. This would provide insight into the severity of iron malabsorption and the etiology of anemia in EED, as well as assessing changes in absorption in children recovering from EED.

SINGLE CELL ISOTOPE METHODS FOR PROBING MICROBIOTA-GUT FUNCTION

Spatially highly resolved isotope imaging emerges as an important complement to common stable isotope-labeling techniques, which essentially measure whole body or population averages that mask relevant differences between individual cells. In working toward a complete understanding of a biological system, it is important to get a more refined understanding of the contributions of its individual parts, the cells, and their interactions with each other (65,66). Use of high-end microspectroscopy and secondary ion mass spectrometry instruments for stable isotope analytics allows dissection of the physiological function and metabolic heterogeneity of subpopulations of cells that constitute a tissue or the diverse species of the complex human microbiota. This is of particular importance for the gastrointestinal tract, where metabolic processes are extremely complex and dynamic

because of the temporally and spatially variable presence of various diet- or host-derived substrates, and the diverse microbial populations and host cells that metabolize those substrates (67). With these single cell stable-isotope methods in hand, one can now start disassembling the manifold synergistic and antagonistic physiological interactions among individual cells of the intestinal microbiota and the host tissue and identifying key cellular populations that determine the flow of nutrients and energy to and from the host. As with any other stable isotope-labeling approach, the type of isotope (mostly ^{13}C , ^{15}N , but also ^{18}O or ^2H) and substrate, and how the labeled substrate is administered determines which physiological function is being investigated. The incorporation of the isotope label into cellular components of a microbial cell is subsequently quantified with specialized instruments, such as a confocal Raman microspectroscopy, which has a lateral resolution of approximately $1\ \mu\text{m}$, or a NanoSIMS, a secondary ion mass spectrometer for parallel detection of up to 7 ion masses and nanoscale spatial resolution. Isotope analysis can be directly combined with fluorescence in situ hybridization for the identification of microbial cells (67). Selected applications include identification of microbes that forage on host-derived $^{13}\text{C}/^{15}\text{N}$ -labeled protein compounds (68) and use of heavy water ($^2\text{H}_2\text{O}$) as a general marker for cellular activity (69). Although these single-cell methods were initially developed and applied in animal models, stable isotope analysis of individual cells can be readily performed on fecal samples or tissue biopsies obtained from patients that are subject to some of the more routine stable isotope tests mentioned above. Single cell analysis by Raman microspectroscopy or NanoSIMS is inherently time-consuming

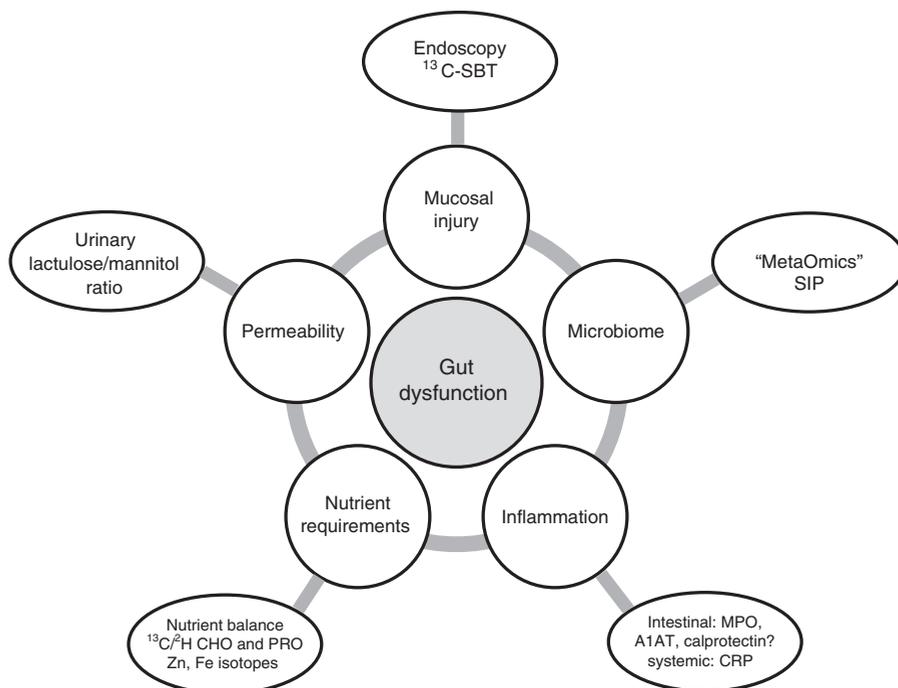


FIGURE 3. Associated with environmental enteric dysfunction (EED) are a host of functional aberrations that potentially allow for diagnostic tests in EED. Only some of these aberrations are suitable for the application of stable isotope technologies. SBT has potential to assess mucosal injury (villus atrophy). Characterizing the composition gut microbiome in EED will benefit from applying the full suite of “omics” technologies (meta-omics). Characterizing the function of members of the microbiota will require stable isotope probing (SIP) and single cell technologies. Developing tools to describe nutrient requirements (carbohydrate [CHO], protein [PRO], and micronutrients [Zn, Fe]) will require development of robust stable isotope protocols. Characterizing inflammation relies on biomarkers such as α -1-antitrypsin (AIAT), myeloperoxidase (MPO), and possibly calprotectin for intestinal inflammation and established biomarkers such as C-reactive protein (CRP) for systemic inflammation. Characterizing gut permeability relies largely on established sugar absorption/urinary excretion tests. Therefore, of significant potential for the application of stable isotope technologies is the assessment of mucosal injury, gut microbiota function, and nutrient requirements.

and thus essentially not tailored to routine diagnostics in gastroenterology. Instead, these advanced research tools will contribute to a high-resolution picture of the complex physiological processes in gastrointestinal tract, particularly into the role of individual members of the resident microbiota in EED.

CHALLENGES AND FUTURE PERSPECTIVES

The potential for stable isotope–based techniques to provide noninvasive, possibly low-cost, and field-deployable diagnostic tests in gut dysfunction is a tantalizing prospect. Significant research is still required to realize this prospect if we are to make use of stable isotopes in a setting such as EED for diagnostic or disease stratification purposes. There are challenges to overcome but the opportunities that could be realized are profound.

Challenges

Integrating high-end, high-cost mass spectrometry technologies in limited-resource settings may be seen as a significant barrier to implementing stable isotope technologies in a community setting. Solutions are beginning to emerge with field-deployable infrared-based techniques for measuring isotopic abundance in select metabolites (mainly CO₂ and H₂O). Careful selection and validation of appropriate tracers for appropriate end-point measurements with robust clinical outcomes is required. Techniques for single cell investigations will remain focused in well-resourced and competent research laboratories, but because such techniques can add considerable insights into microbe and host physiology, they need to be embraced to provide a greater understanding of EED.

Future Perspectives

The adoption of stable isotope–based micronutrient bioavailability assays in intervention studies in EED will continue to add considerable insight into micronutrient use and the efficacy of fortification trials. Research using combinations of tests (stable isotopes and biomarkers) are needed to assess multiple aspects of gut dysfunction (mucosal injury, digestion, permeability/barrier function and microbiota function) simultaneously. The potential for intrinsic labeling of food to dissect how macronutrient uptake and use are affected in EED holds significant promise. These opportunities are outlined in Figure 3. Whether this integrated physiological view has the potential to allow stratification of EED into relative risk of stunted growth remains to be seen, but stratification based on biomarkers has yielded significantly improvements in the management and treatment in other contexts, for example, cancer and cardiovascular disease. Stable isotopes add essential functional measurements to the existing array of biomarkers and have significant potential to be applied in diseases where gut dysfunction has an important role.

Acknowledgments: The authors thank Professor Tom Preston, SUERC, for his constructive critical discussion in drafting this paper and for the picture of isotopic labeling of plants for human studies.

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