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Tests of intestinal mucosal hyperpermeability: Many diseases, many biomarkers and a bright future

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ABSTRACT

The number of disorders now linked to increased intestinal mucosal permeability implies that a substantial percent of the population is affected. Drug interventions targeting reduced tight junctional permeability are being pursued. Although hyper-permeability in itself is not a clinically recognized disease entity, its relationship to disease processes has driven interest in measuring, and even monitoring mucosal permeability *in vivo*. Along with improved knowledge of gut barrier physiology, advances have been made in tests and biomarkers of barrier function. Drawing from our experiences in the past decade, considerations and challenges faced in assessing *in vivo* intestinal permeability are discussed herein, along with indications of what the future might hold.

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Conditions associated with hyper-permeability

There are currently hundreds of original research publications each year exploring intestinal permeability, and the number is increasing. This sustained surge in interest can be explained in part by the increasing number of diseases in which “leaky gut” has been linked or postulated. Within the confines of traditional gastroenterology, hyper-permeability has been correlated with inflammatory bowel disease (IBD) [1,2], celiac disease [3], irritable bowel syndrome (IBS) [4–6], including post-infectious IBS (PI-IBS) following environmental enteropathy [7]. Among the proposed links between permeability and functional gastrointestinal (GI) disorders are alterations in bile acid signalling through farnesoid X receptors [8]. Involvement in liver disease has been reported [9,10], and is thought to play an important role in developing hepatic encephalopathy in liver failure [11]. Extra-GI conditions linked to hyper-permeability include type 1 diabetes mellitus (T1DM) [12,13], obesity [14] and type 2 diabetes mellitus (T2DM) [15]. It has even been described in Parkinson's disease [16,17] and autism [18,19] and juvenile idiopathic arthritis (JIA) [20,21]. Arguments for aberrant gut permeability as a component of pathogenesis of such a broad range of diseases are strengthened by comorbidity studies. In autism, significant co-morbidity exists for IBD and T1DM [22]. Similarly, in JIA, comorbidity has been reported for T1DM [23]. There is also significant comorbidity for obesity in celiac disease in both children [24] and adults [25]. Adding to this, drugs such as NSAIDs provoke hyper-permeability in the small intestine [26] and colon [27], in addition to the stomach [28]. About 5% of the European population can be expected to be taking NSAIDs, with a current trend towards stronger formulations [29]; numbers are thought to be higher in the USA. In summation, all the associated diseases, some with increased incidence, and trends in NSAID use, imply that at any given moment a substantial fraction of society experiences hyper-permeability and this can be expected to increase in the future.

Therapeutic interventions and permeability testing

If hyper-permeability can be detected and corrected, this could lead to downstream health benefits. There are efforts to manipulate permeability, either for enhanced drug delivery by increasing permeability [30–32] or drug safety by reducing permeability [33] or disease intervention [34]. New medicinal chemistries, such as GM-1 conjugates, are exploiting knowledge of gut barrier biochemistry to develop novel delivery approaches for drugs with limited bioavailability due to poor intestinal absorption [35]. Nutritional supplements (e.g., glutamine) [36] and probiotics are being explored [37]. The gut hormone glucagon-like peptide-2 (GLP-2) is an epithelial cell specific intestinal growth hormone. GLP-2 and its analogs are also being explored for capacity to reduce permeability in the hopes of dampening inflammation [38,39]. Metformin is being explored as a treatment for IBS and is thought to protect the gut barrier by blocking activation of mast cells in the

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colon epithelium [40].

As new treatment modalities, drugs and drug delivery chemistries progress, drug safety concerns can also be expected to arise. The potential for hypo-permeability (i.e., failure to increase permeability when physiologically appropriate) and the consequences are not fully understood. Recent evidence indicates that reduced permeability results in severe malnutrition [41]. The proposed mechanism is that insufficient Na^+ permeation through tight junctions from submucosa to lumen results in insufficient lumenal Na^+ for complete absorption of glucose, essential amino acids and fats (through reduction of bile acid reabsorption at distal illeum). These findings are consistent with historical knowledge [42–44]. Hence, permeability tests of the future may need to achieve more than just identifying hyper-permeability. They may need to align with assessments of drug efficacy as well as drug safety.

Mucosal permeability as a dynamic physiological process

There is sufficient *in vitro* data to conclude that it is likely that in the *in vivo* state, dietary and other ingested factors alter permeability as a matter of normal physiology [45,46]. We recently demonstrated *in vivo* synchronous changes in mucosal permeability and motility [47], consistent with dynamic changes in permeability paralleling known changes in motility through digestive phases. Glucose increases permeability, which is thought to occur in part by solvent drag effects [48,49]. Conversely, polyunsaturated fatty acids (PUFA) tend to decrease permeability [50]. More recently, dietary fat was shown to increase permeability [51]. *In vivo* studies have demonstrated that exercise transiently increases small intestinal permeability as measured by lactulose/rhamnose ratio [52]. With a typical meal being a mixture of protein, fat and carbohydrate and varying levels of physical activity during a day, it seems likely that in normal physiology, the gut mucosa experiences regular changes in permeability throughout the day. These changes may need to be explored in more detail in future studies. A new form of permeability test in which the mucosa is challenged by exploiting these dynamic changes might yield more sensitive assays.

Strategies to assess the gut barrier

It is technically possible to study some aspects of the gut barrier in endoscopic biopsies using PCR and Western blot. Studies of permeability in live human biopsies can be achieved with an Ussing chamber [53]. The need for biopsy specimens and the challenges of working with biopsies sets constraints on widespread use. Ideally, the gut barrier would be investigated without endoscopy. Non-endoscopic tests of barrier integrity might be particularly attractive for patient monitoring, as for example, as part of following treatment outcomes.

There is a plethora of validated clinical chemistry analytes that can be accurately measured in feces, blood or urine that ultimately reflect *in vivo* gut barrier function, albeit indirectly. Serum C-reactive protein, fecal hemoglobin and fecal calprotectin are common clinical chemistry analytes that can yield positive findings in the presence of gut barrier dysfunction. Findings of micronutrient deficiencies, such as iron, cobalamin and folate [54] as well as, arguably, vitamin D [55] are common (e.g., ileitis), insomuch as intestinal inflammation drives inflammatory anemia. All of these analytes essentially reflect or result from some aspect of ongoing inflammation. None of these detect the proposed low-grade inflammation of IBS, turn-over of gut mucosal epithelial cells during accelerated cell death or mucosal hyper-permeability. More direct tests of gut barrier function have not yet made their way to routine clinical chemistry. However, several such tests exist and there have been gradual advances in assays as well as biomarker discovery that are likely to make routine testing feasible, while offering added value in clinical investigations.

Among the earliest permeability probes used in humans was ^{51}Cr-EDTA [56]. Lactulose, a disaccharide, has been used to assess paracellular permeability in human small intestine since about the mid 1970's [57]. This was among the earlier members of tests based on sugar permeation in which urinary recovery is evaluated after ingestion. Subsequent studies found evidence that monosaccharides, such as mannitol, reflect small intestinal mucosal surface area [58]. This was supported in studies of celiac and Crohn's diseases in which findings of lower mannitol and/or higher lactulose recovery in urine was found [59,60]. Since then, it has been commonplace to perform small intestinal permeability tests with a combination of a disaccharide and a monosaccharide to obtain a ratio (e.g., lactulose:mannitol ratio), such as to indicate the extent of paracellular permeability referenced against the small intestinal surface absorptive area. An early variant of this test in humans used rhamnose instead of mannitol to obtain a lactulose:rhamnose ratio [61]. Both lactulose:mannitol and lactulose:rhamnose ratios are used to this day for *in vivo* small intestinal permeability tests in humans.

Efforts to study colon permeability were facilitated by the introduction of sucralose to the consumer market as an artificial sweetener (it was approved in the EU in 2004). The 3 chloride groups of this sucrose derivative confer nearly complete resistance to bacterial or eukaryotic metabolism. Sucralose survives in the colon where a normal mucosa prevents it from being absorbed. Any absorbed sucralose passes into urine intact. The chloride groups also confer a weak hydrophobicity allowing for easy separation from other permeability probes or traces of glucose or lactose. The “triple” sugar test selectively assaying small intestine and colon permeability was first evaluated in humans in 2004 [62]. More recently, erythritol has been added to establish a reference for sucralose in colon permeability tests (i.e., sucralose:erythritol ratio) [63].

In some cases, it may be possible to assess the integrity of the epithelium. We have explored the potential use of intestinal fatty acid binding protein (I-FABP) for monitoring epithelium turn-over in the jejunum, ileum and colon in Crohn's disease [64]. In that study, a disconnect between CRP and Harvey-Bradshaw Index (HBI) was seen in about 40% of active Crohn's disease patients; roughly half of these had CRP values within the average for healthy controls, despite HBI over 10. This exemplifies the unmet need for new biomarkers. I-FABP has been investigated as a new biomarker in IBS [65], and it is among the more promising new biomarkers for intestinal ischemia [66]. The number of original research publications per year in which I-FABP was among the analytes has increased markedly in the past 5 years. Currently there about 40 papers each year. It is possible I-FABP will make its way into routine clinical chemistry for use when epithelial injury near the ileocecal region is suspected.

The future

Since 2011, we have pursued rapid, low cost permeability tests in which the readout is fluorescence and there is the possibility for on-site or point-of-care measurement of permeability. In 2010, the human riboflavin transporter was cloned and found to be highly expressed in the small intestine and to be independent of Na^+ co-transport [67]. This led us to explore riboflavin as a new direct fluorescent probe of small intestinal barrier function based on absorption of an actual nutrient [68]. In that same paper, we introduced the potential for bis-boronic viologens (BBVs) coupled to fluorophores to detect and quantify lactulose. We then

demonstrated the potential for combinations of different BBVs with different affinities for different sugars (lactulose and mannitol) in a multiplex format [69]. Most recently, we demonstrated a method by which sucralose can be chemically modified for detection in the fluorescence based BBV assay [70]. Although these fluorescent tests are still under development, we believe strategies such as these hold promise for making permeability tests feasible. A weakness of fluorescence methods in general is the need that urine samples first be cleared of optical interferences from endogenous as well as drug metabolites (e.g., urobilinogen, 5-ASA and azathioprine).

Several developments in ^{19}F-NMR methods suggest this may be a powerful option in the future. These methods may be able to work without any sample clean-up. In principle, blood or urine tubes can be placed directly into the ^{19}F-NMR for readout within 30 min. Benchtop NMRs are now sufficiently sensitive to detect and quantify ^{19}F fluorinated sugars in blood or urine after ingestion. There are very few fluorinated organic compounds that humans could otherwise be exposed to and these would be at very low levels in blood or urine. Interferences in ^{19}F-NMR would be rare. Prices for these instruments have come down to a similar range as plate readers. Synthesis of non-metabolizable, fluorinated monosaccharides [71] and disaccharides [72] have been described. One variant that has been tested in artificial urine used a BBV as the sugar sensor in which the fluorophore component was replaced by ^{19}F [73].

Conclusions

Interest in understanding gut barrier regulation and intervening in this mechanism is increasing. This is paralleled by increasing number of papers in which *in vivo* permeability is investigated. The number of analytes under investigation are also increasing. This occurs in a background in which developments in instrumentation and chemistries are making permeability tests more practical. This raises the tantalizing possibility that routine monitoring of mucosal permeability in patients with chronic conditions will have clinically actionable value that could even be individualized. Multiplexed assays are anticipated in order to accumulate enough information to fully assess the gut barrier.

Research agenda

1. New chemistries, biomarkers and methods are emerging to better assess the gut barrier
2. Studies are needed to characterize sensitivity, selectivity and predictive values
3. Studies are also needed to determine benefits of interventions that protect the gut barrier

Practice points

1. Permeability tests can supplement existing, validated clinical chemistry
2. Modulation of permeability is progressively becoming a treatment strategy
3. As tests become faster, cheaper and more practical, patient monitoring will be feasible

Conflicts of interest

None.

Disclosure statement

Dominic-Luc Webb declares he is an inventor on the following patent filings that relate to chemistries for gut permeability tests described in this publication: US patent 10,274,483 B2 & International PCT SC2014-954-2PCT.

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