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27. PERSISTING CONSEQUENCES OF INTESTINAL INFECTION

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EPITHELIAL STEM CELLS, SELF-RENEWAL AND DIFFERENTIATION IN THE INTESTINE

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SUMMARY

The inner lining of the gut is a simple epithelium that completely self-renews every five days because of the high mechanical and chemical stress. At the basis of the epithelial homeostasis are intestinal stem cells that are located at the bottom of crypts. Progeny is generated on a daily basis to compensate for the loss of differentiated cells that cover the villi. Under normal conditions, active cycling stem cells produce daughter cells that compete with each other for residency in the stem cell niche. Upon niche displacement, a daughter cell will lose stem cell characteristics, migrates out of the crypts and matures into a terminally specialized differentiated cell type. Here, we describe and summarize the recent developments in the identification and characterization of intestinal stem cells.

INTRODUCTION

The primary function of the intestinal tract is the digestion and absorption of food. The gut is anatomically divided into the small intestine and the colon. The inner wall of the gut is covered with a simple columnar epithelium, which performs the primary functions of (i) digestion via the secretion of enzymes, (ii) water and nutrient absorption and (iii) forms a barrier against gut pathogens. In the small intestine, the surface area is enlarged through epithelial protrusions called villi (Figure 1), while the colon has a flat surface epithelium. Proliferative cells reside in the crypts of Lieberkühn, epithelial invaginations into the underlying connective tissue. These crypts harbour stem cells and transit amplifying cells that are direct descendants of the stem cells. The majority of cells that are produced on a daily basis migrate out of the crypts and terminally differentiate into one of the major three specialized cell types of the intestinal epithelium. These are the absorptive enterocytes, mucous-secreting goblet cells and hormone-secreting entero-endocrine cells. Approximately three days after their terminal differentiation, the cells reach the tip of the villus, undergo spontaneous apoptosis and are shed into the lumen of the gut. Paneth cells are the fourth abundant cell type in the intestine and the only differentiated cell type that escapes the upward migration and instead settle at crypt bottoms (*van der Flier* and *Clevers*, 2009). Paneth cells have a function in innate immunity and antibacterial defence (*Clevers*

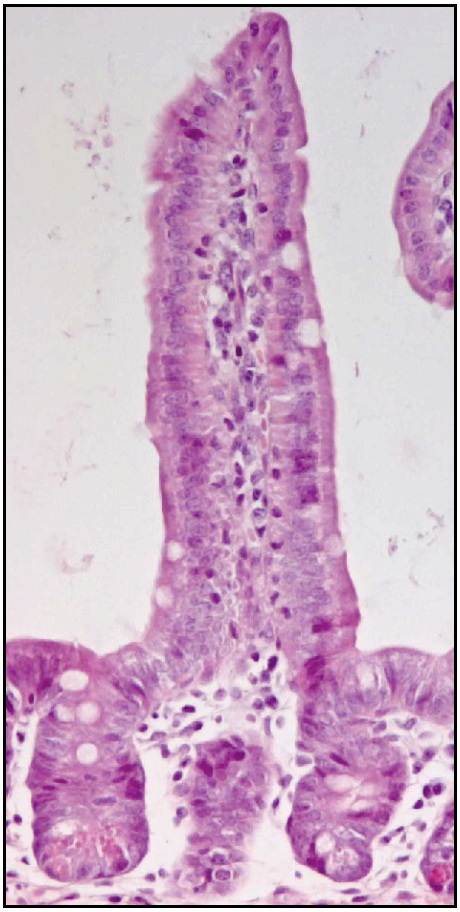

Figure 1: The intestine is lined with a single layer of epithelial cells. The H&E staining is showing the organization of the mouse small intestine in protruding villi and adjacent crypts. Crypts contain stem cells, progenitor cells and Paneth cells while the villus is covered with goblet cells, entero-endocrine cells and enterocytes.

and *Bevins*, 2013). Recently, it was shown that they also play an important role in constituting the intestinal stem cell niche (*Sato* et al., 2011b).

PROLIFERATING INTESTINAL CELLS

The intestinal epithelium is the most vigorously self-renewing tissue present in adult mammals. With the exception of stem cells and Paneth cells, the inner lining of the gut is completely renewed every 5 days. It has been known for decades that multipotent stem cells fuel the proliferative activity of the intestinal epithelium. However, the exact location of these cells was unknown for a long time since molecular markers were missing (*Barker* et al., 2008). In principal, there are only two functional requirements that cells must fulfil in order to define them as stem cells, commonly referred to as stemness.

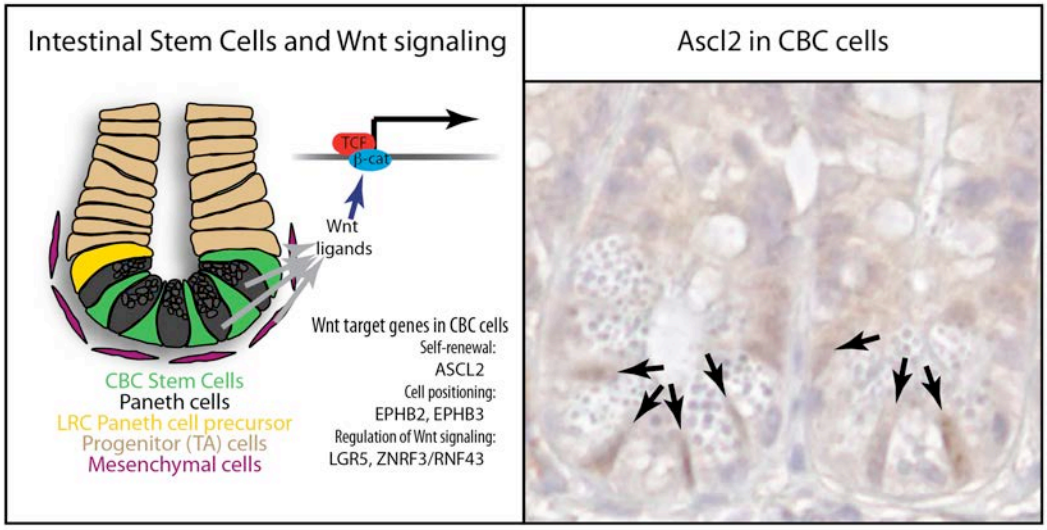

Figure 2: Left panel; schematic representation of small intestinal crypt. Wnt signalling is the driving force behind proliferation and stem cell identity. Right panel; immunohistochemical staining for transcription factor Ascl2 in mouse small intestinal crypts. Ascl2 is specifically expressed in CBC cells (arrows).

First, stem cells are long-lived, preferably throughout the life of an individual. Secondly, stem cells are mulipotent, which means that descendants of stem cells are able to differentiate into all the specialized cell type(s) that are present in a tissue.

WNT PATHWAY

The primary force that drives intestinal epithelial proliferation is the Wnt signalling pathway. Mice that are mutant for the intestine-specific Tcf4 transcription factor fail to establish proliferative crypts (*Korinek* et al., 1998; *van Es* et al., 2012a), while conditional deletion of ß-catenin (*Fevr* et al., 2007; *Ireland* et al., 2004) as well as transgenic expressing of the secreted Wnt inhibitor Dickkopf-1 (*Kuhnert* et al., 2004; *Pinto* et al., 2003) leads to disappearance of proliferative crypts in adult mice. Moreover, malignant transformation of intestinal epithelium is almost invariably initiated by activating Wnt pathway mutations (*Korinek* et al., 1997; *Morin* et al., 1997). Because of this intimate connection between Wnt signalling and intestinal biology, we and others have attempted to unravel the Wnt/Tcf4 target gene program activated in intestinal crypts and colorectal tumours (*van de Wetering* et al., 2002; *van der Flier* et al., 2007; *van Es* et al., 2005). Wnt target gene expression has been identified in three different crypt compartments. Most of the Wnt targets are expressed by the rapidly dividing progenitor cells within the transit-amplifying compartment that consist approximately the top two-third of the crypt. A second group of genes is expressed in post-mitotic Paneth cells that are located at the entire bottom of the crypt. The third

group contains only a very limited number of genes (including *Lgr5*, Ascl2, Rnf43 and EphB2) that are highly expressed in crypt base columnar (CBC) cells (Figure 2). CBC cells are small, undifferentiated cycling cells that are squeezed in between the Paneth cells at crypt bottoms. Already in the early '70, they have been suggested, based on their morphology and location, to be intestinal stem cells (*Cheng* and *Leblond*, 1974).

INTESTINAL STEM CELLS

The exact location and identity of intestinal stem cells has been the subject of an intense scientific debate for years. In addition to the CBC cell as candidate intestinal stem cell, another popular postulation was that the cells that were physically located on top of the Paneth cell compartment, the so-called +4 location (cell position counted from the crypt bottom), functioned as the intestinal stem cells. Rational for this hypothesis was that these cells are label retaining (*Potten* et al., 1974) and extremely radiation sensitive (*Potten*, 1977). DNA label retention by the +4 cells was postulated to be the result of asymmetric segregation of old and new DNA strands (*Potten*, 1977; *Potten* et al., 2002) in order to protect their genome from damage. Asymmetric DNA segregation was suggested to be a stem cell specific phenomenon.

However, definitive prove for stemness needs to be demonstrated by experimental assays, rather than association based on morphology, location, marker expression or any other specifically assigned cell-characteristic. An experimental assay to prove stemness is a transplantation experiment. Hereby a cell population is isolated based of specific presence (or absence) of markers. Once isolated from the donor, the cells are transplanted into a recipient and tested for their ability to give rise to a complete "recovery" effect. For example in bone marrow transplantation experiments, transplanted donor haematopoietic stem cells repopulate the entire blood. A critical note by this kind of assay is that the ability of the cell population to function as stem cells is tested in a challenged/stressed system. Therefore, cell transplantations between individuals or species primarily test the ability of cells to adapt and grow in a foreign milieu, rather than testing the actual behaviour of cells prior to experimentation. Nevertheless, transplantation assays are a powerful tool to test stemness potential, but it is likely that the population of cells with stemness potential is larger than the number of cells that actually do function as stem cells in normal homeostasis.

An alternative method to test stemness is based on genetic marking and is commonly referred to as lineage tracing or fate mapping. Here, the DNA of specific (candidate) stem cells is marked at any specific moment in time. Subsequently, over time all descendants of the marked population inherit the mutation. Moreover, the genetic marking can be visualized via straightforward laboratory techniques and allows experimental testing of the two functional descriptions of stemness. First, over time the genetic marking needs to be detectable in all the cell types that are present in the tissue (multipotency). Second, the presence of the genetic marking needs to be maintained within the tissue for the entire life-time of the organism (self-renewal). The stemness of a specific cell population is tested without the pres-

ence of stress or other injury responses. Therefore, lineage tracing assays test actual stemness, i.e. it identifies cells that function as stem cells in normal homeostasis. However, also with tracing experiments care needs to be taken into account since it is possible that only a limited number of cells within the marked group are the real stem cells. It is likely that the population of cells that contain actual stemness fall within the larger population of cells that consists stemness potential.

LGR5 IS AN INTESTINAL STEM CELL MARKER

Lineage tracing was used to prove that the *Lgr5* gene is specifically expressed in intestinal stem cells and can be used as a stem cell specific marker. In situ hybridization experiments (mapping of mRNA expression patterns) as well as different knock-in alleles revealed expression of *Lgr5* in CBC cells (*Barker* et al., 2007; *Tian* et al., 2011). Most importantly, the genetic inducible activation of *LacZ* expression in *Lgr5*$^+$ CBC cells (the genetic clonal marking) was inherited over time by all the differentiated cell types in the intestinal epithelium, thereby experimentally linking *Lgr5*$^+$ CBC cells to multipotency. Moreover, once *LacZ* expression was activated in CBC cells, the marking could be found during the rest of the lifespan of the animals in the intestinal epithelium, showing that *Lgr5* expressing CBC cells represent long-lived multipotent stem cells of the intestine (*Barker* et al., 2007).

Lineage tracing has also been performed for several markers that were claimed to be +4-cell specific, including: Bmi-1 (*Sangiorgi* and *Capecchi*, 2008), Hopx (*Takeda* et al., 2011), mTert (*Montgomery* et al., 2011) and Lrig1 (*Powell* et al., 2012). However, robust expression of most of these markers was also detected in *Lgr5*$^+$ CBC cells (*Munoz* et al., 2012). A recent study shows that genetic lineage tracing of DNA label-retaining cells identified a rare, non-dividing secretory precursor that co-expresses *Lgr5* and all +4 makers. These cells are located near crypt bottoms where they undergo terminal differentiation over periods of weeks towards Paneth cell lineage. However upon tissue damage, premature secretory precursors can revert their fate back into a cycling, *Lgr5*$^+$ CBC cell (*Buczacki* et al., 2013).

ADDITIONAL INTESTINAL STEM CELL MARKERS

Isolation of *Lgr5* expressing CBC cells using FACS allowed global gene expression analysis (*Munoz* et al., 2012; *van der Flier* et al., 2009). One of the genes enriched in the *Lgr5*$^+$ cells turned out to be the CBC restricted Wnt target Ascl2 (*Jubb* et al., 2006; *Sansom* et al., 2004; *van der Flier* et al., 2007). Ascl2 is one of the mammalian homologous of the Drosophila Achaete-scute complex genes encoding a basic helix-loop-helix transcription factor. Transgenic overexpression of Ascl2 throughout the intestinal epithelium induces crypt hyperplasia and de novo crypt formation on villi. The opposite experiment, induced deletion of Ascl2 from the intestine, results in rapid loss of CBC stem cells. The combined results from these genetic gain and loss of function studies in the intestinal epithelium show that Ascl2

controls the intestinal stem cell fate (*van der Flier* et al., 2009). Other stem cell specific Wnt target genes are Rnf43, an ubiquitin ligase for Wnt receptors (*Koo* et al., 2012) and EphB2, a cell surface marker which expression pattern was utilized to isolate human intestinal stem cells (*Jung* et al., 2011).

NEUTRAL DRIFT COMPETITION BETWEEN MULTIPLE INTESTINAL STEM CELLS

On average, there are around 14 *Lgr5*$^{+}$ CBC cells at the bottom of intestinal crypts that are sandwiched in between similar numbers of post-mitotic Paneth cells (*Snippert* et al., 2010). To be able to map the fate of neighbouring stem cells simultaneously, a multicolour reporter mouse was generated (*R26R-Confetti*). Upon activation of *R26R-Confetti*, each cell inherits one out of four possible fluorescent markers via DNA recombination, i.e. green, yellow, red or blue. This 'colour picking' is completed in a random fashion, is specific per individual cell and inherited by daughter cells. Using the *R26R-Confetti* mouse, the individual behaviour of multiple intestinal stem cells in the same crypt was followed in time. The study revealed that *Lgr5*$^{+}$ CBC cells double their numbers each day by symmetric divisions, after which the daughter cells stochastically adopt stem cell or transit amplifying cell fates based on their relative positioning towards the Paneth cells (niche cells). Due to the limited number of Paneth cells, CBC stem cells compete in a neutral fashion for limited niche space (*Snippert* et al., 2010). As long as *Lgr5*+ CBC (daughter) stem cells are in close proximity to Paneth cells they will maintain stemness. In the case that a (daughter) stem cell is displaced from Paneth cells, i.e. loss of direct contact, they will be primed for differentiation (*Sato* et al., 2011b). This stochastic stem cell model in which cell fate is determined by presence in the Paneth cell niche fits also well with recent data that demonstrated that progenitor cells that initially lost their stemness, can revert towards their stem cell fate upon stress and injury responses (*Buczacki* et al., 2013; *Tian* et al., 2011; *van Es* et al., 2012b).

INTESTINAL ORGANOIDS

Most of the above described stem cell work is based on genetic mouse models. However, it is essential to translate those scientific findings from the mouse to the human situation, for instance via transplantation assays or *in vitro* culturing assays. The culture of individual clones of cells that are derived from various types of progenitor populations has become a widely used method to define the identity and behaviour of (human) stem cell types. Most cultures are named after the tissue they were derived from, for example, neurospheres (*Reynolds* and *Weiss*, 1992), mammospheres (*Shackleton* et al., 2006; *Stingl* et al., 2006) or colonspheres. A few years ago, another *ex vivo* cell culture system has been developed for initially wild-type small intestinal epithelia. It allows long-term growth of so-called mini-guts or intestinal organoids (*Sato* et al., 2009) (Figure 3). In contrast to sphere

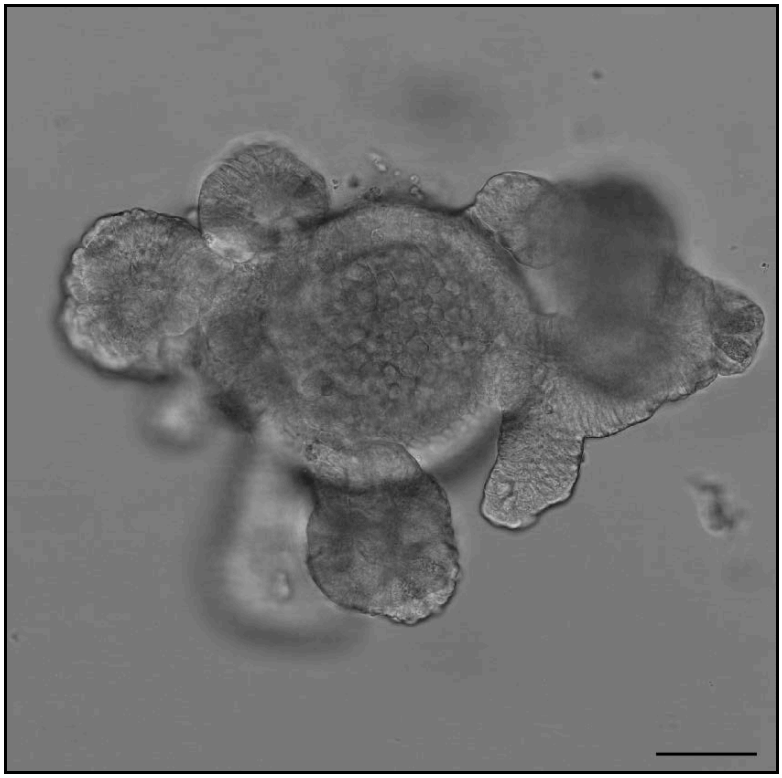

Figure 3: Bright field image of mouse small intestinal organoid. Scale bar is 50 µm.

cultures, organoids are three-dimensional asymmetric cell-cultures that can be grown from primary cells that are isolated from mouse and human intestines (*Sato* et al., 2011a). Organoids are cultured using matrixgel and a medium that contains a cocktail of growth factors such as R-spondin, EGF and Noggin. Important, these organoids share a lot of characteristics with the normal intestinal epithelium, such as the presence of all the different cell types, compartmentalization between progenitor and differentiated zones and correct positioning and migration of cell types along the crypt-villus axes. Since intestinal organoid cultures do not contain mesenchymal cells, the intestinal stem cell niche is provided by Paneth cells (secreting Wnt, EGF and Notch ligands) and the limited number of added growth factors (*Sato* et al., 2011b).

INTESTINAL STEM CELL NICHE

By using neonatal tissues, an alternative long-term multi-lineage intestinal epithelial culture has been established in which mesenchymal parts are present as well (*Ootani* et al., 2009). Mesenchymal niche architecture is also maintained once organoid cultures are initiated from human embryonic stem cells which differentiation is guided towards intestinal fate (*Spence* et al., 2011). Interesting, the mesenchymal layer develops and differentiates along

with the epithelium with the same kinetics, suggesting an intimate signalling crosstalk between mesenchyme and epithelium.

Active Wnt signalling is essential for organoid cultures. In the absence of mesenchymal tissue, Wnt ligands are either produced endogenously by the epithelium (Wnt3A in Paneth cells of mouse small intestine) or need to be added exogenously in the medium. Interestingly however, *Wnt3a* knockout mice don't have an intestinal phenotype, while organoid cultures from these mice fail to develop. To compensate for the lack of endogenous Wnt3a production, Wnt3A mutant organoids can be rescued by addition of Wnt3A or by growing them on primary mesenchymal cells that produce Wnt proteins (*Farin* et al., 2012). Above results illustrate that the mesenchyme surrounding crypts, as well as Paneth cells, function together as the niche by providing the essential signals to the intestinal epithelial stem cells.

CONCLUDING REMARKS

The intestinal epithelial (stem cell) field has developed very rapidly since the identification in 2007 of *Lgr5* as the first definitive stem cell marker. The intestinal epithelium represents a unique model to study adult stem cell biology and lineage specification. The combination of a rapid self-renewing tissue, evident compartmentalization of proliferating and differentiated cell types and a relative simple, repetitive tissue architecture, is ideal for the visualization and identification of stem cell types, cell fate specification and cellular behaviour. In the past, genetic mouse studies have created a wealth of new insights on the biology of the intestinal epithelium. The recently established long term culture conditions of intestinal epithelium, especially mini-organs from human origin, will probably boost the research field for the next generation by providing a variety of possibilities for research and therapeutic applications of intestinal biology.

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IRRITABLE BOWEL SYNDROME: ROLE OF GUT BACTERIA AND BACTERIAL TOXINS

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SUMMARY

Irritable bowel syndrome (IBS) is a common condition affecting 10-15% of the population. While the precise aetiology remains unknown, our group has focused on the contributions of altered gut flora to IBS, including links between acute gastroenteritis and post-infectious IBS (PI-IBS) and the role of small intestinal bacterial overgrowth (SIBO). Breath testing and culture of small intestinal aspirates indicate that SIBO is present in over one-third of IBS subjects and almost two-thirds of diarrhoea-predominant IBS (D-IBS) subjects, but only one-tenth of non-IBS subjects. The success of antibiotic therapies in impacting IBS phenotypes also supports a bacterial role in IBS. Four large-scale multi-centre trials indicate that rifaximin significantly reduces SIBO and non-constipation IBS with little bacterial resistance or reduction of benefit in successive retreatments. To elucidate the mechanisms underlying PI-IBS, we developed a rat model using *Campylobacter jejuni*, the most common identified cause of acute gastroenteritis. This model mirrors many findings in human IBS subjects, including altered stool form more than three months after clearing initial infection, SIBO, increased intraepithelial lymphocytes (IELs), reductions in deep muscular plexus interstitial cells of Cajal (DMP-ICCs), required for normal intestinal motility, and increases in specific mucosal defence mediators. Common to all pathogens causing gastroenteritis is cytolethal distending toxin (Cdt). We developed a rat model using a mutant *C. jejuni* lacking Cdt, which demonstrated significantly ameliorated bowel phenotypes including SIBO and IELs and no reduction in DMP-ICCs, strongly implicating Cdt in the development of IBS phenotypes. Further, early exposure to *C. jejuni* appears to mitigate the acute effects of second infections, which suggested immunity might play a role in IBS development. Our recent data indicating molecular mimicry between Cdt and neural elements of the gut support this hypothesis and may form the basis for future biomarkers and potential therapies directed at the underlying causes of IBS.

INTRODUCTION

Irritable bowel syndrome (IBS) is the most common chronic medical condition in the U.S. accounting for nearly 30% of all gastroenterology related health care costs (*The Lewin Group*, 2001; *Thompson*, 1994) and affecting 10-15% of the population (*Drossman* et al., 1982; *Thompson* and *Heaton*,

1980). This condition does not discriminate by age and the prevalence of this condition is equally common among young adults. These facts make IBS a very important disease state. However, the aetiology of IBS has remained unknown.

Historically, IBS was treated as largely a psychological disorder and anti-depressants were used as treatment for IBS. Our group has validated microbe-mediated theories in the pathophysiology of post-infectious IBS (PI-IBS) and small intestinal bacterial overgrowth (SIBO) and spent the last 10 years characterizing alterations in gut flora that may contribute to IBS.

SIBO AND IBS

Over the last decade, we have developed a new hypothesis in IBS that focused on bloating as a universal complaint in IBS subjects. Through a series of studies, we demonstrated that SIBO, which results from reduced gut motility, is more common in IBS. This was initially controversial as we used breath testing as a surrogate of SIBO. While we recognized the limitations of this technique, it was apparent that the breath test was frequently abnormal in IBS compared to healthy controls. Despite continued controversy, a key meta-analysis by our group has secured that breath testing is important in IBS and that the odds of having a positive breath test in IBS compared to healthy controls is far greater (*Shah* et al., 2010). Since then, two studies have conclusively demonstrated through culture of the small bowel that IBS subjects have higher coliform (colon bacteria) levels than healthy persons (*Posserud* et al., 2007; *Pyleris* et al., 2012).

SMALL BOWEL MICROBIOME IS ALTERED IN IBS

In another aspect of our on-going work to identify the mechanisms responsible for IBS, we have begun to explore how changes in gut microbial populations contribute to IBS. Since September 2009, subjects undergoing upper endoscopy have been recruited to a repository developed by our collaborator Dr. Evangelos Giamarellos-Bourboulis in Athens, Greece. Samples and data obtained include serum, genetic material, subject phenotypes, and aspirates from the small intestine (*Pyleris* et al., 2012). Conventional cultures of these small intestinal samples demonstrated that 39.3% of IBS subjects had SIBO (defined as $>10^3$ cfu/ml), compared to 11.3% of non-IBS subjects (subjects with GI complaints that did not have IBS). Moreover, 60% of D-IBS subjects were found to have SIBO (*Pyleris* et al., 2012). More recently, we have begun to characterize the specific microbial populations present in the small intestine in IBS vs. control subjects. Preliminary deep sequencing analyses suggest that the microbial profile (microbiome) in the small intestine of IBS subjects is markedly different from that in healthy controls, with significantly reduced microbial diversity and overrepresentation of a few specific genera (*Chang* et al., 2013). The overgrowth of these specific microbial populations, possibly in concert with the loss of other microbes with important protective effects, may contribute to the gut symptoms of IBS subjects.

TREATING SIBO IN IBS

While the uncovered connection between IBS and SIBO was important, it was equally important to identify treatment options for those who suffer from IBS. Based on our growing evidence for the connection to SIBO, we initiated a series of studies to test the use of antibiotics in IBS. The first was the use of neomycin in the first double blind antibiotic study (*Pimentel* et al., 2003). Although neomycin successfully improved IBS in the study, neomycin was a poor antibiotic for SIBO. Thus we began looking for an alternate non-absorbed antibiotic. Rifaximin had all the properties that made it ideal for IBS. It was almost completely non-absorbed, had no issues with bacterial resistance, was highly effective for SIBO, was not related to conventional antibiotics (thus would not create cross-resistance) and did not change stool flora. Since identifying this antibiotic, we have conducted four large-scale multicentre trials to examine the efficacy of rifaximin (*Pimentel* et al., 2011a, 2006). The most recent of these were the completion of the Target 1 and 2 trials published singularly in the New England Journal of Medicine. This final phase III study conclusively showed that rifaximin is effective in IBS without constipation and that a 2-week treatment had benefits lasting a minimum of 12 weeks. No previous IBS therapy had this kind of effect, suggesting that we impacted a potential mechanism of IBS. We also examined the effects of retreatment with rifaximin in subjects with non-constipated IBS, and found that more than 75% of subjects who initially responded to rifaximin also responded to subsequent treatments, with no significant reduction in benefit for up to 5 successive retreatments (*Pimentel* et al., 2011b). Furthermore, there was no change in the duration of benefit (median time between treatments) for successive retreatments (*Pimentel* et al., 2011b).

POST-INFECTIOUS IBS

Over the last decade, it has been established that intestinal pathogens play a significant role in the development of IBS. Numerous studies have shown that IBS can be precipitated by an episode of acute gastroenteritis, and that up to 57% of subjects who otherwise had normal bowel function may continue to have altered bowel function for at least 6 years after recovering from the initial acute illness (*Neal* et al., 2002). Based on two recent meta-analyses of this research, approximately 10% of subjects who have documented acute gastroenteritis develop IBS, with a summary odds ratio of 6 to 7 for PI-IBS (*Halvorson* et al., 2006; *Thabane* et al., 2007). As gastroenteritis is extremely common, so-called PI-IBS may in fact constitute a large proportion of IBS cases. Thus, reducing risk factors for IBS development after acute gastroenteritis may have an impact on the incidence of IBS. Although the mechanisms of PI-IBS remain unclear, investigators have identified certain risk factors for the development of IBS after gastroenteritis. The two most significant of these are duration/severity of gastroenteritis and female sex (*Gwee* et al., 1999; *Neal* et al., 1997). Stress, manifest as recent traumatic life events, and a neurotic personality trait were also predictors of PI-IBS (*Gwee* et al., 1999). Evidence of low-grade inflammation is evident in PI-IBS patients.

Rectal biopsies demonstrate mildly elevated intraepithelial lymphocytes and entero-endocrine cells that persisted 12 months after infection with *Campylobacter jejuni* (*Spiller* et al., 2000), which is the most common cause of acute gastroenteritis in the US (*Tauxe*, 1992). Increased rectal lymphocytes also occur in general IBS patients, but to a lesser degree (*Dunlop* et al., 2003). Elevated expression of pro-inflammatory cytokine IL-1β was detected in *C. jejuni* PI-IBS rectal biopsies (*Gwee* et al., 2003) and in *Shigella* PI-IBS recto-sigmoid and terminal ileum biopsies (*Wang* et al., 2004). Thus, acute gastroenteritis may increase the risk of developing IBS in a susceptible individual through persistent low-grade activation of the gut immune system, or possibly through establishment of an intestinal dysbiosis, defined as an alteration of the composition of the gut flora. Animal infection models of PI-IBS will play a key role in characterizing the mechanistic pathways and underlying alterations in this process.

RAT MODEL OF PI-IBS

To study the underlying mechanisms of PI-IBS, we developed and validated a rat model (*Pimentel* et al., 2008) using the human pathogen *C. jejuni* as the infective agent. Over the past 8 years, we have characterized and validated this model, which exhibits phenotypes that closely mimic those seen in human patients with PI-IBS. These include: altered stool consistency that persists three months after the clearance of the acute infection and the development of SIBO (*Pimentel* et al., 2008), as well as increased rectal intraepithelial lymphocytes and reduced numbers of deep muscular plexus interstitial cells of Cajal (DMP-ICCs) (*Jee* et al., 2010). The latter is particularly significant as ICCs and myenteric nerves are known to be required for normal intestinal motility, including phase III of interdigestive motor activity (*Nieuwenhuijs* et al., 1998). Further, evidence suggests that a deficiency of phase III motor activity, which is known to induce SIBO (*Nieuwenhuijs* et al., 1998; *Vantrappen* et al., 1977), is associated with the development of SIBO in IBS (*Nieuwenhuijs* et al., 1998). We have also confirmed alterations in mucosal defence mediators such as TNF-α in post-infectious rats (*Sung* et al., 2013).

ANTIBIOTIC PROPHYLAXIS PREVENTS THE DEVELOPMENT OF IBS-LIKE CHARACTERISTICS IN A RAT MODEL OF *C. JEJUNI* INFECTION

Antibiotic therapy (*Lembo*, 2008; *Pimentel* et al., 2003, 2006; *Sharara* et al., 2006) and the *C. jejuni* vaccine (*Monteiro* et al., 2009) mitigate the effects of gastroenteritis in humans (*Lembo*, 2008; *Pimentel* et al., 2003, 2006; *Sharara* et al., 2006). Since duration of gastroenteritis is a risk factor for IBS (*Neal* et al., 1997), these approaches may be effective in preventing IBS. To determine the effect of prophylactic antibiotic therapy on the development of post-infectious IBS-like symptoms in rats, we gavaged rats with either rifaximin (200mg rifaximin daily for 3 days) and *C. jejuni* 81-176 (5×10^8 cfu, given on day 2) (C+/R+), or with *C. jejuni* alone (C+/R-). The

two groups were then compared for both acute *C. jejuni* colonization, and for the development of post-infectious IBS-like symptoms three months later. We found that rats that received rifaximin prophylaxis (C+/R+) cleared the initial *C. jejuni* infection significantly faster than the C+/R- group (10.3±7.1 days vs. 12.6±5.9 days, $p<0.01$) (*Pimentel* et al., 2011c). Further, we found that after 3 months, the C+/R- rats had a greater persistent variability in stool % wet weight than C+/R+ rats ($p<0.01$), and that the average stool consistency was closer to normal in C+/R+ rats than in the C+/R- rats (*Pimentel* et al., 2011c).

CYTOLETHAL DISTENDING TOXIN (CDT) IS REQUIRED FOR EXPRESSION OF AN IBS-LIKE PHENOTYPE IN THE RAT MODEL

The Cdt toxin is common to all bacterial organisms that cause post-infectious IBS, and thus a potential candidate toxin mediator of IBS. To evaluate the effect of Cdt on the post-infectious IBS-like symptoms in rats, rats exposed to a Cdt knockout (Cdt-) mutant of *C. jejuni* were evaluated for stool form three months after clearing the initial *C. jejuni* infection, and compared to results obtained in rats infected with wild type *C. jejuni* (Cdt+). Stool samples were collected and evaluated over a 3-day period to obtain an average stool form. Stool form at 3 months post-infection was significantly better in the Cdt- group. Furthermore, 42% of the Cdt+ rats had altered stool form 2 out of 3 days, as compared to 18% of the Cdt- rats ($p=0.028$, Fisher's exact test). In addition, the stool wet weight in the Cdt+ rats demonstrated an alternating pattern of wet and dry, such that the variance was 8.4±6.4, compared to 4.2±2.4 for the Cdt- rats ($p<0.001$) (*Morales* et al., 2011; *Pokkunuri* et al., 2012).

To evaluate the effect of Cdt on gut histology, DMP-ICC were also examined in Cdt- rats. Three months after clearance of the initial *C. jejuni* infection from their stool, the ileum was resected and CD117 immunostaining (anti c-kit) was performed on cross-sections of mucosa. The results revealed normal DMP-ICC staining in the ilea of these rats, which is in marked contrast to the reduced numbers and altered appearance of DMP-ICC seen in sections from rats exposed to wild type *C. jejuni* (*Morales* et al., 2011; *Pokkunuri* et al., 2012). Taken together, these data strongly implicate Cdt as directly contributing to the development and severity of PI-IBS phenotypes.

POTENTIAL ROLE OF IMMUNITY IN THE DEVELOPMENT OF PI-IBS

Epidemiologic studies from developing countries indicate that the prevalence of PI-IBS in developing countries is lower than or similar to that in developed countries, despite much higher incidence of *Campylobacter* enteritis, which suggested to us that acquired immunity might play a role in PI-IBS. To begin to determine whether immunity was involved in the effects of *C. jejuni* and Cdt in the gut, we infected 50 rats with *C. jejuni* 81-176 as juveniles, and then re-infected them with the same strain two months later, and

compared the results to those from another 50 rats exposed for the first time as adults. Fewer rats infected for a second time (juvenile then adult (J+/A+)) had detectable stool *C. jejuni* compared to their first infection ($p<0.05$) and compared to the 50 rats that only received *C. jejuni* as an adult (J-/A+) ($p<0.05$). In addition, the number of days of loose stool was less for a second infection than the first (1.58±0.16 vs. 2.50±0.29 days, $p<0.05$) and the overall duration of the second infection was shorter than the first ($p=0.001$). These data suggest that exposure to *C. jejuni* early in life mitigated the acute effects of a second infection (*Sung* et al., 2013).

We next compared the development of SIBO and stool phenotypes during the post-infectious period. Interestingly, 47% (23/49) of J+/A+ rats developed SIBO, as compared to 26% (13/50) of J-/A+ rats ($p=0.019$), but J-/A+ rats that developed SIBO had greater alterations in stool consistency than J+/A+ rats that developed SIBO ($p<0.01$) (*Sung* et al., 2013). These data suggest that while rats exposed to *C. jejuni* early in life were not protected against the development of SIBO, the second infection was better tolerated and resulted in less severe stool phenotypes, perhaps suggesting the development of tolerance to the SIBO. Moreover, for rats who were first exposed as infants and then reinfected, we found that those who had diarrhoea during the first exposure were more likely to develop SIBO than those who did not ($p=0.02$) (*Sung* et al., 2013). This result mirrors findings in humans that suggest a relationship between the severity of acute illness and the likelihood of subsequent development of IBS (*Halvorson* et al., 2006; *Thabane* et al., 2007), and led to our hypothesis that immunity might play a significant role in the development and severity of PI-IBS phenotypes. Recent data generated in our laboratory support this hypothesis, and further suggest that the effects of immunity are the result of molecular mimicry between Cdt and endogenous neural elements of the gut (*Pimentel* et al., submitted). These results may form the basis for future biomarkers and potential therapies that could be directed at the cause of IBS, rather than just treating SIBO.

PERSPECTIVES AND FUTURE DIRECTIONS

In summary, our work to date indicates that alterations in gut microbial populations contribute to IBS and the development of IBS phenotypes. Specifically, we have shown that SIBO, which is caused by reduced gut motility, is present in human IBS subjects, particularly D-IBS subjects, and can be ameliorated using antibiotics such as rifaximin. Further, acute gastroenteritis caused by *C. jejuni* and similar pathogens can precipitate PI-IBS. Using a rat model, we have shown that acute *C. jejuni* infection exhibits altered gut histology including reductions in the DMP-ICC which are required for gut motility as well as increased IELs and immune response mediators, and results in altered stool phenotypes and the development of SIBO in the post-infectious phenotypes. Further, we have shown that these are likely potentiated by the bacterial toxin Cdt. Lastly, recent work by our group indicates that the small intestinal microbiome is markedly different in IBS subjects, with significantly reduced microbial diversity and overrepresentation of a few specific genera, and that immunity may play a significant role in the devel-

opment of IBS, due to molecular mimicry between Cdt and neural gut moieties. Future work will include characterization of the small intestinal microbial signatures in IBS vs. healthy controls, and the development of biomarkers and targeted therapies for IBS based on immune targets. The results will allow us to significantly impact the diagnosis and treatment of IBS.

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FREQUENT SYMPTOMATIC OR ASYMPTOMATIC INFECTIONS MAY HAVE LONG-TERM CONSEQUENCES ON GROWTH AND COGNITIVE DEVELOPMENT

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THE IMPORTANCE OF DIARRHOEA

Diarrhoeal disease represents a continuing public health challenge. This is based on two combined issues: in terms of mortality it ranks as the second most common cause of death in children under 5 years old (*Liu* et al., 2012) and in terms of morbidity it has been associated with long-term deficits in physical (Checkley *et al.* 2008) and cognitive development (*Fischer Walker* et al., 2012).

Diarrhoea as a clinical symptom is defined as increased stool mass due to excess fluid in stool (*Field*, 2003). Most commonly this is assessed by the frequency of loose stools (by convention ≥3 loose stools per day), where "loose" is when a stool takes the shape of its container. Disruption to normal functioning of the gut, where water would normally be reabsorbed (*Woods*, 1990; *Field*, 2003), results in loss of fluids in stool and hence risk of dehydration and death. Whilst there are many causes of diarrhoea the most common – oft assumed cause – is enteric infection (WHO, 1988; *Thapar* and *Sanderson*, 2004). In fact, enteric pathogens were deemed to be responsible for between 12.5-94.3% of the moderate to severe diarrhoea (Kotloff *et al.* 2012) in the first year of life in the recent Global Enteric Multicenter Study (Kotloff *et al.* 2013).

Diarrhoeal symptoms that continue over a number of days is classed into one of three syndromic diarrhoeal diseases (Keusch *et al.* 2006): acute diarrhoeal disease (<7 days), prolonged (7≤ days <14) and persistent (≥14 days) (Moore *et al.* 2010). Mortality and morbidity are associated with higher rates of diarrhoeal disease episodes and in particular with persistent diarrhoea. In general, the burden of diarrhoeal disease is highest in low income countries, though it remains a universal health challenge (*Black* et al., 2003).

Research into diarrhoeal disease as a non-specific syndrome has a long history and correspondingly has produced non-specific health interventions (*Sedgwick* and *Macnutt*, 1910; *Blaise* et al., 2007). Considerable progress has been made in establishing the role of specific enteric pathogens as causes of diarrhoeal symptoms, but perhaps more important is the lack of detail regarding entero-pathogens as a cause of the long-term morbidity in the *absence* of overt diarrhoea.

Here we present preliminary findings based on incomplete data from The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED). Through the analysis of both symptomatic and asymptomatic stool samples we are beginning to quantify the role of asymptomatic infection as a driver of the long-term morbid consequence of the diarrhoeal disease syndrome.

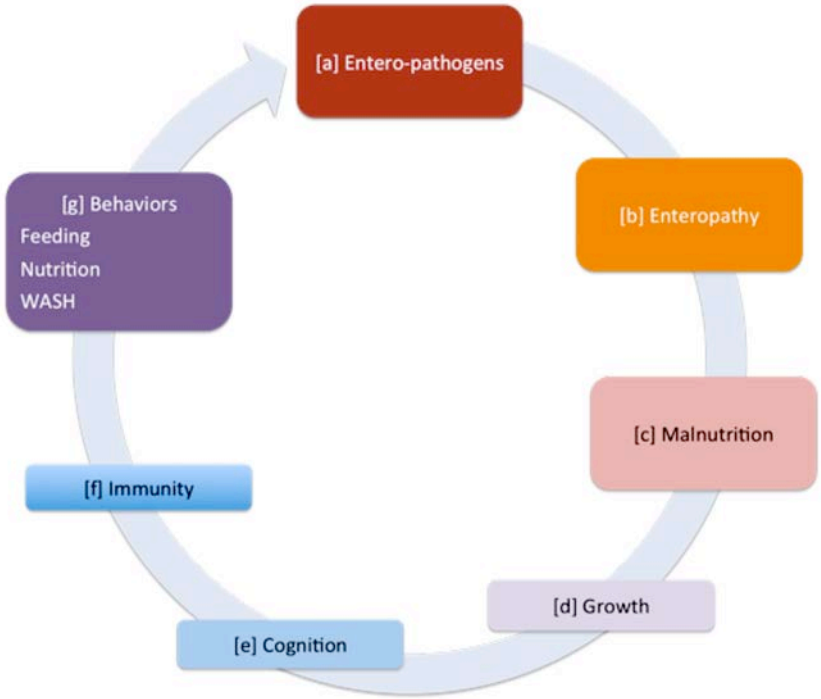

Figure 1: The "vicious cycle" of enteric infection-malnutrition and long-term consequences.

MORTALITY FROM DIARRHOEA AND ENTERO-PATHOGENS

A key detail of the studies that estimate the worldwide burden of diarrhoeal mortality is that they are based, not on measures of diarrhoea as the name of the syndrome might imply, but on enteric infection (*Black* et al., 2010). International Classification of Diseases certificates (WHO, 1990) are restricted just to the range of codes corresponding to 'intestinal infectious disease' (A00-A09). Intestinal infection is one, albeit probably the first, among many possible causes of diarrhoea, but other causes of diarrhoeal symptoms are (for these purposes) ignored. The estimated burden of mortality is, therefore, specifically an estimate of the mortality attributable to enteric infection.

Identifying an enteric infection is considerably harder than observing a visible physiological symptom like diarrhoea. Inevitably with collated certificates of death there is considerable variability in the robustness of reporting and specificity of the cause of death (*Murray* et al., 2007). For example, one classification included in the estimation of the burden of mortality is "presumed infectious origin" and upon closer inspection, some certificates are based on 'verbal autopsy' without laboratory confirmation, which has been estimated to have a positive predictive value of just 0.39 for diarrhoeal disease (*Setel* et al., 2006). Diarrhoea then, in some cases at least, is presumed to be the result of enteric infection in the absence of laboratory confirmation. To make full use of the estimates of mortality, diarrhoea must be assumed to be a reliable indicator of enteric infection.

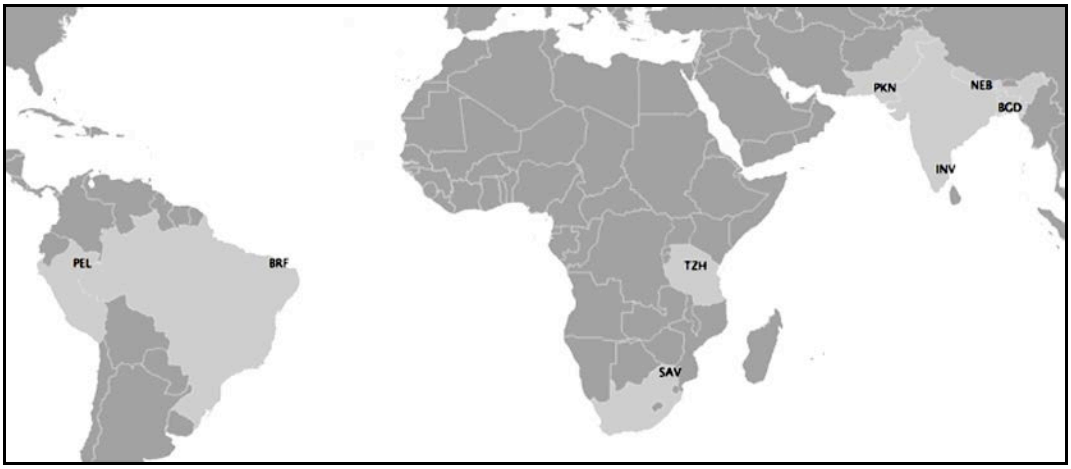

Figure 2: The location of the 8 cohorts of children who make up the MAL-ED study population. PEL Iquitos, Peru; BRF Fortaleza, Brazil; SAV Limpopo, S. Africa; TZH Haydom, Tanzania; PKN Naushahro Feroze, Pakistan; INV Vellore, India; NEB Bhaktapur, Nepal; BGD Mirpur, Bangladesh.

MORBIDITY FROM DIARRHOEA

Only in the most severe cases does diarrhoea lead to dehydration and risk of death (*Snyder* and *Merson*, 1982; *Glass* et al., 1991). Far more common are self-limiting episodes of diarrhoea (*Baqui* et al., 1991). The importance of diarrhoeal disease is not, therefore diarrhoea *per se*, so much as more insidious and cryptic morbidity that is considered so common as to be overlooked (*de Wit* et al., 2001; *MacDougall* et al., 2008). A considerable body of evidence suggests that enteric infection in children can result in a 'vicious cycle of poverty' (*Guerrant* et al., 2008) (Figure 1): (a) infection with enteric pathogens is associated with (b) impaired gut-function(*Salazar-Lindo* et al., 2004; *Petri* et al., 2008; *Viswanathan* et al., 2009; *Costa* et al., 2011) that can then (c) exacerbate malnutrition (*Guerrant* et al., 2008) and restrict the processing of nutrients necessary for (d) physical (*Mondal* et al., 2012) and (e) cognitive development (*Guerrant* et al., 1999; *Niehaus* et al., 2002; *Lorntz* et al., 2006) on top of (f) suppressing immune responses (*Schaible* and *Kaufmann*, 2007), thereby impairing a child's ability to resist (a) recurrent infections (*Moore* et al., 2010) and illness (*DeBoer* et al., 2012) from (g) increasingly 'risky' behaviours/environments. Indeed, it is the exploration of this cycle that has formed the essential hypotheses of the MAL-ED study:

1. Infection with specific entero-pathogens contributes to stunting, wasting, and/or micronutrient deficiencies causing intestinal inflammation and/or by altering the barrier and adsorptive functions of the gut; and,
2. The combination of enteric infections and malnutrition results in growth and cognitive impairments in young children and may lead to impaired immunity as measured by responses to childhood vaccines.

This cycle is predicated on the role of enteric pathogens. Enteric infection results both in physical damage to the gut and to a diversion of metabolic activity away from growth and towards combating infection(s). What is unclear is the relative degree to which infections are reflected by diarrhoeal symptoms.

Table 1: Diagnostic accuracy of diarrhoea as an indicator of enteric infection, using data from all 8 field sites and children from 0-6 months of age

	Mean	CI	N
Sensitivity	0.18	(0.15,0.21)	6,203
Specificity	0.91	(0.89,0.93)	5,966
Accuracy	0.61	(0.58,0.64)	12,169

THE MAL-ED NETWORK

The Malnutrition and Enteric Infections (MAL-ED) network ("Mal-ED") was formed to investigate the aetiology, risk factors, and interactions of enteric infections and under-nutrition and their consequences for child health and development (*Lang*, 2011; The MAL-ED Network, 2013a). This prospective field, clinical and laboratory-based observational study follows cohorts of children from (approximately) birth to 24 months. The population studied come from eight sites in countries with a historic high incidence of undernutrition and diarrhoeal disease (Figure 2).

Enrolment of new-borns began in November 2009 and ended in February 2012. Each MAL-ED Network site has enrolled more than 200 study subjects at regular intervals from near birth (prior to 17 days after birth) to 24 months of age. To reach each site's goal of following 200 children over two years and allowing for dropouts, each site enrolled approximately 10-12 children per month. Active surveillance for illness symptoms is accomplished by visiting each home two times per week. Using harmonized protocols, each site performs a number of tests and assessments, including those for gut function, monthly anthropometry, nutritional intake, cognitive development, micronutrient status, environmental assessment, and vaccine response. Follow-up will cease in February 2014, when the last child enrolled at each site will have turned two years of age.

The data presented here are from the early stages of the MAL-ED project – at the time of writing, data and sample collection has not yet ended – and the analyses here represent tentative preliminary findings based on incomplete data and should be viewed in that context. Given the staggered enrolment and substantial quantity of data being generated through longitudinal surveillance, new data are still being added and at different rates depending both on the nature of the data and on the site. One early finding from MAL-ED is the considerable heterogeneity in the biological data among the field sites – a finding that is consistent across many aspects of the study and requires considerable attention and caution both in analysis and translation into general actionable conclusions.

SYMPTOMATIC INFECTION

The assumption that diarrhoea is a useful indicator of enteric infection is implicit in the majority of public health surveillance. Diarrhoea is logistically easy to identify, though with some variability as to what rate of loose

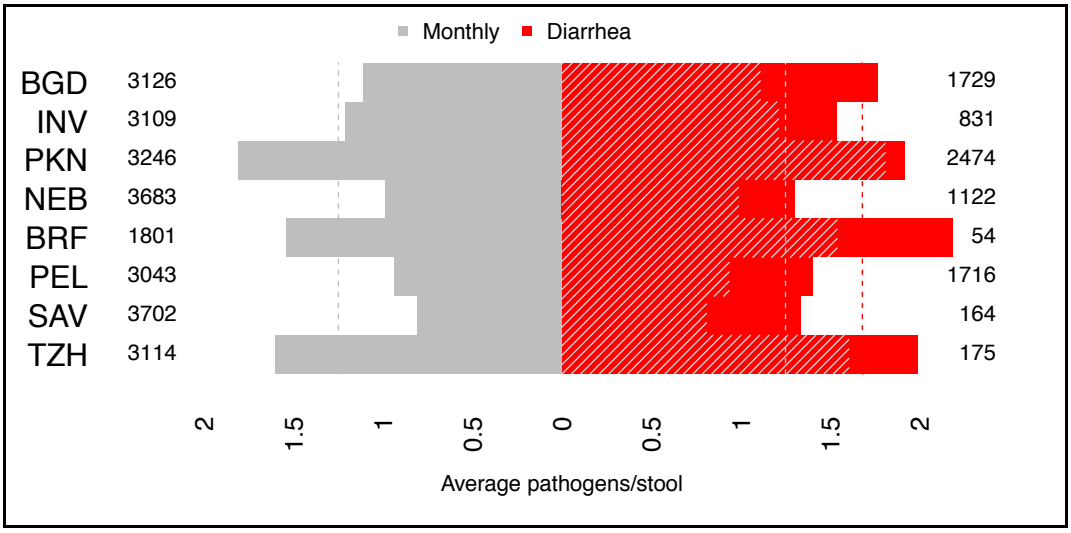

Figure 3: The average number of positive tests for pathogens per stool. Monthly "control" stools are shown in grey and reflected in the hashed bars that overlay the diarrhoeal stools in red. The number of stools tested is given in numbers at the end of each bar. Each bar corresponds to a MAL-ED site (see Figure 1 for site names). This figure is based on stools from children aged 0-12 months.

stools constitutes 'unusual' (*Kosek* et al., 2003). What is consistent, though, is the use of diarrhoea as an indicator for the pernicious morbidity associated with the syndrome.

Experience to date from the MAL-ED network suggests that diarrhoea is, at best, a poor substitute for laboratory confirmed enteric infection. Stools are collected: 1) on a monthly schedule to represent background carriage of pathogenic gut flora in the absence of overt symptoms, and 2) *ad hoc* when a mother declares that a child has had diarrhoea, defined as ≥3 loose stools within 24 hours as recommended by WHO (2006).

A panel of some 57 enteric pathogens is tested in the MAL-ED study including common viruses, bacteria and parasites (The MAL-ED Network, 2013b). Using a generalized estimating equation to account for repeated sampling of the same children and to control for such factors as breast-feeding and age, these tests were used to assess the utility of diarrhoea as a diagnostic test for the presence of enteric infection (*Sternberg* and *Hadgu*, 2001; *Murakami*, 2010). To simplify this assessment, all the various tests including ELISA, microscopy and PCR confirmation were treated as a binary variable such that any test being positive was used to indicate infection. What has emerged from a preliminary assessment in cohort children from 0-6 months of age is a sobering observation that the sensitivity and specificity of diarrhoea as an indicator of enteric infection were 0.18 and 0.91 respectively (Table 1). The low sensitivity reflects a large number of asymptomatic infections that would be classed as false negatives (i.e. no diarrhoeal symptoms despite at least one positive laboratory test for an enteric pathogen). However, the high specificity is the result of the small number of cases with a diarrhoeal (indeed any) stool that did not have a corresponding positive test for a pathogen.

Indeed the paucity of diarrhoea as a diagnostic indicator of enteric infection is evident in the average number of pathogens detected in stools that have been tested so far in MAL-ED (Figure 3). The number of pathogens detected is remarkably similar between monthly and diarrhoeal stools, with an overall average 1.7 pathogens identified per diarrhoeal stool and 1.3 pathogens per monthly "control" stool.

Diarrhoea consequently appears to be an under-estimate of enteric infection. One possible reason for this is that symptoms are a product not of the mere presence of pathogens – a limitation imposed by traditional testing methods – but of the absolute number of any given pathogen or the quantitative combinations of pathogens present. With regard to this latter case, emerging technologies offer exciting possibilities in quantifying pathogen burden based on quantitative PCR (*Operario* and *Houpt*, 2011; *Platts-Mills* et al., 2012).

INFECTION AND GROWTH

Enteric pathogens theoretically have a twofold impact on growth and development: first they damage the intestine by inducing inflammation, decreasing barrier function, decreasing absorptive capacity or combinations of all three (*Field*, 2003); and second, they can divert metabolic energy towards an immune response and away from optimal growth. Evidence of an association between enteric infection and deficits in growth or cognition are somewhat ambiguous – more so considering that many studies have used diarrhoea as a surrogate of infection.

In a recent study, *Lee* et al., (2013) present a simple model of the growth of a child in relation to *Campylobacter* infection. Specifically, they distinguish between those infections that were from symptomatic (diarrhoeal) stools and those from regularly collected asymptomatic ('control') stools. This is an interesting analysis, not least because they make a rare attempt to explicitly differentiate the two types of carriage (another example being changes of weight in relation to *Cryptosporidium* infection (*Checkley* et al., 1997)).

We are developing a similar model though rather than just *Campylobacter*, we are assessing the role of those pathogens that, to date, have been detected in >0.5% of all stools collected (either normal monthly or diarrhoeal samples) amongst the total MAL-ED study population (Figure 4).

The change in a child's linear growth can then be calculated over a defined period. *Lee* et al. (2013) reported a 9-month period in their final model, though we are analysing our data over a range of from 2 to 18 months. Over the corresponding interval, the numbers of positive pathogen tests are summed. This model, thereby, attributes a portion of the change in length over a period of time to infections with a particular enteric pathogen. In the case of infections that result in transient perturbation in growth, with growth deficits that can readily be caught up, the effect of infection is likely to be noted over short duration lags. Pathogens with longer, more persistent effects or repeated infections with one or more pathogens are more likely to be identified in longer intervals over which their cumulative impact becomes apparent.

Individually, each pathogen is comparatively rare and consequently the impact on the change in height over 9

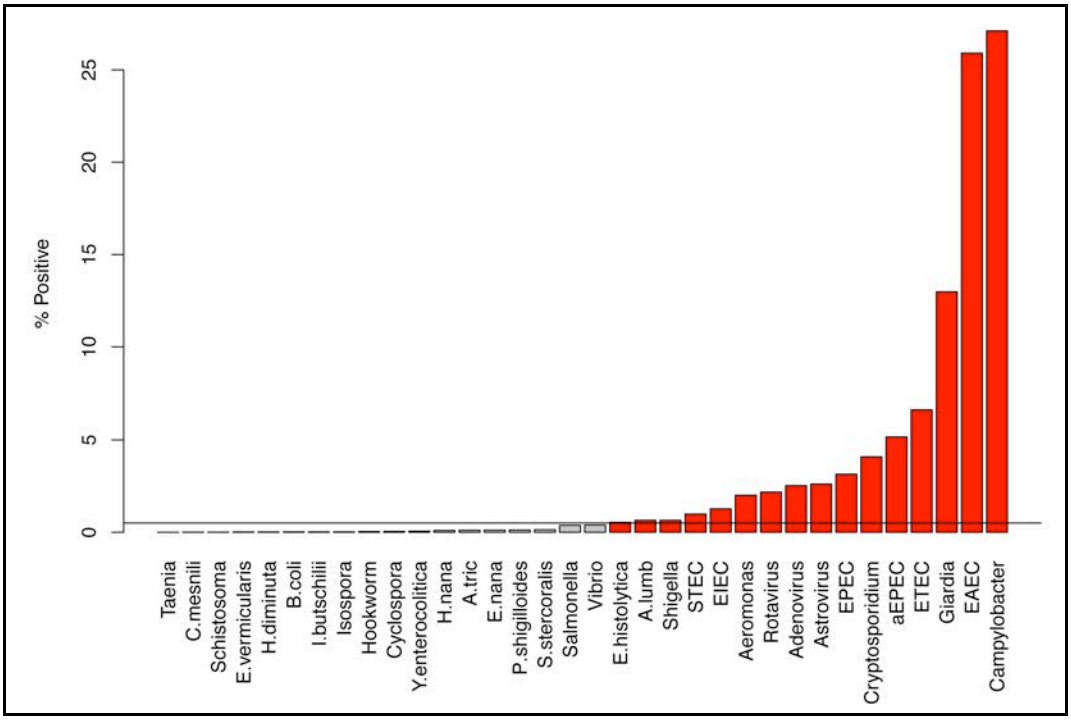

Figure 4: The percentage of stools with positive test results for pathogens in the MAL-ED study. The horizontal line distinguishes those pathogens (in red) that were found in >0.5% of all stool samples and those that were not (in grey). This figure is based on microbiology data from children 0-24 months old, aggregating the currently available data from all 8 field sites.

months was both negligible and statistically non-significant (at 5%). In preliminary analyses, there appear to be four exceptions: (i) STEC is negatively associated with a change in height when detected in diarrhoeal samples (-0.34cm/infection/9 months) as are (ii) symptomatic infections with giardia (-0.05cm/infection/9 months), while (iii) EIEC and (iv) *Campylobacter* in asymptomatic stools appear to be associated with positive (+0.14 cm/infection/9 months) and negative (-0.03 cm/infection/9 months) growth respectively.

When pathogens are pooled, however, both symptomatic (-0.03 cm/infection/9 months) and asymptomatic (-0.01 cm/infection/9 months) infections were statistically significantly associated with decreased growth. Despite the fact that the effect is greater in symptomatic infections, the overall impact of asymptomatic infection is likely to be greater as they are considerably more frequently represented in the data. Similarly, aggregating all infections, regardless of provenance, appears to be statistically significantly associated with negative growth and is just under the mean of the effect of the two stool types.

It is surprising how few analyses have explicitly considered asymptomatic infection in relation to growth, particularly given the number of infections that are unlikely to result in symptoms (*Hellard* et al., 2000) or continued infection once symptoms have resolved (*Tangermann* et al., 1991). Despite this, a model of early MAL-ED data that ignores the type of stool in

favour of a binary 'infected or not', is the most informative model based on the Akaike Information Criterion (AIC) goodness of fit (*Akaike*, 1974) suggesting that it is the presence of pathogen and not symptoms that are of greatest importance to growth.

What remains to be assessed in this type of analysis is the consequence of simultaneous co-infections. In theory the co-incidence of pathogens may have greater impact than pathogens in isolation. From the MAL-ED data, aggregating across the field sites, some 37% of diarrhoeal stools and 17% of the monthly stools have had more than one pathogen detected (most of which had two positive tests, but a maximum of 9 pathogens have been detected in the same stool so far).

INFECTION AND COGNITION

As well as being required for physical growth, nutrients and growth factors regulate brain development during foetal and early postnatal life. Over this period, the brain demonstrates its greatest plasticity, but also its greatest vulnerability to nutrient insufficiency, hence early brain development is crucial for later cognitive development and learning (*Rice* and *Barone*, 2000; *Thompson* and *Nelson*, 2001). Over 200 million children (worldwide) under five years old have been estimated to fail to reach their full cognitive potential, which cascades into poorer school performance and a subsequent reduction in economic productivity (*Grantham-McGregor* et al., 2007). Many factors contribute to this period of cognitive development including, and most relevant here, are nutrient deficiencies and infectious disease (*Walker* et al., 2007).

Through a suite of tests, MAL-ED aims to assess various aspects of cognitive development at several time points over the first two years of life (The MAL-ED Network, 2013c). The developmental assessments are performed at periodic intervals to capture the progression of child development across a range of domains including: a general cognitive assessment (*Bayley*, 2005), memory, motor development, language (*Fenson* et al., 2006), learning, temperament (*Wachs* and *Desai*, 1993), social interactions and the immediate environment (*Caldwell* and *Bradley* 1984). Alongside these child metrics, maternal reasoning (*Raven* and *Court*, 1996) and depressive symptoms (*Beusenberg* and *Orley* 1994) are also assessed.

In much the same fashion as the physiological stages of the vicious cycle, there are conceptual routes by which symptomatic infection (diarrhoea) might influence opportunities for cognitive development beyond the direct interference with nutrition (*Murray-Kolb* and *Beard*, 2009; *Armony-Sivan* et al., 2010; *Lima* et al., 2013) since diarrhoea may also alter infant-caregiver interactions in terms of both quality and quantity (*Lamb* et al., 1999). In addition, nutritional status itself may feedback into the infant-caregiver interaction (*Gardner* et al., 2005).

Cognition is hard to assess, because it is difficult to define a specific measurement(s) (*Siegler*, 1989; *Richardson* and *Richardson*, 2002). This is made more complex because conceptually, cognitive development is a function of other factors that are themselves ambiguous constructs (*Walker* et al., 2007) – for example parental engagement, the child's environment and nutrition. Though intuitively important, these constructs are hard to analyse because they are troublesome to quantify consistently and to pin down to specific

observable metrics. A solution that is often used is to combine observable measurements (for example, individual items on surveys or responses to questionnaires) as multi-dimensional representations of unobserved 'latent' variables (*Hoyle* and *Smith*, 1994; *MacCallum* and *Austin*, 2000).

An example of one solution to examining these thematic variables is given by Amorim et al. (2010). Individual measurements were combined into themes: the sum of sections from the Home Observation for the Measurement of the Environment (HOME) Scale (*Caldwell* and *Bradley*, 1984) represent "parenting style" and the child's "environment" and anthropometric measures as surrogates of "nutritional status". These unobservable constructs were then used to estimate the observed cognitive score measured as the sum of the cognitive and language subscales from the Bayley scales of infant development, 3rd edition (BSID-III) (*Bayley*, 2005).

Amorim et al. (2010) found the nutritional scores to be statistically significant predictors of the cognitive development score. This relationship appears also to hold in early MAL-ED analyses; the enrolment weight and length-for-age, which is an indicator of long-term nutritional status (WHO Working Group, 1986), both appear significantly related to the Bayley score.

The illness and infection variables have not yet been demonstrated to be statistically related to the cognitive score, with the exception that the total days of maternally reported fever that may be associated with a diminished Bayley score. That these variables were not related to cognition does not necessarily mean that they are not important factors. In the context of a structural equation (as used by *Amorim* et al., 2010), measured variables explicitly feed into latent variables, thus specifying the direct and indirect relationships between variables. In this particular model, there are likely to be strong correlations between the non-significant illness factors and the highly significant nutrition factors (for example see the above association between height and infection). As such, there are putative indirect, though not shown here, associations between infection and cognition. It is perhaps noteworthy that the mean estimate of the association with cognition is stronger for the symptomatic infections, which again may (though not demonstrated here) reflect the additional associations expected between diarrhoeal symptoms and caregiver interactions.

THE LEGACY OF DIARRHOEA

Despite the weakness of diarrhoea as an indicator of enteric infection and the lack of studies quantifying the impact of asymptomatic infection, there remains a persistent literature that discusses diarrhoea as a 'cause' of physical and cognitive shortfalls. The reason for this apparent paradox may lie in the naming of the syndrome. Diarrhoea is used as a short-hand for both the overall "diarrhoeal disease syndrome" (indicative of serious gut dysfunction) as well as loose stools. This confusion allows the persistence of language that suggests that diarrhoea (the symptom) is a useful indicator of the syndrome despite the lack of evidence that "loose stools" *per se* are a robust metric of enteric infection. Indeed this language under-estimates the importance of the largely asymptomatic syndrome.

An additive Bayesian network (abn) (*Heckerman* et al., 1995; *Lewis* and *McCormick*, 2012) using the earliest

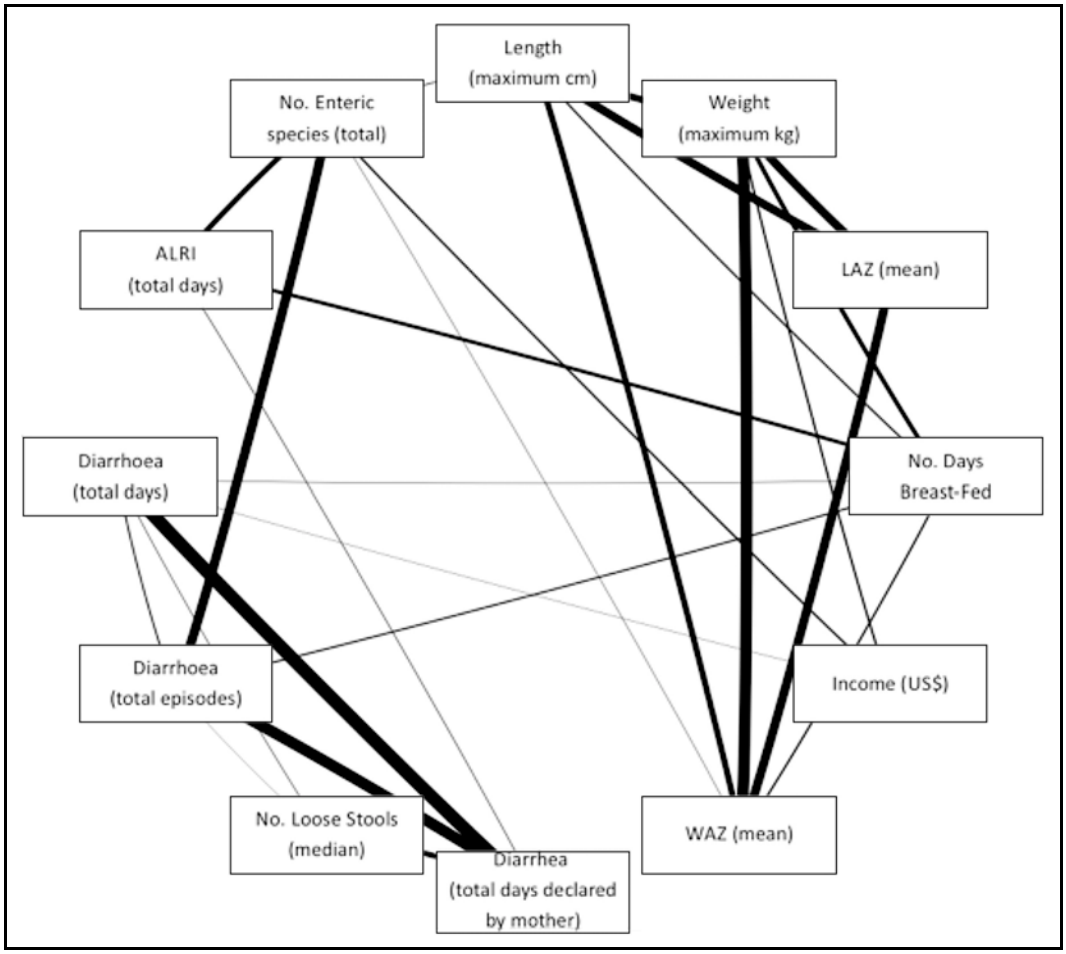

Figure 5: The statistical relationships between variables using an additive Bayesian network (abn). MAL-ED data from all 8 field sites for children at 6 months of age were used, with random effects for the field site. Line width indicates the strength of statistical association.

available data was constructed in an effort to agnostically identify relationships among variables being studied in MAL-ED. The abn was used for structure discovery, identifying a single globally optimal directed acyclic graph (DAG) that describes the data (*Koivisto* and *Sood*, 2004; *McCormick* et al., 2013). The abn is a multivariate model, treating each variable as both a potential predictor of every other variable included in the analysis as well as a response. Unlike traditional models (such as a generalized linear model), the abn avoids the temptation to fallaciously combine the results of individual regression models that have a single (presumed independent response variable) (*Hand* et al., 1997). Furthermore it better reflects the co-dependency of the many interacting components of a system that is by definition a collection of related variables. This allows a statistical structure to emerge from the data themselves that identifies, not only the immediate relationship between a suite of variables and a single outcome, but also the relationships between the

many variables that are both directly and indirectly associated within a system (*Lewis* and *Ward*, 2013).

What emerges from MAL-ED data of the first 6 months of life is that, within these preliminary data, there appear to be no direct associations between diarrhoeal symptoms and anthropometric measures (Figure 5). This makes sense in the context of the analyses above. What also makes sense is the presence of indirect statistical associations between diarrhoeal symptoms, enteric pathogens (not distinguishing between symptomatic and asymptomatic) and anthropometry (mean WAZ and maximum length by 6 months old). This putative pathway is particularly interesting because it reflects what is widely expected from the diarrhoeal disease syndrome even if the disease symptoms are not reliable indicators.

What were missing from the available data at the time of the analysis, were measures of cognitive development and nutrition. The latter would appear central to the manifestation of the diarrhoeal disease – the assumption is that the syndrome impacts the efficient processing of nutritional input, for example through physical damage to the intestine, the rapid egestion of food before nutrients are absorbed. Such data are being collected within the MAL-ED study, however, and when available, will be added to this emerging model. Based on other studies, it is perhaps unlikely that the impacts on growth become detectable (above the between-site heterogeneity) until children are older (*Checkley* et al., 1998; *Lima* et al., 2000; *Moore* et al., 2001). As additional data become available it will be possible to reassess this early observation.

CONCLUSIONS

There is a considerable body of evidence supporting a conceptual model in which growth and cognitive development are negatively affected by undernutrition. A prime cause, aside from poor quality or low quantity of food, is the interference with optimal gut function by enteric infection. Within this cycle, enteric infection is frequently referred to as diarrhoeal disease, however this belies the importance of asymptomatic infection.

Enteric infection resulting in diarrhoea is relatively uncommon compared to an overwhelming number of asymptomatic infections or continued carriage (whether or not deleterious) observed in the settings studied in MAL-ED. The question is whether or not symptoms are important contributors to growth outcomes? As indicators of gut dysfunction and to indicate the risk of dehydration leading to hospitalization or death, symptomatic diarrhoea is clearly useful. However, preliminary evidence from this longitudinal prospective study suggests that any enteric infection– symptomatic or not – contributes to decreased growth. The impact of each infection is relatively small and slow to emerge, with the significance of each infection becoming more apparent as the lag since the infection increases. These results may change when examining the complete data from MAL-ED and in the broader context of growth outcomes over the entire 24 months of the study are modelled.

Likewise, any association between enteric infection and cognitive development may become clearer as more data become available from older children. Even so, early data from MAL-ED hint

that the role that entero-pathogens play is indirect - acting on nutrition and thereby influencing cognitive development. Such a hierarchy of interactions is likely to be most clearly identified by accounting for the many interlinked associations in the wider syndrome.

The diarrhoeal disease syndrome is something of a misnomer that has distorted the expectations that diarrhoea (the symptom) is a reliable indicator of gut infection and/or dysfunction. In fact, enteric infection is what is commonly understood when referring to "diarrhoea" and that seems to, based on preliminary observations noted here, have long-term consequences for growth and cognitive development, whether or not those infections are associated with overt diarrhoea.

ACKNOWLEDGMENTS

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UTILIZATION OF THE US DEPARTMENT OF DEFENSE MEDICAL SURVEILLANCE SYSTEM AND SERUM REPOSITORY FOR ASSESSING LONG-TERM HEALTH CONSEQUENCES OF ACUTE ENTERIC INFECTION

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INTRODUCTION TO DODSR AND DMSS

Second only to randomized controlled trials (RCTs), prospectively designed cohort studies represent the best way to explore the association between exposure and outcome in human populations. However, given practical limitations of sample size requirements, the high frequency of lost-to-follow-up, the relative rarity of some outcomes and other funding and logistical constraints, such epidemiologic investigation is rarely feasible. In absence of this study design, one begins to explore the potential for other, population-based data sets from which cohort studies can be reconstructed. One such available data source is available within the confines of the United States Department of Defense (US DoD). The objective of this paper is to review aspects and elements of the system maintained by the US DoD and how they can be utilized to explore novel associations including the persisting consequences of intestinal infection.

The active duty US military represents a large, uniquely healthy, young, active subset of the general US population and is one amenable to epidemiologic studies. In 1986, the US Army established a data repository with the stated purpose of supporting HIV screening, care and research (*Rubertone* et al., 2002). Seven years later, the scope of this system expanded to include all illness and injuries of importance for public health and/or the DoD. Additionally, self-completed pre- and post-deployment health surveys were initiated in the 1990s to document exposures during deployment as well as general changes in health subsequent to deployment (*Moore* et al., 2010). These data are linked within the Defense Medical Surveillance System by social security number to longitudinal demographic, medical encounter, vaccination and deployment data on all active duty service members.

 There were no financial conflicts of interests among any of the authors. The study protocol was approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects. This study was conducted under support of the Military Infectious Disease Research Program.

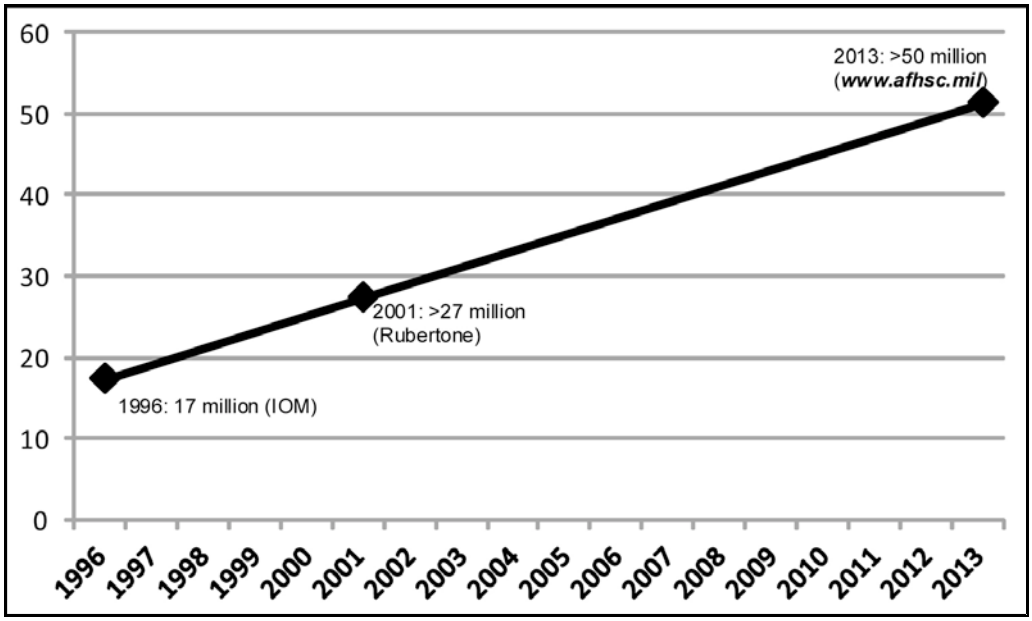

Figure 1: Estimated number of serum samples in DoD serum repository.

Almost simultaneously to the establishment of the serum repository, in 1985, the US DoD implemented a Human Immunodeficiency Virus (HIV) screening program in which remnants of sera collected from recruits that tested negative for HIV was archived (*Moore* et al., 2010). Subsequently, the sera were transported to a single facility and the serum repository was born. In the 1990s, in response to illnesses affiliated with participation in the first Gulf War, the DoD expanded serologic screening by implementing pre- and post-deployment blood draws for serum archival to identify potentially important exposures during deployment. The serum repository has grown exponentially since that time and at present houses over 56 million serum samples from potential recruits, active duty and former active duty service members (Figure 1). These serum samples can be linked (via social security number) to data within the Defense Medical Surveillance System (DMSS).

While these are invaluable resources with fairly limited access, it is useful to explore how these data and samples have begun to be utilized to explore the association between acute infection and chronic gastrointestinal (GI) sequelae, what additional questions are pending and how archives within the US DoD can be utilized to answer some of these questions. Additionally, full acknowledgement and evaluation of the strengths and limitations of the DMSS and the Department of Defense serum Repository (DODSR) related to assessing the link between acute enteric infection and post-infectious sequelae is necessary to identify knowledge gaps that need filling with supplementary data and other data/repository systems available. The objective of this report is to summarize the key findings to date utilizing the DMSS/DODSR, to outline expanded areas of research in which they can be utilized to explore novel hypotheses on infection and chronic GI sequelae and begin to frame the mechanism(s) by which these studies could be conducted.

Table 1: List of gastrointestinal conditions that do not meet the standard for appointment, enlistment or induction with relevant ICD-9 and CPT codes (DOD instruction 6130.03)

Site/Condition:	**Exclusionary conditions:**
Oesophageal Disease:	(1) Current or history of oesophageal disease (530.0-530-9), including but not limited to ulceration, varices, fistula, or achalasia. (2) Gastro-Oesophageal Reflux Disease (GERD) (530.81), with complications. (a) Stricture or B-ring. (b) Dysphagia. (c) Recurrent symptoms or esophagitis despite maintenance medication. (d) Barrett's esophagitis. (e) Extra-oesophageal complications; reactive airway disease; recurrent sinusitis or dental complications. (3) History of surgical correction (fundoplication or dilation) for GERD within 6 months (45.89). (4) Current or history of dysmotility disorders, to include diffuse oesophageal spasm, nutcracker oesophagus, non-specific motility disorder, and achalasia. (5) Eosinophilic oesophagitis. (6) Other oesophageal strictures, for example lye or other caustic ingestion
Stomach and Duodenum:	(1) Current dyspepsia requiring medication; or history of dyspepsia lasting 3 or more consecutive months and requiring medication within the preceding 12 months. (2) Gastric or duodenal ulcers: (a) Current ulcer or history of treated ulcer within the last 3 months. (b) Recurrent or complicated by bleeding, obstruction, or perforation within preceding 5 years confirmed by endoscopy. (3) History of surgery for peptic ulceration or perforation (533.0-599.9). (4) History of gastroparesis. (5) History of bariatric surgery of any type (e.g., lap-band or gastric bypass surgery for weight loss). (6) History of gastric varices.
Small and Large Intestine:	(1) Current or history of inflammatory bowel disease, including but not limited to indeterminate (558.9), Crohn's disease (555), ulcerative colitis (556), or ulcerative proctitis (556.2). (2) Current infectious colitis not otherwise specified (009.1). (3) Current or history of intestinal malabsorption syndromes (579.9), including but not limited to celiac sprue, pancreatic insufficiency, post-surgical and idiopathic (579). Lactase deficiency does not meet the standard only if of sufficient severity to require frequent intervention, or to interfere with normal function. (4) Current or history of gastrointestinal functional and motility disorders within the past 2 years, including but not limited to pseudo-obstruction, megacolon, history of volvulus, or chronic constipation (564.0) and or diarrhoea (787.91), regardless of cause, persisting or symptomatic in the past 2 years.

Table 1 (continued): List of gastrointestinal conditions that do not meet the standard for appointment, enlistment or induction with relevant ICD-9 and CPT codes (DOD instruction 6130.03)

Site/Condition:	Exclusionary conditions:
Small and Large Intestine:	(5) History of gastrointestinal bleeding (578), including positive occult blood (792.1), if the cause has not been corrected. Meckel's diverticulum (751.0), if surgically corrected more than 6 months prior DOES meet the standard. (6) Current or history of irritable bowel syndrome (564.1) of sufficient severity to require frequent intervention or prescription medication or to interfere with normal function. (7) History of bowel resection (CPT 44202-44203). (8) Current or history of symptomatic diverticular disease of the intestine (562). (9) Personal or family history of familial adenomatous polyposis syndrome or hereditary non-polyposis colon cancer syndrome.
Hepatic-Biliary Tract:	(1) Current acute or chronic hepatitis, hepatitis carrier state (070), hepatitis in the preceding 6 months or persistence of symptoms after 6 months, or objective evidence of impairment of liver function. (2) Current or history of cirrhosis (571), hepatic cysts (573.8), abscess (572.0), or sequelae of chronic liver disease (571.3). (3) Current or history of symptomatic cholecystitis (575.10), unless successfully surgically corrected; postcholecystectomy syndrome; or other disorders of the gallbladder and biliary system (576). Cholecystectomy DOES meet the standard if performed more than 6 months prior to examination and patient remains asymptomatic. Endoscopic procedure to correct choledocholithiasis, if performed more than 6 months prior to examination and patient remains asymptomatic, MAY meet the standard. (4) History of sphincter of Oddi dysfunction. (5) Choledochocyst. (6) Primary biliary cirrhosis or primary sclerosing cholangitis. (7) Current or history of pancreatitis, acute (577.0) or chronic (577.1). (8) Pancreatic cyst. (9) History of pancreatic surgery. (10) Current or history of metabolic liver disease, including but not limited to hemochromatosis (275.0), Wilson's disease (275.1), or alpha-1 anti-trypsin deficiency (273.4). Gilbert's syndrome DOES meet the standard. (11) Current enlargement of the liver from any cause (789.1).
Anorectal:	(1) Current anal fissure or anal fistula (565). (2) Current or history of anal or rectal polyp (569.0), prolapse (569.1), stricture (569.2), or faecal incontinence (787.6), within the last 2 years. History of removal of juvenile or inflammatory polyp DOES meet the standard. (3) Current haemorrhoid (internal or external), when large, symptomatic, or with a history of bleeding (455) within the last 60 days.

Table 1 (continued): List of gastrointestinal conditions that do not meet the standard for appointment, enlistment or induction with relevant ICD-9 and CPT codes (DOD instruction 6130.03)

Site/Condition:	Exclusionary conditions:
Abdominal Wall:	(1) Current hernia (except for small or asymptomatic umbilical hernias), including but not limited to uncorrected inguinal (550) and other abdominal wall hernias (553). (2) History of open or laparoscopic abdominal surgery (CPT 22900-22999, 43500-49999) during the preceding 6 months (P54). Uncomplicated laparoscopic appendectomies (CPT 44970) meet the standard after 3 months.
Obesity:	History of any gastrointestinal procedure for the control of obesity (CPT 43644-43645, 43770-43775, 43842-43848, 43886-43888) or artificial openings, including but not limited to ostomy (V44).

POPULATION DEMOGRAPHICS

One of the most unique attributes of the Department of Defense repository is the population from which the data are obtained. As of February 28, 2013, there were 1.39 million active duty service members with an average age of 28.8 years (Defense Manpower Data Center; DMDC). This very healthy, physically active subset of the general population is one in which assessment of the risk of chronic GI sequelae following from infection is beneficial as it is generally free of oftentimes confounding co-morbidities. Specifically, all United States Department of Defense recruits are subject to detailed physical exams and medical history prior to being processed for recruit training. The specific requirements are outlined in the Department of Defense Medical Standards for Appointment, Enlistment, or Induction. The current list of gastrointestinal conditions that do not meet the standard for appointment, enlistment or induction, updated 28 April 2010 under Department of Defense Instruction 6130.03 are included in Table 1 and highlight the differences between this and the general population. Conversely, studies in this population often lack external validity to the general population which may limit extrapolation of data to other dissimilar populations.

Age is a known risk factor for many of the chronic GI outcomes currently linked to IGE including IBS, IBD and coeliac disease. With the overwhelming majority (approximately 80%) of active duty personnel aged 18-35 (DMDC), estimates of relative risks may be confounded by age. Specifically, risk of PI-IBS has been shown to decrease with increasing age (*Marshall* et al., 2006; *Neal* et al., 1997). Studies to date are too limited to assess whether age serves as a confounder or an effect modifier of the risk associated with antecedent exposure and studies of active duty personnel have included age as a matching variable between cases and controls. However, matching on this potential important covariate precludes any assessment of its impact on the association between infection and outcome among this uniquely young population.

The overwhelming majority (85.4% as of Sept 2012) (DMDC) of active duty military personnel are male compared to an estimated 50.2% of the general US population (2010 US census). Importantly, being of female gender has been repeatedly associated with an increased risk of FGD including IBS, functional dyspepsia and functional constipation (*Mearin*, 2011). Incidence of these functional outcomes in military populations based on these data is lower than what has been reported elsewhere using similar methodology. As such, studies should ensure matching on gender for assessment of most PI-FGD. Furthermore, stress has a role in IBS risk and may confound or modify the effect of enteric infection on FGD risk in a population that is frequently deployed into what are likely broadly considered to be stressful environments (*Drossman*, 2011). Additionally, gender has been indicated as a risk factor for more pathological conditions such as IBD and coeliac disease (*Green* and *Cellier*, 2007; *Karlinger* et al., 2000). Despite the potential lack of external validity, numerous large cohort studies have elucidate novel findings of public health importance; examples include the Framingham Heart Study (*Dawber* et al., 1951) and those of smoking and mortality among a cohort of physicians (*Doll* and *Hill*, 1954; *Doll* and *Pike*, 1972).

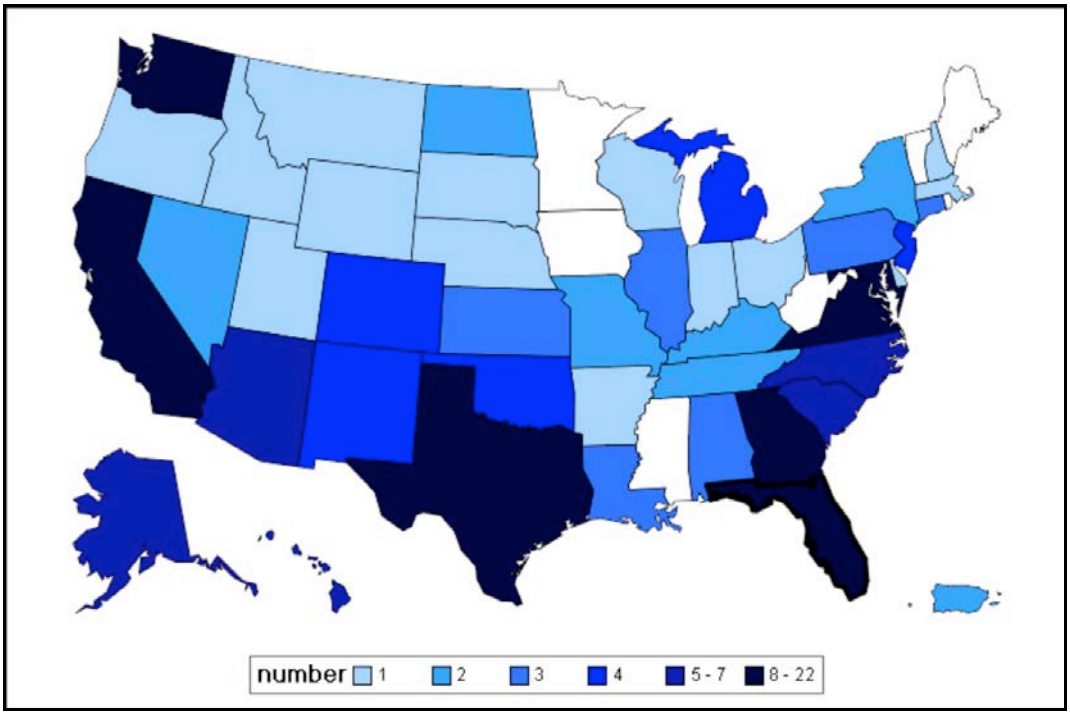

Figure 2: Map of the Number of Military Treatment Facilities in the US and Puerto Rico.

ACCESS TO CARE

One of the factors limiting the utilization of medical encounter databases in other populations is variability in access to medical care. For example, studies within large provider networks (Kaiser, Aetna, etc.) within the United States have frequently reported findings within their covered population; however, it is unclear if the covered and non-covered populations in those regions are comparable or whether covered individuals sought care outside of their provider network (*Koebnick* et al., 2012; *Stephenson* et al., 2005). In contrast, active duty military personnel have equal and unfettered access to healthcare at any one of the 184 US- (and Puerto Rico-) based military treatment facilities (MTFs) (Figure 2) or the 31 locations internationally (http://www.tricare.mil/mtf/main1.aspx; accessed 5/10/13). In a reference population followed for an average of 3.7 years, the median number of outpatient medical encounters per subject was 29 (interquartile range: 14, 56) for just under an estimated 8 outpatient medical encounters annually per active duty service member (*Porter* et al., 2012). These encounters included general health physicals, vaccinations, procedures, illness and injuries handled in an ambulatory setting. As expected, the number of inpatient visits was much lower (annually <1 per service member).

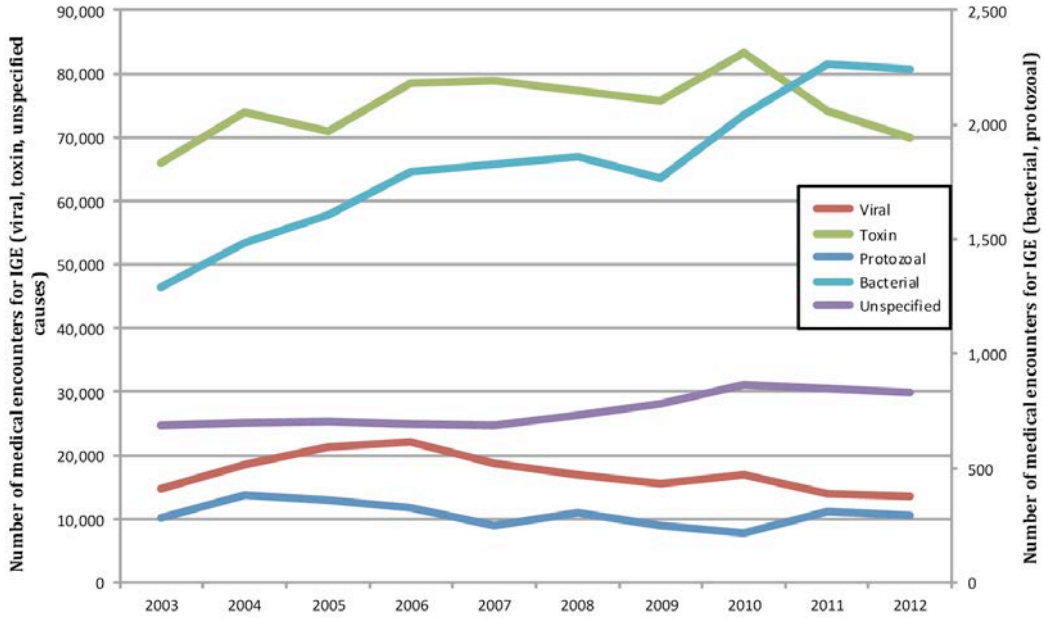

Figure 3: Number of medical encounters for infectious gastroenteritis among active duty US military personnel 2003-2012.

EXPOSURES OF INTEREST

The under-reporting phenomenon of acute gastroenteritis is well-appreciated and has been documented by the US Centers for Disease Control and Prevention (CDC) most recently in estimates of foodborne-related illness in the US (*Scallan*, 2011a,b). The DMSS is no different from other passive reporting systems and it is anticipated that estimates of infectious (and/or toxin-mediated) gastroenteritis rates significantly underestimate true incidence. Nonetheless, over the past 10 years (2003-2012), there have been 1.8 million documented medical encounters for acute gastroenteritis of presumed infectious/toxin-mediated origin (Figure 3), the overwhelming majority (99.2%) of which occurred in an outpatient setting. Current studies of the post-infectious consequences of acute infection have most frequently focused on specific bacterial pathogens including Campylobacter, Salmonella, Shigella and entero-haemorrhagic *E. coli*; thus an assessment of the estimated minimum number of available relevant exposures for these pathogens is warranted. As shown in Figure 4, medical encounters associated with these 4 pathogens have remained relatively stable over the past 10 years with a total number of just under 3,000 total encounters. Importantly, these documented medical encounters are likely to greatly underestimate the actual population exposed to these pathogens (*Porter* et al., 2012a).

Importantly, the above events are limited to those occurring at a MTF and do not include the numerous exposures that occur during military deployment and/or assignment in regions of the globe with high rates of travellers' diarrhoea. While deployment to those regions may serve as a surrogate of exposure, prior studies have been limited in their ability to link increased

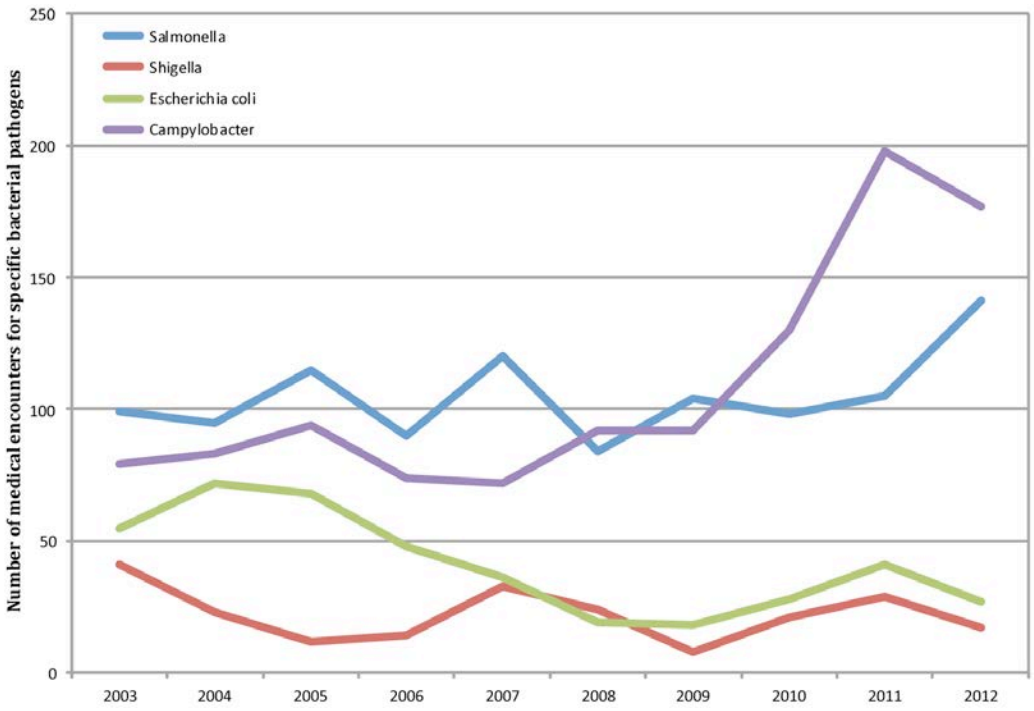

Figure 4: Number of medical encounters for Salmonella, Shigella, Campylobacter and *E. coli* O157:H7 among active duty US military personnel 2003-2012.

sequelae risk with deployment to high TD regions (*Porter* et al., 2011a, 2012b, 2013a). This may be due to a potential healthy worker effect or non-specific in the exposure (or in this case the exposure surrogate) thus biasing effect estimates toward the null hypothesis of no association.

A potential mechanism to circumvent this limitation is to utilize deployment health assessments designed to document exposures occurring during deployment. US Department of Defense policy mandates the collection and maintenance of deployment health data (DoDI 6490.03, Deployment Health, 11 Aug. '06). One mechanism utilized to facilitate these mandates is the pre- and post-deployment health assessments (DD 2795 and 2796, respectively). These self-assessments are completed electronically, maintained by the AFHSC and can be linked with the serum specimens as well as demographic and medical encounter data. Data obtained as part of these forms include deployment information (e.g., country, duration), general assessments of in-theatre and post-deployment health and exposures of interest during deployment. While this form has undergone several iterations, most recently in SEPT 2012, the version preceding the current edition collected data on potential IGE. In 2011, Porter et al described an increased odds of self-reported diarrhoea/vomiting among FGD cases compared to matched controls both of whom were first-time deployers with only one deployment during the surveillance period (*Porter* et al., 2011b). While lacking sensitivity and specificity, these data sources open the door for obtaining important deployment-specific exposures.

Table 2: Epidemiologic studies of post-infectious sequelae conducted utilizing data obtained from DMSS

Reference	Exposure(s)	Outcome(s) and estimated relative risk/odds ratio	
Curry et al., 2010	Infectious gastroenteritis	Reactive arthritis: Nonspecific arthropathy:	4.4 (2.2, 8.7) 1.8 (1.5, 2.1)
Porter et al., 2008	Infectious gastroenteritis	Crohn's disease: Ulcerative colitis:	1.5 (1.2, 2.0) 1.4 (1.1, 1.7)
Porter et al., 2011a	Infectious gastroenteritis	Constipation: Dyspepsia: Functional diarrhea: Irritable bowel syndrome:	2.2 (2.0, 2.3) 2.4 (2.1, 2.7) 6.3 (4.4, 8.9) 3.7 (3.4, 4.1)
Porter et al., 2011b	Diarrhea/vomiting during deployment	Constipation: Dyspepsia: Irritable bowel syndrome: Any FGD:	1.9 (0.9, 3.9) 6.8 (2.9, 15.4) 6.3 (2.5, 15.4) 2.9 (1.8, 4.8)
Porter et al., 2012a	Infectious gastroenteritis[a]	Crohn's disease Ulcerative colitis	1.5 (0.4, 6.3) 3.5 (1.4, 9.0)
Porter et al., 2012b	Norovirus	Constipation: Dyspepsia: IBS: GERD:	1.3 (0.9, 1.8) 1.4 (0.8, 2.5) 0.7 (0.3, 1.5) 1.4 (1.1, 1.8)
Porter et al., 2013c	Campylobacter Salmonella Shigella Yersinia	Constipation: Dyspepsia: IBS: GERD:	1.6 (1.3, 2.0) 1.3 (1.0, 1.9) 2.9 (2.2, 3.8) 1.6 (1.4, 1.8)
Riddle et al., 2012	Infectious gastroenteritis	Celiac disease:	2.1 (1.4, 3.0)
Riddle et al., 2013	Campylobacter[b]	Celiac disease:	3.5 (0.7, 18.0)

IBS=Irritable bowel syndrome; FGD=Functional gastrointestinal disorder
[a] Among personnel with IBS
[b] No increased risk observed following Shigella, Salmonella or Yersinia

Importantly, deployment not only increases the potential risk of exposure, but also likely increases the risk of psychological stressors, independent risk factors of functional bowel disorders. It has been hypothesized that stressful events, such as deployment and/or combat situations may modify the effect of infection of FGD risk (*Drossman*, 2011). This was corroborated by the 2011 study by Porter et al (although not statistically significant) (*Porter* et al., 2011b). Further supporting the link between military exercises and functional symptoms are the results of a recent study of Singapore military personnel in which increased intestinal permeability during combat training corresponded to an increase in post-training GI complaints (*Li* et al., 2013). Recognition of this potential effect modification and development of methods to accurately capture levels of deployment-related stress are important to ensure appropriate interpretation of results in this population.

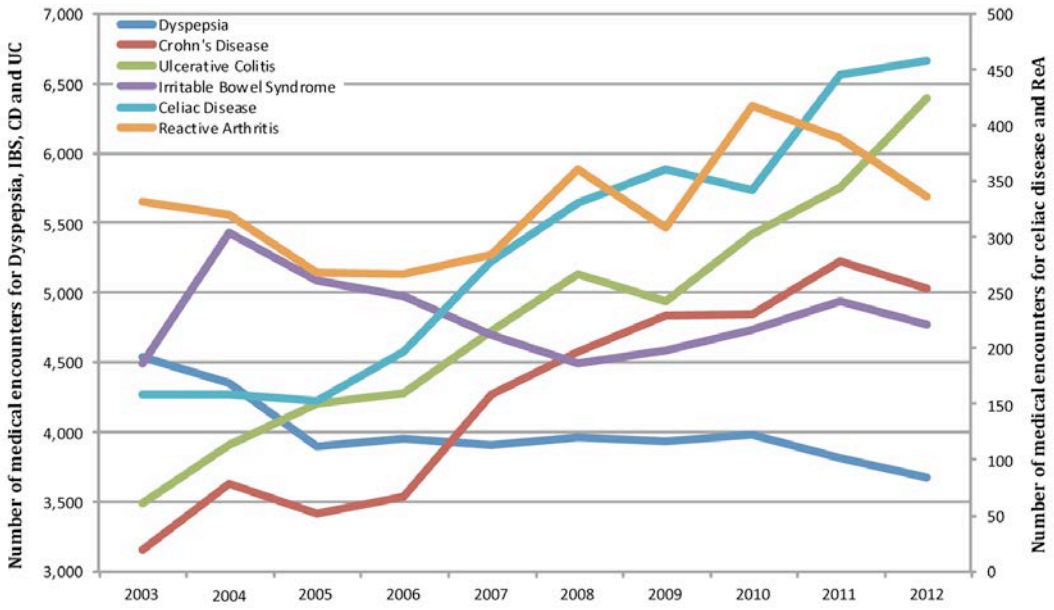

Figure 5: Number of medical encounters for adverse health outcomes associated with enteric infection among US military personnel 2003-2012.

OUTCOMES OF INTEREST

Of equal importance to the numbers of available exposures are the outcomes of interest for studying the long-term impact of acute gastroenteritis. A recent study on the incidence of FGD among this population from 1997-2007 estimated rates of any FGD of approximately 231 per 100,000 person-years (/100K p-y) with the highest rate observed for constipation (127/100K p-y) followed by IBS (66/100K p-y) and dyspepsia (49/100K p-y) and (*Porter* et al., 2011a). Similar estimates over comparable time periods have been made for IBD (29.2/100K p-y) (*Porter* et al., 2008), coeliac disease (3.6/100K p-y) (*Riddle*, 2012) and reactive arthritis (4.1/100K p-y) (*Curry* et al., 2010). Importantly, these outcomes are associated with numerous medical encounters annually (Figure 5). Over the last 10 years, there have been 40,000-50,000 medical encounters each for dyspepsia, CD, UC and IBS and 3,000-4,000 each for coeliac disease and reactive arthritis. This represents as substantial population from which to identify well-defined cases and retrospectively follow the disease progression from pre-onset to present.

LIMITATIONS

The limitations of medical encounter data systems have been described previously and an exhaustive delineation of the limitations specific to the DoD system is beyond the scope of this review. Nonetheless, Table 3 outlines some of the potential areas of bias inherent in generic systems as well as the DMSS. Importantly, studies assessing the quality of medical encoun-

Table 3: Potential sources of bias inherent in generic medical encounter repositories as well as the DMSS (modified from *Schneeweiss* and *Avorn*, 2005)

Generation of typical medical encounter data	**DMSS**	**'Other' systems**
Population:	- Well-defined demographic data for 1.4 million currently active duty service members - Approximately 10 million total service members - Dissimilar to general population - Unique occupational exposures	- Demographic data of total population may be unavailable - System dependent (Kaiser, NHANES, country-wide)
'Ill' patient seeks care:	- Potential variability in care-seeking behaviour across demographic characteristics - Potential use of serum repository to document outcomes among those not seeking care - Frequent well-visits and vaccinations - Data linkable across global MTFs - Lacks medical encounters during deployment • pre-deployment health assessments • Post-deployment health assessments	- Lack of insurance in specific populations may decrease care-seeking behaviour - No ability to document outcomes among those not seeking care - Potential variability in care-seeking behaviour across demographic characteristics - Care 'out of network' • ER visits • During travel
Medical examination/ history:	- Medical history electronically available - Medical examination data not available	- Medical history may/may not be electronically available - Medical examination data may/may not be available
Procedures & testing:	- CPT codes readily available (potential for miscoding)	- CPT codes may/may not be available (potential for miscoding)
Diagnoses:	- ICD9-CM codes readily available (potential for miscoding) - Can be validated by serologic testing for specific outcomes	- ICD9-CM codes readily available (potential for miscoding)

Table 3 (continued): Potential sources of bias inherent in generic medical encounter repositories as well as the DMSS (modified from *Schneeweiss* and *Avorn*, 2005)

Generation of typical medical encounter data	**DMSS**	**'Other' systems**
Interventions (pharmacy):	- Pharmaceutical data not readily linked with medical encounter data - Over the counter medications not routinely available - Free samples not documented	- Over the counter medications not routinely available - Free samples not documented
Outcomes:	- Out of military (theoretically linkable with Veteran's Administration data) - All other administrative changes documented in system	- Lost to follow-up • Provider/network change • Death • Other
Research purposes:	- Supported through AFHSC (with DoD collaborator) - Human subjects' protection - Methods exist to remove patient identifiers	- Limited to those with network-specific access

ter data to identify exposures and outcomes of interest have been conducted to varying degrees of success. Specific to the DMSS, Payne et al validated anthrax vaccination history and found relatively highly levels of positive and negative predictive values (*Payne* et al., 2007). While no studies assessing the PPV of the exposures and outcomes of interests related to post-infectious sequelae of acute enteric infectious have been published to date, efforts are underway by the authors to explore the utility of these ICD-9 codes to identify incident outcomes. The on-going study utilizes a total of 1750 subjects with a medical encounter in which an ICD9-CM code specific for one of a variety (IBS, Crohn's disease, ulcerative colitis, reactive arthritis, non-specific mono-arthropathy, coeliac disease, infectious gastroenteritis) of clinical outcomes is documented (250 patients/outcome). The medical chart of each identified patient is then obtained and data elements extracted to allow for adjudication by a third party. Completion of the adjudication process will enable the estimation of a positive predictive value associated with each ICD9-CM code with $^{+}/_{-}$ 5%. The results of this study will support on-going efforts within and external to the DoD regarding outcomes of interest and the utilization of electronic medical records to identify novel associations.

UTILIZATION OF SERUM REPOSITORY

Perhaps one of the greatest strengths of the DoD system and an element that sets it apart from other medical encounter systems is the availability of sequential serum samples that can be linked to the demographic, deployment, vaccination and medical encounter data. A quick PubMed® search indicates that investigators have utilized the serum repository to identify serologic risk factors for disease (*Levin* et al., 2012; *Munger* et al., 2013), genotypic factors associated with disease (*Scher* et al., 2011) and temporal changes in antibody profiles preceding disease onset (*Arbuckle* et al., 2003). Despite these significant advancements in disease understanding brought about through utilization of the serum repository, these, and similar studies have only begun to scratch the surface of the potential utility of these serum samples. Specifically, revolutionary advances in genomics, proteomics and metabolomics have ushered in the systems' biology era and utilization of the DoD's serum repository, has the potential to transform our understanding of disease processes and, related to post-infectious sequelae of enteric infection, sample testing may elucidate novel mechanisms by which acute infection may lead to prolonged adverse health outcomes.

Specifically related to potential sequelae of enteric infection, genomic analyses have identified that genes associated with intestinal barrier and responses to enteric pathogens were associated with an increased risk of PI-IBS among subjects with affected by a waterborne outbreak of entero-haemorrhagic *E. coli* and *Campylobacter* (*Villani* et al., 2010). Metabolomics have been utilized to identify baseline differences in IBS patients compared to controls and to subsequently measure the impact of probiotics on the metabolome and subsequent clinical improvement of IBS patients (*Hong* et al., 2011). Genetic biomarkers have shown

similar importance for Crohn's disease with CARD15/NOD2 combined with bacterial infection shown to increase disease risk (*Vaiopoulou* et al., 2012). While not specific to post-infection IBD, novel disease biomarkers such as IL-6, IL-23, ASCA and pANCA have expanded our understanding of disease patho-etiology (*Yau* et al., 2013). While omics-based studies of samples obtained from the DoD serum repository are not readily available in the peer-reviewed literature, the accessibility of these serum samples combined with new technologies and platforms with which to conduct novel assays has the opportunity to enhance our understanding of the inter-relational association between the human genome, proteome and metabolome and further our understanding of the pathophysiology of the sequelae of acute enteric infection.

Importantly, these samples are not without limitations which have been highlighted previously and include storage temperature (-30°C), available aliquot volume (50 cc), number of freeze/thaw cycles and potential gaps in the cold chain from specimen collection to storage (*Moore* et al., 2010). Furthermore, from a research perspective, the temporality of specimen collection around an event(s) of interest may be sub-optimal. For example, we recently conducted a sero-epidemiologic study which required the last serum sample prior to initiating basic training during which an exposure of interest (norovirus outbreak) had occurred. The mean time from sample collection until the exposure of interest was approximately 6 months. Despite this (and other) limitation, one of the most unique attributes of the DODSR is its longitudinal nature which enables measurements of seroconversion and/or the development of novel biomarkers not present in previous (or subsequent) samples.

OTHER LARGE EPIDEMIOLOGIC DATABASES

As alluded to previously, the US military is not the sole source of medical encounter data. Country-wide systems exists globally as do those that are specific to given managed care organizations or those established to enable long-term cohort studies. For example, Norway has a universal healthcare system in which all citizens have unrestricted access to healthcare and in which medical encounters at hospital-based clinics are documented (*Lofthus* et al., 2005). In 2012, a total of 1.7 million patients received care on at least one occasion at one of the public hospitals, approximately 500K of which were associated with an in-patient stay (https://www.ssb.no/en/pasient). A subset of these data are querable online (https://www.ssb.no/en/helse).

This registry is inherently encrypted with no link to personal identification information. However, encrypted data can be linked to other sources of data utilizing identifiable information (*Bakken* et al., 2012). While an invaluable resource to researchers, these data are not without limitations. Specifically, linking data for single individuals across multiple years is difficult as identifiable information is often replaced with a unique identifier specific to the year in which care was sought (*Lofthus* et al., 2005). Additionally, for specific years, available diagnoses may be limited; furthermore, it is unclear if procedure codes are incorporated into the available data (*Lofthus* et al., 2005). Similar systems exists in other Nordic countries including Den-

mark (*Andersen* et al., 1999), Finland (*Sund*, 2012) and Sweden (*Ludvigsson* et al., 2011) with recent efforts to combine registries across countries to further expand the available study population (*Furu* et al., 2010; *Olsen* et al., 2010).

In addition to country-wide registries, other population-based cohort studies have been prospectively designed to allow research among a cross-section of populations. One specific study to note is the National Health and Nutrition Examination Study (NHANES). This study, initiated in the early 1960s, is designed as a survey-based cohort study on specific populations and health-related topics. Since 1999, approximately 5,000 persons are surveyed annually for demographic, socioeconomic, dietary and other health-related information as well as medical examinations to include dental, psychological assessments and laboratory tests. Data obtained from NHANES have been utilized in a multitude of research studies involving infectious and non-infectious diseases (*Tuteja* et al., 2008). In addition to epidemiologic data, NHANES also maintains a biorepository with plasma, serum and purified DNA that can be linked to health-related data. However, these samples are not collected on a longitudinal basis as are the DODSR samples. Furthermore, these data are inherently different than those described in the US military system and the Nordic countries in that they are obtained for the sole purpose of research while the medical encounter data are collected as part of routine medical care. Complete understanding of these differences, some of which are outlined in Table 3, is key to ensuring appropriate data interpretation and extrapolation to other populations.

CONCLUSION

The US DOD medical encounter databases and serum repository are invaluable assets with the ability to modify our understanding of many disease processes. Historically, well-designed epidemiologic studies have been at the fore of linking exposures with outcomes and have enabled estimates of outcome risk, identifying host- and pathogen-specific risk factors and understanding the timing from exposure to outcome. Studies of the post-infectious sequelae of enteric diseases are no different as highlighted by several systematic reviews on the epidemiologic evidence (*Halvorson* et al., 2006; *Thabane* et al., 2007; *Poropatich* et al., 2010; *Deising* et al., 2013; *Pike* et al., 2013; *Porter* et al., 2013b). While these studies are have enhanced our appreciation of the acute/chronic disease link, additional research is needed. The OMICs revolution has paved the way for the utilization of the DOD SR linked with relevant medical encounter data to further enhance our understanding of disease patho-etiology. Importantly, these studies must be accompanied by improved mechanistic preclinical studies, which to date suggest multifactorial biological processes leading to PI-FGD. Together these studies have the potential to begin unravelling the complexity of post-IGE sequelae and revolutionize our understanding of the long-term impacts of acute infection.

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AN ENTERIC TRIANGLE: PROTOZOAN INFECTIONS, LINKS TO ENVIRONMENTAL ENTEROPATHY AND THE POTENTIAL INFLUENCE OF THE INTESTINAL BACTERIAL MICROBIOME ON THIS INTERACTION AND OVERALL HEALTH IN THE DEVELOPING WORLD

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SUMMARY

Lasting changes to the host intestinal mucosal surface may be caused by faecal enteropathogens such as *Entamoeba histolytica,* one of the causes of host diarrhoea, which when it repeatedly and frequently occurs instigates a subclinical condition, environmental enteropathy (EE), characterized by blunting of intestinal villi and intestinal inflammation. Amoebiasis and EE occur in the context of the host's intestinal bacterial microbiome and the persistent changes driven by enteropathogens could be modulated by the composition of these intestinal bacterial communities. In this work, we will explore the long term changes to the host by protozoan infections such as *E. histolytica* in man and model organisms such as mice, how the intestinal bacterial microbiota and probiotic organisms might influence overall health and infections with these protozoa, and finally the role these interactions might have in health and wellness in the developing world where enteric diarrheal disease is endemic. We hypothesize that interactions between the host's immune system, protozoan infections and the intestinal microbiome might influence EE and in turn vaccine failure and perhaps exacerbate nutritional deficits, increasing the risk of malnutrition in a food insecure household.

ENTERIC INFECTIONS AND ENVIRONMENTAL ENTEROPATHY

For many years our laboratory has tracked the medical histories of a large cohort of children living in the Mirpur slums of Dhaka, Bangladesh. Here, colonization with parasites is a major cause of diarrheal illness, which is a significant source of morbidity and mortality in the developing world. Colonization with *Giardia lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica* likely underlie 58 million cases of childhood diarrhoea (*den Hartog* et al., 2013). Furthermore, diarrheal disease is a primary cause of mortality in children less than five years of age in these nations and accounts for nearly two million deaths annually (WHO). However, in tracking these children, it has also become apparent that these infections in early life may have lasting influences on health, particularly growth, later nutritional status, susceptibility to further infection and perhaps even influence vaccine

efficacy (*Korpe* and *Petri*, 2012; *Mondal* et al., 2012; *Kotloff* et al., 2013). This cohort, and many others in developing tropical nations, has a high incidence of environmental enteropathy (EE), a subclinical condition caused by constant faecal–oral contamination that is characterized by blunting of intestinal villi and other histopathological abnormalities of the intestine such as increased crypt lengthening and intestinal inflammation, including lymphocyte and monocyte infiltration. EE is believed to significantly contribute to malnutrition and stunting in these populations by preventing normal development of the intestine. Much nutrient absorption occurs at the tips of intestinal villi and this is not possible in severely damaged and inflamed intestines (*Fagundes-Neto* et al., 1984, 1994; *Korpe* and *Petri*, 2012). Our laboratory has shown a correlation between *E. histolytica* driven childhood diarrhoea and later stunting (*Mondal* et al., 2006). In turn, malnourished children have higher rates of infection by *E. histolytica* and *Cryptosporidium* (*Korpe* and *Petri*, 2012; *den Hartog* et al., 2013a). Thus repeated colonization with enteropathogens, including these protozoa, likely instigates a feedback loop of poor nutrition and stunting. While E. *histolytica* and *Cryptosporidium* do influence diarrheal diseases, they have not specifically been shown to cause EE in man. However, *E. histolytica* has been shown to instigate many of the characteristics of EE in murine models of amoebiasis (*Houpt* et al., 2002a; *Mondal* et al. 2012; *Verkerke* et al. 2012; *den Hartog* et al., 2013a). *Houpt* et al. (2002b) have shown, for instance, that injection of *E. histolytica* trophozoites into C3H/HeJ mice leads to chronic caecal infection in the majority of mice. They demonstrated that infected mice had histological changes including crypt hyperplasia, epithelial ulceration, and submucosal inflammatory infiltration that were reminiscent not only of human amoebiasis but also human EE. Thus, we have hypothesized that repeated infections with *E. histolytica* and other protozoans in children might contribute to EE by eliciting long lasting changes to their intestinal mucosa and to the infiltrating populations of immune cells present. Our laboratory is currently exploring how this organism and other enteropathogens might influence the long term sequelae of EE. However, while entertaining this notion, it is useful to understand the context of intestinal *E. histolytica* infection.

Initial infection occurs after ingestion of faecally contaminated water or food containing *E. histolytica* cysts which then undergo excystation in the lumen of the small intestine. The amoeba trophozoite then feeds on resident bacteria and possibly the intestinal epithelium and in rare cases, it may cause systemic amoebiasis by invading the intestinal mucosa and traveling to the blood stream, liver or brain (*Petri* and *Singh*, 1999; *Haque* et al., 2003; *Verkerke* et al., 2012). However the intestinal lumen is densely populated by a community of bacteria that may have a significant influence on the host's immune response at baseline, and during amoeba infection, as well as the virulence of the amoeba itself (*Mirelman* et al., 1983; *Noverr* and *Huffnagle*, 2004; *Frederick* and *Petri*, 2005; *Maslowski* and *Mackay*, 2010; *Cho* and *Blaser*, 2012). These interactions may in turn also influence the hosts nutritional status and ability to mount a successful immune response to later infections (*Mondal* et al., 2012). Thus when considering the persistent effects of protozoan infection and EE, it is also pertinent to examine the contribution of the intestinal bacterial microbiota to human health.

INFLUENCE OF COMMENSAL BACTERIA ON MAN AND MICE

The normal flora of the human gastrointestinal tract is a large, complex community of bacteria that is composed of at least several hundred species and consists of 10^{12} bacteria per gram of large bowel content. There are far more bacterial cells than there are eukaryotic cells in the human body and these organisms form a symbiosis that influences many aspects of human physiology including the composition of the metabolome, which is the complete set of body wide small-molecule metabolites including hormones, chemokines, cytokines and other signalling molecules (*Arumugam* et al., 2011; *Siezen* and *Kleerebezem*, 2011; *Cho* and *Blaser*, 2012). However, understanding the mechanisms by which the human microbiota influences the metabolome, immune system and nutrition is an emerging science. In studying the composition of the microbiota it has become clear that, while there is some stability on a phyla and genera level within human populations, there is much variability in the milieu of specific bacterial species between individual humans. Enterotypes are classification units based on the bacterial composition of the gut microbiome and have been utilized to describe these shared groups of bacterial phyla and genera in humans and to study correlations with diseases that might be influenced by the microbiome. These enterotypes are independent of ethnic background but appear to be influenced to some degree by the composition of diet (*Arumugam* et al., 2011; *Wu* et al., 2011). How many enterotypes are present is yet to be determined, however there are at least three (*Arumugam* et al., 2011). They are defined largely by the variation in the levels of three genera, *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3) but have contributions from other genera (*Arumugam* et al., 2011). These enterotypes can further be represented by analysing clusters of bacterial families and this analysis method shows that these first two enterotypes are primarily characterized by the presence of *Bacteroides* and *Prevotella*, whereas the third cluster is mostly characterized by related groups of the family *Clostridiaceae*, and unclassified *Lachnospiraceae*. Several studies have suggested that specific enterotypes might be associated with inflammatory responses in the intestine and deregulation of normal metabolic controls. Specifically, intestinal autoimmunity, colitis, and obesity, have been associated with enterotypes 2 and 3 above, however, which specific organisms might influence these diseases is still a point of contention (*Arumugam* et al., 2011; *Giongo* et al., 2011; *Siezen* and *Kleerebezem*, 2011). Studies of type 1 diabetes have further demonstrated that there are distinct differences in the microbiome of infants that developed the disease compared to those that did not, with decreased microbial diversity and a higher proportion of the *Bacteroidetes* phyla compared to *Firmicutes* in the group with the disease (*Giongo* et al., 2011). It has also recently been shown that members of the *Bacteroidetes* and *Firmicutes* are heavily involved in metabolism of complex carbohydrates in the intestine (*Flint* et al., 2012). Thus, the composition of the intestinal bacterial microbiota might significantly influence intestinal inflammation, metabolism and nutrition in man. However, the bulk of these studies have taken place in the developed world. Changes in the microbiome that might underlie the development of EE are not well described.

Mouse models have demonstrated

more directly that the microbiome can have a significant influence on the function, structure and composition of the immune system and intestine as well as malnutrition. Gnotobiotic, or germ free, mice have structural defects in peyers patch formation in the small intestine, decreased or absent IgA production, few intra epithelial lymphocytes and a systemic defect in T regulatory cell induction, all of which is reversed once the animals are recolonized with a normal murine faecal microbiota (*Umesaki* and *Setoyama*, 2000; *Jiang* et al., 2004). Components of the intestinal microbiota such as *Lactobacillus casei* and bacteria present on vegetables and in the soil such as *Lactobacillus plantarum* and *Bifidobacterium bifidum* can also drive Th1 and T regulatory helper cell induction which may be antagonistic to inflammatory Th2 helper cell driven pathologies such as asthma and eczema and have been shown to be protective in colitis models (*Feleszko* et al., 2007; *Schwarzer* et al., 2011). The microbiome can also have a significant influence on nutritional status in murine models. Smith et al. have recently shown that kwashiorkor, a type of severe acute malnutrition, may be influenced by the gut microbiome. They observed that when the microbiome from malnourished versus healthy Malawian twins was transplanted into gnotobiotic mice the kwashiorkor microbiome instigated marked weight loss in recipient mice, as well as altered the metabolism of carbohydrates and amino acids, when compared to the microbiome from the healthy twins (*Smith* et al., 2013).

Another striking example of a specific component of the bacterial microbiota influencing physiology and the immune responses is the role segmented filamentous bacteria (SFB) play in immune maturation in the intestine of mice. SFB are commensal, uncultivable, obligate gut tropic, members of the *Clostridiaceae* that have been shown to drive potent IgA induction and Th17 helper cell induction in the intestine (*Davis* and *Savage*, 1974; *Ivanov II* et al., 2008; *Gaboriau-Routhiau* et al., 2009; *Kuwahara* et al., 2011). Colonization with SFB has been shown to exacerbate or influence a number of intestinal and extra intestinal models of human disease in mice, including colitis, autoimmune myelitis, arthritis and type 1 diabetes via Th17 cell induction (*Nutsch* and *Hsieh*, 2012). The bacteria, while uncultivable, are well described both genetically and morphologically in mice and a number of studies have suggested that they may be present in humans as well (*Davis* and *Savage*, 1974; *Child* et al., 2006; *Kuwahara* et al., 2011; *Yin* et al., 2012; *Caselli* et al., 2013; *Jonsson*, 2013). SFB colonization also strongly influences the composition of the intestinal microbiota through its unique kinetics of colonization and influence on the immune system. For a short time after weaning, the bacteria becomes the dominate species within the murine gut and has been shown to outcompete enteropathic bacteria species (*Chase* and *Erlandsen*, 1976; *Heczko* et al., 2000; *Stepankova* et al., 2007; *Kuwahara* et al., 2011). The ability of SFB, a component of the mouse, and possibly human, normal flora, to strongly influence the basic makeup of the immune system as well as colonization by other bacterial species illustrates the interconnectedness of individual bacterial species, the broader microbiome and health. However the influence of the intestinal bacterial microbiome is by no means limited to other bacterial species and may have profound influences on protozoa virulence and colonization and in turn amoebiasis, diarrheal disease and EE.

INTERACTIONS BETWEEN BACTERIA AND PROTOZOA

As mentioned previously, many protozoa inhabit the intestine for a significant portion of their lives, and in doing so interact intimately with other organisms present there (*Mirelman* et al., 1983). *Berrilli* et al. (2012) have recently highlighted some of the interactions between intestinal microbial communities, probiotics and bacterial pathogens and many types of parasite infections. Protozoa, including *E. histolytica* , are both influenced by the presence of other enteropathic and probiotic bacteria and influence the composition of the broader intestinal microbiota. *Galván-Moroyoqui* et al. (2008) have explored the effect of co-culture of trophozoites from *E. histolytica* and *E. dispar* with the enteropathogenic bacteria strains *Escherichia coli* (ETEC), *Shigella dysenteriae* and a commensal *Escherichia coli* on epithelial cell monolayers. In doing this they determined that phagocytosis of pathogenic bacteria augmented the cytopathic effect of *E. histolytica* on the cell monolayer as well as increased expression of the adherence lectin, Gal/GalNAc, on the amoeba's surface. Thus, interactions with enteropathic bacteria in humans might serve to increase the virulence of *E. histolytica* during amoebiasis. *E. histolytica* colonization in turn also influences the composition of the microbiome. *Verma* et al. (2012) have shown that during amoebiasis there is a significant decrease in absolute quantification of *Bacteroides*, *Clostridium coccoides*, *Clostridium leptum, Lactobacillus* and *Campylobacter* and an increase in *Bifdobacterium,* while there was no change in *Ruminococcus* compared to healthy patients. These works suggest that some of the pathology that results during amoebiasis might be driven by a disregulated microbiome or cross talk between enteropathic bacteria and protozoa and the intestinal immune system, particularly the intestinal epithelium. In fact, there are several works suggesting that probiotics, particularly *Lactobacillus* species, might be protective in the context of protozoan infections (*Preidis* et al., 2011; *Travers* et al., 2011). Thus a decrease in protective, commensal, lactobacillus species during *E. histolytica* infection might influence the severity of disease. One murine study has shown that daily administration of *Lactobacillus acidophilus*, a bacteria common in yogurt, for one week in *Giardia lamblia* infected BALB/c mice significantly reduced *G. lamblia* infection burden in those mice. Disease severity was also significantly decreased. Histological analysis of the intestine showed that probiotic administration protected mice against parasite induced mucosal damage and decreased intestinal villous atrophy (*Shukla* et al., 2010). Thus probiotic interventions might provide an attractive avenue to decrease intestinal damage in populations in which repeated intestinal protozoal infections occur (*Shukla* et al., 2010). Further understanding of how protozoa influence and are influenced by the intestinal microbiome, enteropathogens and probiotics would thus be informative in designing microbiome based interventions for diseases such as EE and malnutrition.

ENTERIC INFECTIONS, HEALTH AND EXPLORATION OF THE MICROBIOME IN THE DEVELOPING WORLD

The Petri laboratory has long sought to find connections between enteric infections, diarrheal disease and diseases with persistent effects such as EE and

malnutrition in order to develop targeted interventions that might decrease the burden of disease in the developing world. In our field site in Mipur, Dhaka, we followed children for the first year of life with every-other-day home visits and surveyed enteropathogens in diarrheal and monthly surveillance stool samples. We also measured intestinal barrier function by endocab antibodies, which correlate with translocation of bacterial LPS into blood, and measured nutritional status and stunting by anthropometry. In this study we found that diarrhoea co-occurred with infections caused by several organisms including enteric protozoa (amoebiasis, cryptosporidiosis, and giardiasis), rotavirus, astrovirus, and enterotoxigenic *Escherichia coli* (ETEC). We also observed that malnutrition was present in 16.3% of children at birth and 42.4% at 12 months of age and that children that were malnourished at birth had increased *Entamoeba histolytica*, *Cryptosporidium*, and ETEC infections as well as more severe diarrhoea. The children who became malnourished at 12 months of age were also much more likely to have prolonged diarrhoea and intestinal barrier dysfunction, a mother without education, and low family expenditure (*Korpe* and *Petri*, 2012; *Mondal* et al., 2012; *den Hartog* et al., 2013b).

Our laboratory, along with many other collaborators, has also recently begun exploring links between malnutrition, EE and oral polio vaccine failure. This is particularly important as vaccines for polio (OPV) and rotavirus are far less effective in poor children in the developing world and the underlying cause for this failure is largely unknown. Based on our previous experience we thus hypothesized that failure of oral vaccines such as OPV might be due to EE and be driven by inflammation from endotoxin exposure. We tested this hypothesis in the Mirpur cohort of children by measuring responses to oral poliovirus vaccine in children who received a minimum of three doses of OPV by age 6 months. We observed that diminished antibody responses to OPV were associated with malnutrition, increased serum endocab levels, and shorter breastfeeding duration. We also examined potential immune mechanisms that might underlie vaccine failure in a smaller subset of these children and found that children with OPV failure exhibited globally reduced cellular responsiveness to a range of cytokine stimulations, as well as elevated pro-inflammatory cytokine expression. These data indicated that oral vaccine failure in these children is influenced by a combination of malnutrition, gut barrier dysfunction in early childhood, and is associated phenotype (unpublished data/abstract). These studies further highlighted the complex interrelationship of malnutrition, protozoan and enteric infections, diarrhoea and vaccine failure in infants in low-income settings and the persistent effects of these problems. Thus, identification of probiotics, or particular enterotypes, that are protective during protozoan infection might help mitigate destruction of the intestinal barrier and significantly improve health outcomes in these populations.

Unfortunately, the contribution, if any, of specific components of the intestinal bacterial microbiota to EE and vaccine failure in the developing world is not currently well described. However given the profound influence that intestinal bacteria such as *Lactobacillus*, *Bifidobacterium,* SFB, and enteropathic E. *coli* (ETEC) can have on host immunity, parasite burden and virulence in mice and man, and the relative inexpensiveness of probiotic interven-

tions, it is certainly an area of study that should be pursued. Interestingly, one such recent study has shown a link between the composition of the bacterial microbiota and the effectiveness of an oral typhoid vaccine in a small group of individuals. In this study the composition of the microbiome did not influence the ability of responders to mount an immune response to oral typhoid vaccination, but those with a more complex microbiota mounted a more robust, multiphasic IFN-y response to oral vaccination. Many different organisms represented by operational taxonomic units (OTUs) were found to differ between individuals displaying robust responses and those with late, less robust responses. However the vast majority of these OTUs were classified within the order *Clostridiales* (*Eloe-Fadrosh* et al., 2013). Another recent paper has shown that the presence of some commensal *Clostridium* related species is decreased during autoimmune colitis in children (*Michail* et al., 2012). Thus the composition of the microbiota, and *Clostridia* related organisms, or commensals that induce immune responses similar to these bacteria, may very well be an important factor influencing homeostasis of the intestine and the success of vaccination in the developing world.

FINAL THOUGHTS

Infection with enteropathogens such as the protozoa *Entamoeba histolytica* likely contributes to the development of environmental enteropathy and induces lasting effects on the intestinal mucosa that may negatively impact nutritional outcomes and vaccine success in millions of children each year. These pathogenic organisms live in the context of the intestinal bacteria microbiota and interactions with these species may significantly influence the virulence, and infectivity, of those protozoa as well as the host's ability to mount protective responses against future infections. A better understanding of the interactions between these organisms and the intestinal microbiota, which might include probiotics commonly found in many foods, may lead to cost effective treatments that could significantly decrease the burden of enteropathogens in the developed world.

LITERATURE

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COELIAC DISEASE

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SUMMARY

Coeliac disease, causes chronic inflammation of the proximal intestinal, is an increasingly common disorder impacting health and nutrition. It is also a model disease straddling gut epithelial responses and systemic autoimmunity. It is a disease about which we know much, but also presents mysteries. Why does it occur at any age and why is it increasing. Why do most individuals who carry the genetic predisposition and eat gluten but don't get the disease? It differs substantially from the more classic inflammatory bowel diseases of Crohn's disease and ulcerative colitis. Those latter diseases primarily affect the distal small intestine or colon and rarely the upper intestine. Furthermore, coeliac disease is a response to dietary glutens whereas the microbiota play the dominant role for triggering IBD. The colon and distal small intestine are filled with large numbers of bacteria and other microbiota, the proximal small intestine by contrast is almost sterile. It does contain some microbiota and is a target for pathogenic invasive organisms. The other difference is that the proximal small intestine is dealing primarily with ingesta, not resident microbiota. Indeed, there are further differences between the colon and the small intestine. The colon, which contains the largest resident biomass of our ecology, has built up effective barriers to the microbiota which when breached can result in devastating inflammation. By contrast, the small intestine is a far more permeable organ with its primary role being digestion and absorption. There is more cross talk between the luminal contents and the mucosal immune system. Hence, regulation of the inflammatory response within the small intestine is even more crucial to permit normal digestive functioning to occur than in the colon. The microbiota and coeliac disease may intersect in several ways. The microbiota may be inherently different in children/adults at risk for coeliac disease. Furthermore, pathogenic infections may perturb the intestinal milieu to an extent that individuals genetically prone to coeliac disease will lose tolerance to gluten. The gluten-free diet may change the microbiota that may affect the host. This review summarizes what is known about the aetiology, epidemiology, diagnosis, treatment, as well as the microbiology of coeliac disease.

INTRODUCTION

Coeliac disease is a chronic inflammatory condition predominately affecting the proximal small intestine (*Murray* et al., 2008). It occurs in people who have

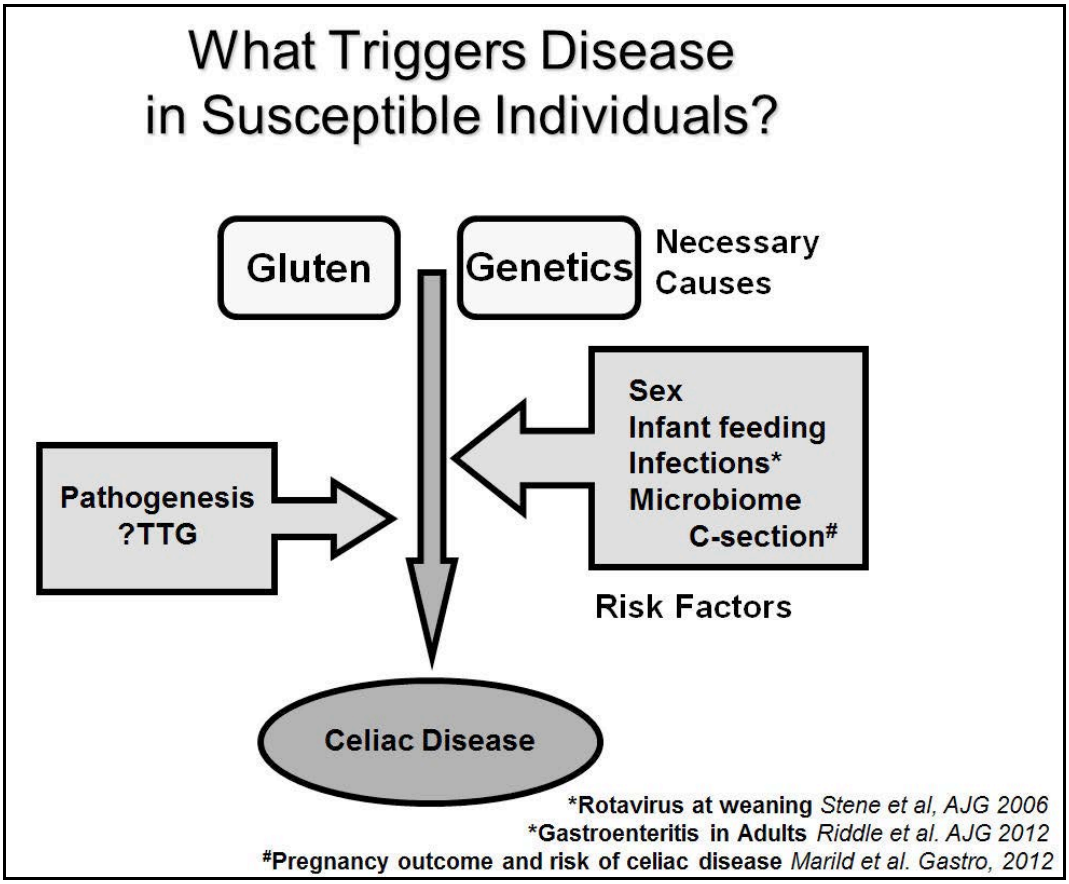

Figure 1: Coeliac disease has two required co-factors: 1) genetic predisposition, and 2) environmental factors, primarily the ingestion of gluten.

a specific genetic type, in particular the MHC class 2 gene pairs encoding the HLA molecules DQ2 or DQ8. Individuals must be exposed to dietary gluten for the disease to occur and, in most patients, the disease regresses and eventually heals when gluten is removed from the diet (*Murray* et al., 2004). The disease mostly affects Caucasians; however, this broadly affects many ethnic and racial groups across Europe, the Middle East, North Africa, India, and the Near East. It also affects Caucasians living in other areas, such as North America, South America, and Australia (*Rubio-Tapia* and *Murray,* 2007). The damage to the intestine produces inflammation as well as impacts the digestive and secretory function of the intestine. This leads to a wide variety of symptoms that can impact digestive function and also impact extra-intestinal sites (*Reilly* et al., 2012). While the rate of diagnosis of coeliac disease has increased dramatically (*Ludvigsson* et al., 2013), this rate of diagnosis probably still greatly underestimates the proportion of patients affected, with most patients remaining undiagnosed (*Rubio-Tapia* et al., 2012). The treatment of coeliac disease is based on avoidance of dietary gluten and, when diagnosed and treated in childhood, usually healing is prompt and complete. However, patients diagnosed as adults heal much less com-

monly and often take much longer to heal (*Lebwohl* et al., 2013). This review will focus on what is known about the triggering factors for coeliac disease, the impact of the microbiome on coeliac disease, as well as the potential impact of gluten on the microbiome and gut function.

AETIOLOGY

Coeliac disease affects individuals who have acquired genetic predisposition as well as ingesting gluten, the storage protein from wheat, barley and rye (Figure 1). If all that was required for coeliac disease to occur was the carriage of the appropriate risk factors as well as the ingestion of sufficient gluten on a daily basis, then fully 30% of the Caucasian population would develop the disease. However, between 1-2% of Caucasian population developed the disease, indicating that there must be other factors responsible for triggering the disease (*Walker* et al., 2010).

Genetic Basis of the Disease

Coeliac disease is associated primarily with the HLA genes initially thought to be Class 1 genes A1B8, but subsequently identified to be the HLA molecules DQ2 or DQ8. These are encoded by DQA1'05:DQB1'02XX and DQA1'03XXDQB1'0302, respectively. The carriage of one or other gene pairs is essential for the development of disease; however, this is not sufficient for the disease to occur. The known HLA genes contribute probably no more than 50% of the genetic familial risk for coeliac disease (*Bevan* et al., 1999). Siblings who carry the HLA type have an increased risk of coeliac disease compared to those who do not (*Murray* et al., 2007). Several genome-wide association studies (GWAS studies), now incorporating many thousands of patients and controls, have identified many other gene loci that are associated with risk of disease (*Garner* et al., 2009). These other loci are close to genes that regulate immune response and inflammation predominately (*Dubois* et al., 2010). Some of these genes may regulate immune responsiveness to microbial stimulation of the innate system. However, the attributable risk of coeliac disease to these other loci is relatively low, probably contributing no more than 10-15% of additional genetic risks. It is likely that coeliac disease is the result of the major HLA susceptibility genes combined with several other common genetic polymorphisms that increase immune responsiveness. Many of these genes associated with these loci are common to other inflammatory conditions, such as rheumatoid arthritis, Crohn's disease and type 1 diabetes, though there are some interesting genes that are negatively associated with particularly type 1 diabetes. The precise risk within family members varies depending on how close genetically the proband is to the patient. Monozygous twins have the highest concordance rate of 80%. Note, this is not 100% as there must be some environmental triggering differences between even identical twins. Siblings who share HLA risk factors have a greater risk than parents or children of patients with coeliac disease (*Book* et al., 2003). Second-degree relatives likely have a lower risk of coeliac disease (*Fasano* et al., 2003).

Dietary Gluten

The primary and required environmental factor for coeliac disease is dietary gluten. Gluten in this context represents the stored proteins from wheat, barley,

and rye, often given the term prolamines. These storage proteins provide the nitrogen store needed for seed germination. The original work identifying the protein fraction of these grains as being deleterious was performed in a series of challenge and withdrawal studies done in children in the Netherlands during and subsequent to the 2nd World War (*Dicke,* 1951). It was these seminal observations that laid the groundwork for the modern treatment of coeliac disease. Since that time, further work was done to identify the most immunogenic fragments of gluten, and these fragments are characterized by a large percentage of amino acids that glutamines and prolines. Particular motifs characterized by sequences of glutamines interspersed with prolines appear to be particularly immunogenic (*Jabri* and *Sollid,* 2006). In addition to their immunogenicity, is also their resistance of endopeptidase activity within the intestine. Within the human intestine, endopeptidases failed to break down some of the most immunogenic fragments of the gluten-derived proteins, particularly a gliadin. A specific 33-mer peptide or a gliadin is especially resistant to digestion. This same 33-mer peptide contains within it 3 very immunogenic peptides that, when each is complexed with the T cell, are presented to the T cells within the small intestine by the HLA molecule on the antigen-presenting cells, produce a very potent inflammatory response. The binding characteristics of these peptides can be greatly enhanced by deamidation of specific glutamine peptides, particularly glutamine amino acids (*Molbert* et al., 1998). Deamidation occurs in response to transglutaminase enzyme effects. Transglutaminases are enzymes present within the intestine and elsewhere. They are expressed constitutively but are especially expressed in the context of inflammation. There are also microbial transglutaminases present within the gut lumen and some have been used in food processing. The transglutaminases act to remove the amine side chain specific glutamines in the gliadin peptides. When these amine groups are removed, so-called deamidation, it renders the peptide far higher binding affinity to the class 2 HLA molecule binding site (*Shan* et al., 2002). By so binding, it then results in dramatically increased affinity to the T cells, which then produce both proliferate and trigger an inflammatory cascade within the intestine. Further complicating matters is the very rich genetic material of these grains. Wheat used for bread is hexaploid and has multiple repeat regions where the genes for these storage proteins. Hence, there are many (probably 50-100) epitopes within each wheat protein that can produce coeliac-responses from T cells derived from the small intestine. Children seem to respond more to native gliadin peptides than do adults and, in general, the deamidated peptides produce a much more potent effect than do the native, non-deamidated peptides. The peptides operant in DQ2 positive coeliacs differ from those in DQ8 positive coeliacs.

Triggers

It has also become clear from epidemiologic studies that coeliac disease can occur at any age (*Lohi* et al., 2007). Patients, in particular children, at genetic risk don't necessarily develop immune responses to gluten immediately. This may occur at discontinuous times over the 1st years of life. In addition, patients with type 1 diabetes who are often screened regularly for coeliac disease may develop coeliac disease later at the approximate age of 10 or 11, despite having been negative for some years previously despite being on a gluten-containing diet. Most intriguingly, el-

derly patients who were negative for coeliac disease may subsequently turn positively (*Norris* et al., 2005; *Vilppula* et al., 2009). The fact that patients, who apparently have tolerated gluten, then can lose tolerance to gluten and develop coeliac disease suggests that there may be environmental triggers. These factors may start at birth. Two studies have suggested that children born by Caesarean section are at increased risk of developing coeliac disease in childhood (*Marild* et al., 2012; *Decker* et al., 2010). Children born in the summer months who will likely wean in the winter are more prone to the disease (*Ivarsson* et al., 2003). While not studied in this largely epidemiologic study, it is well known that route of delivery affects the gut microbiota and even humoral immunity (*Huurre* et al., 2008).

In addition, coeliac disease may also be associated with intrauterine growth retardation and neonatal infections (*Sandberg-Bennich* et al., 2002). After birth, further exposures may be important for the occurrence of coeliac disease; in particular, many studies on breast-feeding have suggested that the failure of overlap between breast-feeding and the introduction of gluten might increase the risk of coeliac disease in childhood. Indeed, the now well-described Swedish epidemic of infant coeliac disease was ascribed to a combination of an abrupt termination of breast-feeding with a dramatic increased concentration of gluten in follow-on formula (*Ivarsson* et al., 2002). Recurrent rotavirus infection alone was initially thought to be an independent risk factor for coeliac disease occurring in infancy. However, subsequent work has demonstrated that perhaps it is recurrent rotavirus infection coinciding with cereal introduction that truly increases the risk for developing coeliac disease (*Stene* et al*.,* 2006).

Work has been done showing that there are changes in the microbiota in coeliac disease. For example, there are changes in the population of microbiota on the duodenal mucosa. One study has suggested increased bacterial adherence to the duodenal mucosa of rod-like bacteria in coeliac disease (*Ou* et al*.,* 2009). It is not known if this phenomenon is primary or secondary to the damaged epithelium and loss of mucus that occurs in the context of coeliac disease. Reductions in the proportions of Lactobacillus and Bifidobacterium species have also been reported in the faecal samples from treated coeliac patients (*Nistal* et al., 2012). Another group has shown that there are differences in Bacteroides species and pathogenic features in coeliac patients as compared to healthy controls (*Sanchez* et al., 2012) based on faecal samples.

These results seem to be contradictory, but there are many explanations as to why they are not. The first explanation is that the majority of earlier identification analyses were based on culture screening methods; whereas today, the majority of studies are based on the sequencing of specific regions of the 16srRNA gene. The second explanation is that some studies evaluate duodenal biopsies, while others evaluate faecal samples, both of which would be sampling different niches that different bacteria can survive. Thus, all of these studies have provided valuable information as to how the composition of the intestinal microbiome of coeliac patients are different from healthy controls; the next step is to determine if any of these changes are causative for or consequential to intestinal damage.

Previous studies have suggested that two different types of situations could trigger intestinal inflammation in coeliac disease. The one type would be decreased levels of anti-inflammatory

bacteria such as Lactobacillus and Bifidobacterium, the other, an increase in inflammation promoting bacteria. In one study, *Bacteroides fragilis* was increased in both untreated and treated coeliac patients, and the *Bacteroides fragilis* associated virulence factors, bft and MPII , were also increased in the coeliac patients as compared to healthy controls (*Nistal* et al., 2012). All of these studies demonstrate then, that dysbiosis of the intestinal microbiota is present in coeliac patients and that this may indeed be causative as opposed to consequential. Potentially then, a combination of probiotics and targeted removal of specific intestinal bacterial species would greatly improve the health of coeliac patients.

There also appears to be differences in immunoglobulin binding of bacteria within the gut. A recent paper suggested that in patients with coeliac disease, both treated and untreated, there is reduction of immunoglobulin-bound bacteria within the intestine as compared to normal. It is interesting that patients with selective IgA deficiency, who do not secrete any IgA, are at greatly increased risk of coeliac disease, with about 10% of patients developing coeliac disease. Recent cases and work by Naval Medical Research in the Department of Defense has suggested that coeliac disease may be more likely to be diagnosed after an infectious gastroenteritis event (*Verdu* et al*.,* 2007; *Welander* et al., 2010; *Riddle* et al., 2012). It can be speculated that these infectious events may result in temporary inflammation activation of stress responses within the small intestinal mucosa, stimulate the innate immune system, which will then drive the adaptive response. Excess numbers of bacteria can also be seen in the duodenum of patients with unresponsive coeliac disease or even before treatment (*Tursi* et al., 2003). Also, co-occurrence of an inflammatory insult, such as rotavirus in childhood, along with the introduction of a novel peptide, such as gluten, especially one to which the child is genetically susceptible to react to, may result in activation of dendritic cells and presenting cells towards a pro-inflammatory response, thereby skewing naïve T cells to become effector T cells producing inflammation as opposed to T-reg cells. It is also possible that in patients who have already established tolerance to gluten that this tolerance could be broken in the context of severe on-going inflammation. Furthermore, wheat and like cereals contain other substances that can incite innate responses (*Junker* et al. 2012) and indeed do so in ways that simulate an LPS-like effect of bacteria (*Yamazaki* et al., 2008*)*.

Uptake of Gliadin Molecules

There are two primary pathways by which the peptides are thought to be taken up. One is an active pathway transcellularly stimulated by IFN-γ through the aberrant expression of IgA receptors on the surface (*Bethune* et al., 2009). In this circumstance, IgA-bound gliadin is taken up and transported through the enterocyte into the basolateral surface where the gliadin peptide is released and then processed by antigen-presenting cells and presented to the T cells responsive to gliadin. The other pathway is the paracellular pathway. It is has been shown the gluten acutely increases gut permeability (*Lammers* et al., 2008). It causes disruption of intercellular tight junctions and this is likely modulated through the release of a peptide called zonulin, also recently shown to be prehaptoglobin 2 (*Tripathi* et al., 2009). The zonulin appears to cause uncoupling of tight junctions that may allow the transit of some gluten peptides in a paracellular pathway, but also allow access of other antigens, particu-

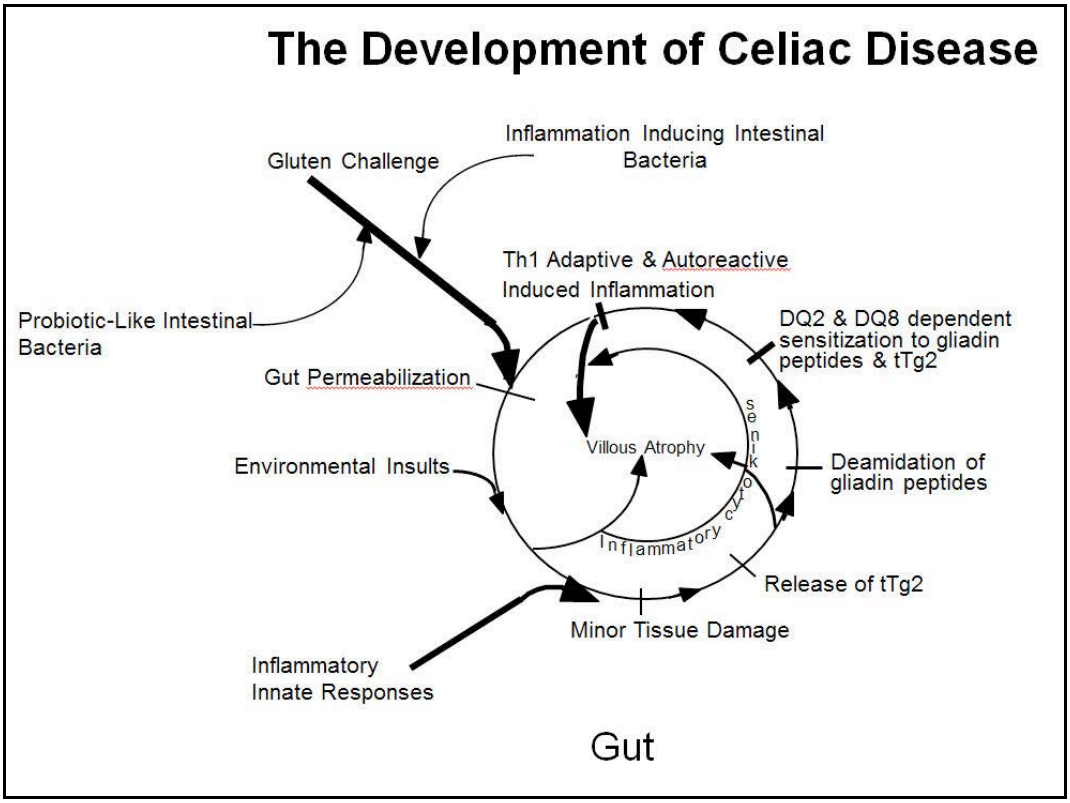

Figure 2: The maelstrom of inflammation that leads to established coeliac disease requires many conspirators affecting both the innate and adaptive parts of the immune system. Reprinted with permission from: Nehra, V., Marietta, E., and Murray, J.A.: Coeliac disease. In: Encyclopedia of human nutrition (Caballero, B., Allen, L., Prentice, A., Eds.). Elsevier, Oxford, 1, 407-418 (2006).

larly bacterial antigens that may excite an innate and amplify an adaptive response to gluten (*Jabri* et al., 2005). Recent clinical trials have shown potential benefit of larazotide acetate, a modulatory inhibitory of gluten's effects on permeability (*Kelly* et al., 2012). This permeability can be exacerbated by the use of an agent that increases inflammation and blocked by larazotide acetate (*Natividad* et al., 2009).

IMMUNOPATHOGENESIS

The immunopathogenesis of coeliac disease involves several cell types within the intestine (*Jabri* et al., 2009). The enterocyte or epithelial cells lining the intestine become distressed and express aberrant class 1 molecules on their surface. These also can express IL15. Intraepithelial lymphocytes are predominately CD8+ T cells, and these cells, while generally thought to have a regulatory effect, in the context of coeliac disease can become cytotoxic and can express NK receptors on their surface (*Meresse* et al., 2004). These NK receptors interacting with the class 1 molecules on the enterocyte can cause enterocyte injury. These intraepithelial enterocytes also respond to IL15, either

produced from the lamina propria or by enterocytes on the surface in response to stress. Key essential ingredients to coeliac disease pathogenesis are gluten-responsive CD4 cells in the lamina propria. These CD4 cells respond to specific gluten peptides presented in the context of class 2 molecules and produce a cascade of cytokines characterized by INFγ and to a lesser extent IL2 and TNF-α. These cytokines drive a cascade of responses that produce both cellular as well as humoral responses (Figure 2). The cellular responses further cascade, drawing in macrophages and activating other cells, including a complement system, neutrophils, eosinophils and mast cells. These combination cells like lead to the destruction of the architecture of the small intestine, resulting in increased thickening or crypt hyperplasia, villous atrophy, disruption of enterocyte function and absorption, increased secretion, and consequent inflammation and malabsorption. Metalloproteases are elaborated, which further alter the architecture. The inflammatory response also includes a potent humoral response and antibodies directed against the deamidated gliadin peptides as well as against tissue transglutaminase are also elaborated within the intestinal mucosa they are secreted, and also increased in circulation. The IgA isotype antibodies are more commonly expressed, particularly directed against the autoantigen tissue transglutaminase. However, for gliadin or deamidated gliadin antibodies, the IgG is equally expressed, suggesting that the primary target for the humoral response is the exogenous antigen and not the autoantigen. Making coeliac disease further different from many autoimmune diseases, with which it shares genetic predisposition, is the observation that the antibodies diminish in quantity once the exogenous antigen is removed. Indeed typically patients who have been on a gluten-free diet for >1 year will become IgA negative. Other antibody responses are often seen. These are antibodies directed at microbial antigens present within the intestine. For example, false positive ASCA antibodies are seen in the context of coeliac disease and usually these diminish or disappear with treatment of the patient with a gluten-free diet, suggesting that these are seen because of injury.

DIAGNOSIS

Coeliac disease is typically detected first by serologic tests. The primary serologic test and most accurate serologic test is the tissue transglutaminase IgA antibody with sensitivities of approximately 95% and specificities of 95% (*Rostom* et al., 2005; *Hadithi* et al., 2007). The performance of this test has been further refined in that patients with extremely positive tests >10 times the upper limit of normal are essentially almost guaranteed to have coeliac disease (*Klapp* et al., 2013). The test which immediately preceded tissue transglutaminase was the endomysial Ig antibody test done by indirect immunofluorescence. This test has a very high specificity, virtually 100% in most laboratories, but a sensitivity that is quite variable, likely due to variability in laboratory methods and interpretations (*Li* et al., 2009). Accompaniment of the tissue transglutaminase IgA test is a measurement of total IgA. This is performed in order to detect those patients with selective IgA deficiency that not only are at greatly increased risk of coeliac disease but for whom the stand-

ard IgA-based tests will be negative even in the setting of coeliac disease (*Rubio-Tapia* et al., 2013). For patients who have known or suspected selective IgA deficiency, deamidated gliadin IgG or tissue transglutaminase IgG tests can be performed, though their sensitivity is not perfect for coeliac disease (*Rashtak* et al., 2008a). Tissue transglutaminase IgG is not useful in the context of normal IgA levels (*Rashtak* et al., 2008a). Antibodies directed against native gliadin antibodies are not of any additional benefit and indeed are fraught with greatly reduced specificity and can negatively impact the accuracy of diagnostic testing when included in diagnostic panels for coeliac disease (*Rubio-Tapia* et al., 2013). All of the antibodies have reduced sensitivity when the patient reduces gluten intake (*Rashtak* et al., 2008b).

Intestinal biopsies showing the classic features of villous atrophy, crypt hyperplasia, and increased intraepithelial lymphocytes are still regarded as the mainstay of diagnosis. Adequate numbers of samples should be taken (*Lebwohl* et al., 2012). Recent guidelines promulgated by the European Society for Pediatric Gastroenterology and Hepatology and Nutrition have suggested that in patients meeting certain very strict criteria, a biopsy may be unnecessary to confirm the diagnosis (*Husby* et al., 2012, 2013; *Rubio-Tapia* et al., 2013).

TREATMENT

Once the disease is detected and confirmed by biopsy, then treatment is usually instituted. Treatment includes a strict, gluten-free diet. While theoretically simple, there are many challenges to be able to adhere to a gluten-free diet long-term. Patients are usually evaluated for any vitamin deficiencies that may require repletion. Most common is iron deficiency, calcium, vitamin D and other fat-soluble vitamins. B12 deficiency can affect over 20% of patients with coeliac disease. It is unclear if this mandates parenteral B12 for life as is often necessitated in patients who have B12 deficiency due to ileal resection or pernicious anaemia (*Murray* and *Ross*, 2004).

In adult patients diagnosed with coeliac disease, bone density is typically performed as diminished bone density, either due to osteomalacia or osteoporosis, is very common. Fracture risk is increased in patients with coeliac disease prior to diagnosis, and the rate does not apparently drop in follow-up (*Jafri* et al., 2008).

PROGNOSIS

Most patients diagnosed as children will promptly respond to a gluten-free diet and will resume normal growth and development. As long as they remain on a gluten-free diet, they will remain healed and well. Adult patients diagnosed with coeliac disease heal much more slowly and are at increased risk of complications and increased mortality, especially within the 1st year of diagnosis (*Rubio-Tapia* et al., 2010; *Lanzini* et al., 2009). This seems to be a relatively modest increase in overall mortality in diagnosed coeliac disease. Patients with undiagnosed coeliac disease, however, may be at greatly in-

creased risk of mortality with an almost 4-fold increase in mortality over 45 years (*Rubio-Tapia* et al., 2009). The increase in mortality associated with coeliac disease is not universally found and variances such as the rate of detection of coeliac disease, in particular geography, may impact the likely long-term mortality. Early detection couple with vigorous treatment with a gluten-free diet is most likely to result in a good outcome.

FUTURE TREATMENTS

Several new treatments are in development for coeliac disease given that we know much about the processes involved. Treatments directed at altering the nature of wheat itself, detoxifying the wheat within the gastrointestinal tract or even before ingestion, binding the peptides within the intestine to prevent presentation to the immune system (*Liang* et al., 2010). The modulation of the permeability or prevention of increased permeability induced by gluten, as well as the degradation of the immune peptides within the gut by potent endopeptidases all are in pre-clinical or even clinical trials (*Gass* et al., 2007; *Cerf-Bensussan* et al., 2007; *van den Broeck* et al., 2009). Other approaches, such as an immunotherapy approach to try to reduce tolerance to gluten, are also under study (*Senger* et al., 2003; *Vickery* et al., 2009; *Keech* et al., 2009). Other targets, such as blocking the deamidation by tissue transglutaminase and blocking the binding of the peptides to DQ2 or DQ8, are also areas that have sparked development of agents in preclinical study (*Xia* et al., 2007; *Klock* et al., 2011). Also, use of worms to skew the immune response away from a coeliac-like response has been used in humans (*Daveson* et al., 2011).

PREVENTION

Coeliac disease has dramatically increased in prevalence over the last 50 years (*Rubio-Tapia* et al., 2009). This is almost certainly due to environmental factors. Identifying and abrogating these environmental factors is crucial if we are to stem the tide of coeliac disease. Whilst it affects still somewhat just less than 1% of the population of the U.S., it now affects over 2% of other Caucasian populations such as Sweden and Finland, rates that appear to be increasing (*Dube* et al., 2005). It is vitally important to identify the factors that have led to this increase so that this increase can be mitigated. Several approaches to the prevention of coeliac disease are under study. The Prevent CD trial primarily in Europe and North America addresses the issue of timing of introduction and quantity of gluten in the infant diet. Other studies looking at using systems biology approach to address the role of the microbial community on tolerance to gluten is also under study. It is interesting to note that the phenomena of oral tolerance require the presence of microbiota within the gut. Studies looking at the co-administration of beneficial bacterial with gliadin molecules have also been studied and may prevent or reverse gluten sensitization (*Huibregtse* et al., 2009).

SUMMARY

In summary, coeliac disease is an increasingly common chronic disease affecting primarily the upper small intestine associated with significant morbidity and mortality. It is often overlooked, but presents both opportunities and challenges for understanding of the interaction between the environment and immune system, and may present an example of a disorder that can be the result of triggering of loss of homeostasis due to food or microbial influences within the intestine.

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ACUTE ENTERIC INFECTIONS ALTER COMMENSAL MICROBIOTA: NEW MECHANISMS IN POST-INFECTIOUS INTESTINAL INFLAMMATORY DISORDERS

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SUMMARY

This chapter discusses how acute enteric infections may lead to post-infectious complications. Particular emphasis is given to infections with *Campylobacter jejuni* and *Giardia intestinalis*, two of the most common causes of enteric infections worldwide. The review provides a critical discussion of the biology of the human intestinal microbiota. Chronic post-infectious sequelae of these infections include malnutrition, stunting, failure to thrive, and impaired cognitive functions. They may also cause post-infectious disease at extra-intestinal sites, including the joints, the skin, the eyes, the lungs, the heart, the muscles, the kidneys, and the central nervous system. Findings from recent and ongoing research suggest that enteropathogen-induced disruptions of the commensal microbiota may at least in part play a role in triggering the sequence of events that result in these presentations. These disruptions include a promotion of the transcellular and paracellular translocation of non-invasive commensal bacteria, as well as disruptions of the composition and structure of microbiota biofilms.

INTRODUCTION

Diarrhoeal disease resulting from enteric infections remains one of the major causes of morbidity and mortality worldwide. Each year, an estimated 2 to 2.5 million children under the age of 5 years die from the 1.4 billion yearly diarrhoeal episodes in the paediatric population of developing countries (*O'Ryan* et al., 2005). Approximately 20 parasitic, viral, and bacterial pathogens are known to be the most common causative aetiologies (Table 1). Infection most commonly occurs through ingestion of contaminated food or water, or through direct faecal oral infection. Host factors, such as the host's nutritional and immune status, as well as environmental factors, like co-infections, are important in determining symptom severity. Indeed, some enteropathogens worsen the outcome of concurrent infections while others, like *Giardia intestinalis*, may partly protect children against diarrhoeal disease in developing countries (*Moore*, 2001; *Jensen* et al., 2009; *Veenemans* et al., 2012). Intestinal parasitic helminths are known to posses potent immune-regulating properties that may help attenuate tissue damage (*Maizels* et al., 2003), but overall, the mechanisms directing the clinical outcome of co-

Table 1: Enteropathogens most commonly reported as causes of acute diarrhoea worldwide. Most of these have been reported to have long-term sequelae via mechanisms that remain incompletely understood.

Parasites:	*Giardia intestinalis* (syn. *lamblia*, or *duodenalis*) *Cryptosporidium hominis* and *C. parvum* *Entamoeba histolytica*
Bacteria:	*Campylobacter jejuni* and *C. coli* Diarrhoeagenic *Escherichia coli* (ETEC- enterotoxigenic; EPEC - enteropathogenic; STEC - shiga-toxin producing; EAEC; enteroadherent; EIEC - enteroinvesive) *Salmonella* sp. *Shigella* sp. *Vibrio cholera* *Aeromonas* sp.
Viruses:	Rotavirus Norovirus Sapovirus Astrovirus Enteric adenovirus

infections and polyparasitism remain obscure. In developed countries of the World, public media warn that prevalence of enteric infections seems to increase. Indeed, the Wall Street Journal (3/15/2012, Martin) reported that according to CDC estimates, every year approximately 48 million Americans become ill through contaminated food, and 3,000 die. In its "National Briefing," the New York Times (3/15/2012, A20, Grady) reported that the CDC observes that "Deaths from gastrointestinal infections more than doubled in the United States from 1997 to 2007, to more than 17,000 a year from 7,000 a year.

Adding to the raising concerns caused by acute enteric infections, recent observations indicate that post-infectious complications may arise following exposure to a variety of enteropathogens, including *Campylobacter jejuni,* diarrhoeagenic *Escherichia coli, Salmonella* sp.*, Shigella* sp.*, Cryptosporidium parvum,* and *Giardia intestinalis*. In developing countries, acute diarrhoeal disease caused by these enteropathogens can lead to failure to thrive, stunted growth, and impaired cognitive functions. Recent studies following outbreaks of intestinal infections, and large retroactive cohort studies, also found that these infections may be responsible for chronic fatigue syndrome, arthritis, irritable bowel syndrome (IBS), and flare-ups in patients with Inflammatory Bowel diseases (IBD) (*Rodriguez* and *Ruigomez*, 1999; *Riddle* et al., 2001; *Berkman* et al., 2002; *Gradel* et al., 2009; *Kalischuk* and *Buret*, 2010; *Moore* et al., 2011; *Wensaas* et al., 2012). As a result, from being a leading cause of global child death, infectious diarrhoea now appears to have become a key source of lifelong morbidity (Table 2). The causes of the post-infectious clinical manifestations due to enteric infections, even after complete elimination of the enteropathogen, remain obscure. However, the commonality of these post-infectious disorders raises the intriguing possibility that they may share at least some of their basic biological mechanisms, hence offering great potential for the identification of novel therapeutic targets. This chapter elaborates on

Table 2: Examples of post-infectious complications and disorders reported following enteric infections, with *C. jejuni, E. coli, Salmonella* sp*., Shigella* sp*., Cryptosporidium parvum, G. intestinalis*, or viral enteropathogens.

Affected site:	Disorder/condition:	References:
Intestine:	Food allergies	Farthing et al., 1986; Hardin et al., 1997; Di Prisco et al., 1998
	Post-infectious irritable bowel syndrome	Rodriguez and Ruigomez, 1999; Spiller et al., 2000; Riddle et al., 2001;; Dizdar et al., 2007; Stark et al., 2007; Thabane et al., 2007; Gradel et al., 2009; Hanevik et al., ,2009; Marshall et al., 2010; Robertson et al., 2010; Wensaas et al., 2012; Buret et al., 2013; Simren et al., 2013
	Flare-ups in inflammatory bowel diseases	Kalischuk and Buret, 2010; Riddle et al., 2001; Swidsinsky et al., 2009; Reiff and Kelly, 2010; Buret et al., 2013
	Coeliac disease	Riddle et al., 2001
Joints:	Arthritis	Keat, 1991; Borman et al., 2001; Carlson and Finger, 2004; Schiellerup et al., 2008; Scher and Abramson, 2011
Skin:	Urticaria/Pruritus/Dermatitis	Di Prisco et al., 1998; Giacometti et al., 2003; Pietrzak et al., 2005
Eyes:	Iridocyclitis/Choroiditis/Retinal haemorrhages	Pettoelo-Mantovani et al., 1990; Corsi et al., 1998
Lungs:	Asthma,	Di Prisco et al., 1998; Han et al., 2012
	Obstructive lung disease	Hity et al., 2010
Heart:	Endocarditis	Miki et al., 2005
Muscles:	Hypokalaemic myopathy	Cervelló et al., 1993; Addiss and Lengerich, 1994; Genovese et al., 1996
CNS:	Impaired cognitive function	Guerrant et al., 1999; Berkman et al., 2002; Niehaus et al., 2002; Simsek et al., 2004; Koruk et al., 2010; Forsythe and Kunze, 2013; Bergman and Graham, 2005
	Autism	Mulle et al., 2013
	Guillain Barré Syndrome (paralysis)	Willison and O'Hanlon, 2000; Willison, 2005
Kidneys/ Urethra:	Haemolytic uremic syndrome	Delans et al., 1984
Entire body:	Stunting	Farthing et al., 1986; Guerrant et al., 1999; Berkman et al., 2002; Simsek et al., 2004; Ignatius et al., 2012
	Chronic Fatigue Syndrome	Mørch et al., 2009; Naess et al., 2012; Wensaas et al., 2012

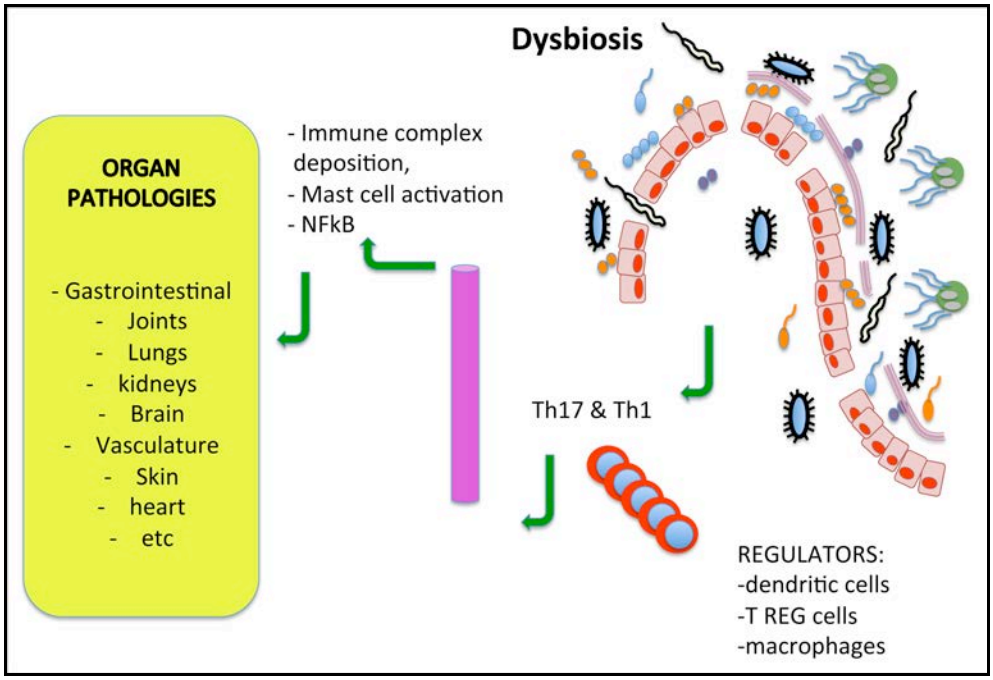

Figure 1: Hypothetical mechanisms, inferred from published research, by which acute enteric infections may lead to post-infectious, chronic complications. Enteropathogens, including *Campylobacter jejuni* or *Giardia intestinalis* cause microbiota biofilm dysbiosis, and disruptions of gut homeostasis and barrier function (*Kalischuk* and *Buret*, 2010; *Cotton* et al., 2011; *Buret* et al., 2013). These effects may be further compounded by a direct breakdown of the mucus barrier via mucin degradation by the enteropathogen (*Macfarlane* et al., 1999, 2005; *Moncada* et al., 2005; *Derrien* et al., 2010). In turn this causes proliferation of autoreactive cells like Th17 and Th1 lymphocytes, and release of pro- inflammatory cytokines (including IL-17, IFN-γ, TNF, etc.). Local dendritic cells, macrophages, and T Regulatory cells play a central role in regulating these processes. As the effects reach the circulation, this leads to immune complex deposition, tissue mast cell degranulation, NF-κB activation, and/or other yet unknown processes that cause organ pathology and disease (*Hardin* et al., 1997; *Hity* et al., 2010; *Philpott* et al., 2011; *Scher* and *Abramson*, 2011; *Han* et al., 2012; *Forsythe* and *Kunze*, 2013; *Mulle* et al., 2013).

recent findings suggesting that acute enteritis may disrupt the intestinal homeostatic balance between the intestine of the host and its own microbiota, and that these alterations in turn may be responsible for initiating the sequence of events culminating in post-infectious symptomatology. Because *Campylobacter jejuni* and *Giardia intestinalis* are some of the most common causes of bacterial and parasitic enteritis worldwide, the chapter will emphasize on findings using these two disease models primarily (*Savioli* et al., 2006; WHO: Campylobacter. Fact sheet N°255, October 2011 http://www.who.int/mediacentre/factsheets/fs255/en/).

BIOLOGY OF THE INTESTINAL MICROBIOTA

Intestinal microbiota and host have evolved to live in tolerant commensalism. Indeed, host immunity and gut physiology are shaped by the microbiota, which itself is modulated by host immune, genetic, dietary and other

environmental factors. Recent evidence also indicates that disruptions of these microbiota influences homeostasis to cause disease at extra-intestinal sites, including asthma, obstructive lung disease, arthritis, and even disorders of the central nervous system such as autism (*Hity* et al., 2010; *Scher* and *Abramson*, 2011; *Han* et al., 2012; *Forsythe* and *Kunze*, 2013; *Mulle* et al., 2013). Much of the findings remain association-based, and cause-to-effect relationship studies are now sorely needed. Over 70% of the gut microbiota have not yet been cultured or classified, but new culture-independent techniques have established that these microbial communities are host- and GI tract location-specific, and that they play a key role in health (*Zoetendal* et al., 2008; *de Weerth* et al., 2013; *Lepage* et al., 2013). Indeed, recent advances in sequencing, metagenomics, and bioinformatics technology have found that the estimated 10^{14} human gut microorganisms, weighing a rough total of 3 pounds, contain an overall genome size 150 times larger than that of the human genome (*Zoetendal* et al., 2008; *de Weerth* et al., 2013; *Lepage* et al., 2013). Moreover, these studies found that gene exchanges between representatives of the gut microbiota were in fact much more common than previously anticipated (*Zoetendal* et al., 2008). This raises the question as to whether or not such exchanges may also be common between enteropathogens and the normal microbiota. In the early stages of life, the gut microbiota undergoes some degree of shifting, but overall, in the later parts of life, it remains stable in the absence of major disturbances of the host's health conditions and diet (*Dethlefsen* and *Relman*, 2011). To date, the phylogenetic core of the human microbiota is thought to be composed of 60-70 highly prevalent species (*Tap* et al., 2009). Loss of diversity of the microbiota, which may occur via mechanisms that remain unclear, has been associated with disease (*Frank* et al., 2007). Taken together, these new insights from DNA sequence-based analyses of gut microbial communities suggest that the microbiome represents a key environmental factor that can influence disease manifestation (Figure 1). How in turn acute enteric infections may be at the source of pathogenic microbiota disruptions has become a very intriguing part of this puzzle.

The population of industrialized countries, with their characteristic high fat, high protein diets, harbour different microbiota than those living in rural areas of developing countries, with a polysaccharide-rich diet (*De Filippo* et al., 2010)). The differences mainly reflect an increased representation of *Bacteroidetes* in the latter group, a group of bacteria known for its high genetic ability to hydrolyse xyloses. The relative sensitivity of these distinct microbiota to enteropathogens, and how in turn disruptions in their respective flora may differentially regulate post-infectious disorders, is unknown.

Microbial communities colonizing the gut do so in a gradient, from few organisms in the oesophagus and stomach, to the much more heavily colonized colon (*Hokins* et al., 2002). Ever since the late 19th century, when Robert Koch's studies in Germany developed the germ theory of disease, bacteria were envisioned as single cells that float or swim through some kind of watery habitat, within the human body, or in the environment in which they lived. With the giant progresses made in microbiology since then, and in the large part only since the 1960's,we now understand that the swimming bacteria in typical laboratory cultures act nothing like the ones encountered in nature. Indeed, many of these organisms do not,

in the real world, spend much time swimming freely as isolated cells. Instead, they stick to wetted surfaces in organized colonies that form a complex multi-species slime-enclosed film, allowing them to withstand environmental flushing forces, as well as resist against antimicrobials and competing microbes. These slime-producing communities are known as bacterial "biofilms" (*Hall-Stoodley* et al., 2004). The slimy coating in which they live contains more than 90% water and complex microbially secreted exo-polysaccharides that confer viscosity. The GI tract, with its constant luminal flow and high nutrient availability make it an ideal site for bacteria to live as biofilms. While we now understand that gut microbiota do indeed live in a biofilm phenotype, much remains to be learnt on how the integrity and physiology of these communities may bear on gut homeostasis and disease (*Von Rosenvinge* et al., 2013). Importantly however, as these biofilms live in very close proximity to host epithelial cells, they are the microbial communities most likely to interact with host physiology, immunity, and metabolism. As such, more so than studies on faecal microbiota, research now needs to investigate the bacterial biofilms living on the mucosal surface of the intestine, as it is likely to shed key elements of our understanding of host-gut microbiota interactions. Indeed, biofilm microcolonies exist on the intestinal mucosal surface in healthy people, and these bacterial populations are different from those living in the intestinal lumen (*Macfarlane* and *Macfarlane*, 1992). The layer of mucus coating the epithelial surfaces of the gut prevents most microorganisms from reaching and/or persisting on the mucosal surface. However, mucus glycoproteins, including mucins, represent an important source of carbohydrates for the saccharolytic biofilm bacteria, particularly at site where fermentable carbohydrates may be in small supply, such as in the colon (*Macfarlane* et al., 1992). This breakdown of mucin appears to be a cooperative undertaking by the microbiota, meant to benefit the entire bacterial biofilm community (*Macfarlane* and *Macfarlane*, 1992). Intriguingly, intestinal biofilm bacteria living on mucin differ metabolically and phylogenetically from those living in a planktonic state (*Macfarlane* et al., 2005). Together, these data demonstrate the physiological significance of the biofilm mode of life of the intestinal microflora. A number of reports have now established that patients with IBD and IBS have a reduced microflora diversity, and an apparent reduction in the commensal phyla *Firmicutes* and *Bacteroidetes*, as well as increased numbers of *Proteobacteria* (*Reiff* and *Kelly*, 2010; *Simren* et al., 2013). Furthermore, recent evidence from fluorescent *in situ* hybridization labelling (FISH) indicates that mucosal bacterial colonies live in closer proximity to the epithelial lining in IBD than in normal individuals (*Swidsinsky* et al., 2009). Whether this results from a more "adherent-invasive" phenotype in the bacteria found in patients with IBD requires further clarification. Indeed, involvement of the commensal microflora in the initiation, persistence, and flare-ups of IBD has been suggested as early as 1971 (*Hill* et al., 1971). However, much remains to be learnt about the role of microbiota biofilm disruptions in the aetiology of post-infectious complications following acute enteric infections. Moreover, research is needed to assess the effects of enteropathogens on gut biofilm integrity, in an attempt to identify new mechanisms that lead to the severe intestinal and extra-intestinal post-infectious complications of acute enteric infections.

CAMPYLOBACTER JEJUNI

Campylobacter jejuni is one of the most prevalent causes of human bacterial enteritis in the World (*Moore*, 2001; *O'Ryan* et al., 2005; Public Health Agency of Canada, URL: http://www.phac-aspc.gc.ca/id-mi/az-index-eng.php). About 90% of human campylobacteriosis is caused by *C. jejuni*, and the remaining 10% is induced predominantly by *C. coli.* In the paediatric population of developing countries however, diarrhoeagenic *E. coli* (EPEC, ETEC, STEC, EAEC, and EIEC) remain the most common bacteria detected during diarrhoea, and these bacteria represent 30-40% of acute diarrhoeal episodes in children (*O'Ryan* et al., 2005). Though polymicrobial infections involving *Campylobacter* appear to be less common in developed countries, the bacterium is frequently isolated with other enteropathogens in the developing World; co-infecting organisms include *Escherichia coli*, *Salmonella sp.*, *Shigella sp.*, *Giardia intestinalis*, and *rotavirus* (*Coker* et al., 2002; *Janssen* et al., 2008).

Inadequate hygiene represents a significant contributor to campylobacteriosis, as is the case for infections with *Shigella* sp.*, Salmonella* sp., and other enteropathogens. *C. jejuni* lives as a non-pathogenic enteric bacterium in poultry and cattle. Upon ingestion of contaminated water or food (e.g. raw milk), infected humans exhibit a range of symptoms varying from mild to severe diarrhoea. Clinically therefore, acute campylobacteriosis is not readily distinguishable from other enteric infections. These clinical features typically arise 2-4 days post-infection and are indicative of the inflammatory response that is occurring (Public Health Agency of Canada, URL: http://www.phac-aspc.gc.ca/id-mi/az-index-eng.php). Histological examination of affected intestinal tissues reveals a broad spectrum of tissue alterations, and, most commonly, infiltration of neutrophils into the lamina propria secondary to NF-κB activation (*Wassenaar* et al., 1999). The disease seems to be less severe in developing countries (*Coker* et al., 2002).

Pathogenesis

The host-pathogen interactions responsible for inciting inflammation remain incompletely understood (*Janssen* et al., 2008). Tissue damage appears to be largely due to the effects of cytotoxins, and/or host-cell invasion (*Wassenaar* et al., 1999; *Russell* et al., 1993). Recent findings suggest that a *C. jejuni* lipoprotein (JlpA) promotes epithelial adhesion, and the subsequent induction of pro-inflammatory signalling (*Jin* et al., 2003). Moreover, *C. jejuni* produces cytolethal distending toxin (Cdt), a DNase-like toxin produced by several species of bacteria. This holotoxin is composed of three subunits: CdtB belongs to the family of DNase I-like nucleases and is the active subunit, while the CdtA and C subunits help deliver CdtB into target cells (*Bang* et al., 2001). Upon epithelial invasion by *C. jejuni*, Cdt translocate into the nucleus, where it activates the G1 and/or G2/M checkpoint responses, resulting in cell cycle arrest, and ultimately cell death via mechanisms that are poorly understood (*Whitehouse* et al., 1998; *Bang* et al., 2001). The effects of Cdt have primarily been described for lymphocytes and monocytes, in which it appears to induce apoptosis (*Hickey* et al., 2005). However, Cdt has also been shown recently to induce non-apoptotic death of endothelial cells (*Bielaszewska* et al., 2005). In addition, *C. jejuni* is thought to produce several other cytotoxins besides Cdt, including shiga-like toxin,

haemolysin, and hepatotoxin (*Wassenaar* et al., 1997). These cytotoxic factors require further characterization, and their possible implication in *C. jejuni*-induced epithelial injury is poorly understood. *C. jejuni* flagella represent another important virulence factor, as they promote motility through the mucus layer, and are important in the adherence to and invasion of epithelial cells (*Wassenaar* et al., 1991; *Konkel* et al., 2004; *Christensen* et al., 2009). Interestingly, strain-dependent induction of epithelial cell oncosis by *C. jejuni* correlates with invasion ability, but may occur independently of Cdt (*Kalischuk* et al., 2007). Regardless, the acute stage of the infection is able to break the intestinal barrier, which in turn allows luminal antigens to stimulate subepithelial immunity. In the acute stage of infection, *C. jejuni* also disrupts protective TLR-9 signalling in epithelial cells (*O'Hara* et al., 2012). These alterations in turn prime the intestine for heightened inflammatory injury upon mild pro-inflammatory stimulation, via mechanisms that remain incompletely understood (*O'Hara* et al., 2012).

Microbiota disruptions by Campylobacter sp.

C. jejuni facilitates the translocation of non-invasive, commensal bacteria, both paracellularly as well as transcellularly. The latter occurs by hijacking the host lipid raft pathway as well as via epithelial M cells (*Lamb-Rosteski* et al., 2008; *Kalischuk* et al., 2009, 2010). On-going research has also uncovered that *C. jejuni* is able to directly disrupt the composition and integrity of human microbiota biofilms, which may lead to a loss of tolerance by the host to its own commensal microbiota (*Buret* et al., 2013). Interestingly, the effects of *C. jejuni* on microbiota biofilms can be duplicated with *G. intestinalis*, but not with a commensal *E. coli* (*Buret* et al., 2013). Further research is warranted to clarify the sequence of events responsible for the post-infectious complications of campylobacteriosis, which will help unravel common pathways through which acute enteropathogens cause post-infectious inflammatory disorders.

Post infectious complications

Long after the *C. jejuni* infection has been cleared, some individuals experience abnormal bowel patterns (more frequent, watery stools; or fewer hard/lumpy stools) that may persist for years (*Riddle* et al., 2001; *Marshall* et al., 2010). These abnormalities may be associated with intestinal enteroendocrine cell hyperplasia, and low-grade inflammation, including an increase in CD3 lymphocytes and proliferation of intraepithelial lymphocytes (*Spiller* et al., 2000).

The post-infectious complications caused by acute campylobacteriosis include Guillain-Barré syndrome, reactive arthritis, Reiter syndrome (an inflammatory disease with either conjunctival or urethral inflammation), Irritable Bowel Syndrome, flare-ups in patients with Inflammatory Bowel Diseases, and possibly celiac disease (*Keat* and *Rowe*, 1991; *Riddle* et al., 2001; *Coker* et al., 2002; *Janssen* et al., 2008; *Marshall* et al., 2010). On rare occasions, *C. jejuni* infection may also cause haemolytic-uremic syndrome, a well-known consequence of infection with enterotoxigenic *Escherichia coli* (*Delans* et al., 1984). Another rare extra-intestinal complication of campylobacteriosis is endocarditis (*Miki* et al., 2005). Intriguingly, the symptoms of post-infectious arthritis appear to be similar regardless of the infecting bacterial species, indicating a role for factors common to a range of pathogens (*Schiellerup* et al., 2008). Guillain-Barré Syndrome may develop in ap-

proximately 1 in 1,000 infected individuals infected with *C. jejuni*, and is a serious autoimmune neurological disorder; symptoms may range from weakness of extremities to complete paralysis and respiratory insufficiency (*Willison*, 2005). The majority of patients may recover completely within 6 to 12 months (*Willison*, 2005). Guillain-Barré Syndrome is thought to occur because of molecular mimicry between the lipo-oligosaccharide of the *C. jejuni* cell envelope, and sugar moieties on nerve gangliosides (*Willison* and *O'Hanlon*, 2000). In turn, antibodies raised during infection with *C. jejuni* may cross-react with nerve gangliosides in some individuals, leading to the demyelinization of nerves, and subsequent degeneration of axons (*Willison*, 2005).

Irritable bowel syndrome (IBS), is the most commonly diagnosed functional gastrointestinal disorder by gastroenterologists, and is characterized by abdominal hypersensitivity and abnormal bowel movement (diarrhoea and/or constipation). It is a common long-term consequence of acute gastroenteritis caused by a variety of enteropathogens, including *C. jejuni*, *Samonella* sp., diarroeagenic *E. coli*, and *Giardia intestinalis* (*Riddle* et al., 2001; *Thabane* et al., 2007; *Ohman* et al., 2010). Altered motility patterns as well as abdominal pain in post-infectious IBS have been associated with mast cell secretions such as mast cell tryptase and serotonin (5-hydrocytryptamin, 5-HT), also released from enterochromaffin cells (*Cenac* et al., 2007; *Cremon* et al., 2011).

Finally, it was recently demonstrated that acute gastroenteritis with *C. jejuni*, diarrhoeagenic *E. coli*, or *Salmonella* sp. may lead to the initiation and or exacerbation of Inflammatory Bowel Diseases (IBD; Crohn's Disease and ulcerative colitis), themselves associated with rheumatic manifestations, further linking gut disturbance to osteoarticular disorders (*Gradel* et al., 2009; *Rodriguez-Reyna* et al., 2009). The mechanisms remain unclear. Several inflammatory factors implicated in IBD implicate the NF-κB pathway. In keeping with a prominent role for microbes in the pathogenesis of IBD, a variety of bacterial products, including bacterial lipopolysaccharide (LPS), are also potent activators of this pathway. Polymorphic mutations of NOD2 (also called CARD15), which acts as an intracellular sensor of bacteria-derived muramyl dipeptide (a component of Gram-positive and Gram-negative bacterial cells walls) are the product of the IBD1 gene mutation present in some patients with IBD, and significantly increase disease susceptibility by altering the NF-κB pathway (*Ahmad* et al., 2002; *Podolsky*, 2002). The cytosolic NOD2 receptor may also activate the NF-κB pathway upon exposure to LPS which may have entered the cytoplasm via mechanisms that have yet to be elucidated (*Ahmad* et al., 2002; *Podolsky*, 2002). The increased numbers of *E. coli* and *Proteobacteria* detected in the intestinal mucosa of IBD patients may heighten exposure to pathogenic products such as lipoproteins, proteoglycans, and LPS. Other components of the resident microflora, within the stressed and inflamed environment of the IBD intestine, may also activate host inflammation via mechanisms that are incompletely understood (*MacPherson* et al., 2004). In addition, upregulated expression and/or polymorphic mutations of receptors for LPS, e.g. TLR-4, have been found in epithelial cells of IBD patients (*Cario* et al., 2000). LPS has the ability to break the intestinal barrier (*Qi* et al., 2005; *Yu* et al., 2005; *Chin* et al., 2006). It has also been recently reported that peripheral blood monocytes from IBD patients

exhibit increased TLR2 expression, and this is correlated with a marked increase of TLR-2 -mediated TNF-α production (*Cantó* et al., 2006). The notion that circulating LPS and anti-endotoxin antibodies can be found in the plasma of IBD patients is consistent with a breach in the epithelial barrier (*Gardiner* et al., 1995). Activation of TLR-4 appears to be implicated in the development of pathology during infectious colitis (*McKay*, 1999; *Cario* et al., 2000). Interestingly, a recent study also demonstrated that *C. jejuni* infection disrupts TLR-9 signalling, which makes the intestinal mucosa more prone to inflammatory injury (*O'Hara* et al., 2012). Furthermore, *in situ* examination of biopsies from patients with IBD revealed the increased uptake of non-invasive, commensal *E. coli* via the follicle-associated epithelial M cells, a phenomenon known to be facilitated by *C. jejuni* (*Keita* et al., 2008). These invading commensal *E. coli* were shown to co-localize with dendritic cells, which correlated with increased levels of the pro-inflammatory cytokine TNF-α. Disruptions of the intestinal barrier by enteropathogens may permit luminal material, including commensal bacteria and/or their products, to activate baso-lateral pro-inflammatory sensors like TLR's which otherwise may have been inaccessible. Therefore, luminal factors capable of breaching epithelial integrity, and/or altering the polar distribution of TLR's, may predispose the intestine to heightened intestinal inflammation in a susceptible host. Findings from on-going research indicate that *C. jejuni*- or *Giardia*-induced disruptions of the microbiota biofilm composition and integrity may help trigger a sequence of events that may lead to post-infectious complications (*Buret* et al., 2013). A better understanding of the disruptions to the resident microflora and deregulated bacterial recognition secondary to acute *C. jejuni* infection may shed new light on the mechanisms responsible for the initiation and/or exacerbation of inflammation in IBD patients.

Taken together, findings have started to establish processes through which campylobacteriosis may lead to post-infectious sequelea. These include inflammatory disorders in the gut, but may also affect extra-intestinal sites, including the central nervous system, the lungs, the kidneys, the eyes, the joints, and even the heart. This characteristic is shared with the post-infectious complications caused by a variety of other enteropathogens, further supporting the hypothesis of common pathogenic pathways. At least part of these processes appear to be triggered by enteropathogen-induced disruptions of the host microbiota biofilms. More research needs to identify the mechanisms through which *Campylobacter*, and other enteropathogens, may trigger events in the microbiota and the intestinal mucosa that ultimately set the stage for chronic inflammatory disorders in the gut, as well as at extra-intestinal sites.

GIARDIA INTESTINALIS

Giardiasis, caused by *G. intestinalis* (synonymous *G. lamblia* or *G. duodenalis)*, is the most common waterborne parasitic infection of the human intestine worldwide, and was recently included in the World Health Organisation's Neglected Disease Initiative (*Savioli* et al., 2006; WHO: Guidelines for drinking water quality, 3rd Edition http://www.who.int/water_sanitation_health/dwq/gdwq3/en/). The prevalence of human giardiasis is highest in devel-

oping countries, where it ranges from 20% to 100% of the population, versus its prevalence of 3-7% in developed countries (*Jensen* et al., 2009; *Ankarlev* et al., 2010). People infected with *Giardia* may develop a broad range of clinical manifestations, ranging from asymptomatic infection, to acute or chronic diarrhoeal disease associated with abdominal pain and nausea (*De Filippo* et al., 2010; *Cotton* et al., 2011). Most infections are self-limiting, although re-infection and chronic infection can occur. Recent evidence indicates that *G. intestinalis*, like *C. jejuni* and other enteropathogens, is responsible for chronic post-infectious complications, via mechanisms that remain obscure.

Pathogenesis

Pathophysiology in giardiasis occurs without invasion of the small intestinal tissues by the trophozoites, and in the absence of any overt inflammatory cell infiltration, with the exception of a modest increase in intraepithelial lymphocytes; some of the acute pathology, which involves a diffuse shortening of epithelial microvilli, is caused by activated CD8+ lymphocytes (*Buret* et al., 1992; *Scott* et al., 2004). As is the case for enteric infections caused by *Campylobacter* sp., diarrhoeagenic *E. coli*, *Salmonella* sp., and others, the pathophysiology of acute diarrhoea in giardiasis implicates a disruption of the intestinal barrier function. In giardiasis, heightened rates of enterocytes apoptosis, intestinal barrier dysfunction, activation of host lymphocytes, shortening of brush border microvilli with or without coinciding villous atrophy, disaccharidase deficiencies, small intestinal malabsorption, anion hypersecretion and increased intestinal transit rates all seem to contribute to disease (*Buret* et al., 1992; *Chin* et al., 2002; *Scott* et al., 2004; *Troeger* et al., 2007; *Koot* et al., 2009; *Cotton* et al., 2011). Whether these effects may be further compounded by degradation of local mucins by *Giardia*, as it was found for other enteric microbes like *E. histoloytica*, requires further investigation (*Macfarlane* and *Macfarlane*, 1999; *Macfarlane* et al., 2005; *Moncada* et al., 2005; *Derrien* et al., 2010). As is the case with other enteropathogens, induction of apoptosis in enterocytes by *Giardia* represents a key component in the pathogenesis of the infection (*Chin* et al., 2002; *Panaro* et al., 2007; *Troeger* et al., 2007; *Buret* et al., 2013). The mechanisms responsible are unknown, and the identification of a *Giardia* "enterotoxin" has remained elusive. *Giardia*-mediated increases in intestinal permeability result from alterations to the apical junctional complexes, including disruptions of F-actin, zonula-occludens (ZO)-1, claudin-1, and α-actinin, a component of the actomyosin ring that regulates paracellular flow, under the control of epithelial myosin light chain kinase (*Teoh* et al., 2000; *Scott* et al., 2002; *Cotton* et al., 2011; *Maia-Brigagão* et al., 2012). The mechanisms leading to loss of intestinal barrier function caused by *Giardia* sp. are shared among a broad range of enteropathogens (*O'Hara* and *Buret*, 2008).

Microbiota disruptions by Giardia

Bacterial components of the microbiota from patients with symptomatic giardiasis appear to heighten *G. intestinalis* virulence in gnotobiotic mice, via unclear mechanisms (*Torres* et al., 2000). Little is known of the effects of acute giardiasis on the human commensal microbiota. But findings from on-going studies indicate that indeed, *G. intestinalis* is able to disrupt the composition and integrity of human intestinal microbiota biofilms, in a fashion similar to what *C. jejuni* does (*Buret* et al.,

2013). More research in this area will help identify common pathways through which acute enteritis may lead to long term inflammatory disorders by disrupting commensal bacterial biofilms, as well as how these may lead to extra-intestinal complications.

Post-infectious complications of giardiasis

Infection with *Giardia* sp. may lead to food allergies, negatively affect nutritional and growth status, and impair cognitive function in humans (*Farthing* et al., 1986; *Berkman* et al., 2002; *Niehaus* et al., 2002; *Ettehad* et al., 2010; *Ignatius* et al., 2012). Recent evidence also indicates that 5-10% of patients diagnosed with giardiasis will develop post-infectious irritable syndrome and functional dyspepsia, long after clearance of the parasite (*Dizdar* et al., 2007; *Stark* et al., 2007; *Hanevik* et al., 2009; *Robertson* et al., 2010). When the infection persists for months, microscopic duodenal inflammation may develop (*Hanevik* et al., 2009; *Mørch* et al., 2009), further underscoring the need for rapid parasitic elimination to reduce the risk of chronic complications in giardiasis. Infection with *Giardia* can cause iron deficiency anaemia, micronutrient deficiencies, protein-energy malnutrition, which all have been linked to growth and cognitive retardation (*Simsek* et al., 2004; *Koruk* et al., 2010). Studies conducted in Brazil and Peru found that diarrhoeal disease occurring in the first 2 years of life negatively correlates with cognitive function, verbal fluency, and physical fitness, and may lead to long-term growth faltering (*Guerrant* et al., 1999; *Berkman* et al., 2002). Long-term sequelae of wasting and/or stunting often include general behavioural and developmental consequences that present as failure to thrive, which has also been linked to giardiasis (*Berkman* et al., 2002; *Bergman* and *Graham*, 2005; *Ettehad* et al., 2010). The persistence of infection and its association with diarrhoea are key factors associated with growth disturbance and failure to thrive, and diarrhoea caused by enteric infections in early childhood has become a predictor of stunting (*Berkman* et al., 2002; *Botero-Garcés* et al., 2009; *Ettehad* et al., 2010). In giardiasis and cryptosporidiosis, as well as other enteric infections, diarrhoea may lead to poor cognitive function by causing zinc and iron micronutrient deficiencies, as well as defects in the anti-oxidant system, which may all affect neuroplasticity (*Ajjampur* et al., 2011). Moreover, diarrhoea during early childhood was also found to impair visual-motor coordination, auditory short-term memory, information processing, and cortical cognitive function (*Guerrant* et al., 1999; *Ajjampur* et al., 2011). Combined with these complications, some individuals may develop post-giardiasis fatigue and musculoskeletal pain (*Naess* et al., 2012). Viral, bacterial, as well as parasitic pathogens have the ability to cause chronic fatigue syndrome. Recent studies have reported a high prevalence of post-infectious fatigue following a giardiasis outbreak in Bergen, Norway, in 2004 (*Dizdar* et al., 2007; *Hanevik* et al., 2009; *Mørch* et al., 2009; *Robertson* et al., 2010; *Naess* et al., 2012; *Wensaas* et al., 2012).

Beyond its long-term consequences on intestinal and overall metabolic parameters, giardiasis, like campylobacteriosis, and other enteric infections, also has the ability to cause post-infectious complications at extra-intestinal sites (*Cantey* et al., 2011). Sites affected include the eyes (*Pettoelo-Mantovani* et al., 1990; *Corsi* et al., 1998), the joints (*Borman* et al., 2001; *Carlson* and *Finger*, 2004), the skin (*Hardin* et al., 1997; *Di Prisco* et al., 1998; *Giacometti* et al., 2003; *Pietrzak* et al., 2005),

and on rare occasions, the muscles (*Cervelló* et al., 1993; *Addiss* and *Lengerich*, 1994; *Genovese* et al., 1996). These patterns of chronic post-infectious consequences again are similar to what has been reported for other enteric infections (Table 2).

CONCLUSIONS

Recent findings clearly demonstrate that the health consequences of enteric infections go far beyond their acute diarrhoeal symptoms, as they can lead to severe chronic post-infectious intestinal inflammatory disorders, failure to thrive, and serious growth and cognitive impairment. Moreover, the chronic sequelae may also cause post-infectious disease at extra-intestinal sites, including the joints, the skin, the eyes, the lungs, the heart, the muscles, the kidneys, and the central nervous system. The mechanisms responsible for these long-term effects remain obscure. However, findings from recent and ongoing research suggest that enteropathogen-induced disruptions of the commensal microbiota may at least in part play a role in triggering the sequence of events that result in these presentations. These disruptions include a promotion of the transcellular and paracellular translocation of non-invasive commensal bacteria, as well as disruptions of the composition and structure of microbiota biofilms. These in turn contribute, at least in part, to the activation of the host autoimmune reactions that are implicated in the production of post-infectious complications.

Beyond the well-established need to better understand host-pathogen cross-talks, as well as interactions between the host and its microbiota, these observations lay the foundations for future research into enteropathogen-microbiota interactions. This research will help better understand gut homeostasis, and will help unravel new pathophysiological pathways.

The direct benefits of microbiota are not well understood mechanistically. Most information is derived from the use of probiotics, and more recently the use of faecal microbiota transplant (FMT). With the recent decision of the Federal Drug Administration (U.S.A.) to call FMT a “drug” and hence to only allow its use under an Investigational New Drug application, (with a recently implemented exception for the treatment of *Clostridium difficile* infection), mechanistic insights into the now well-established beneficial effects of FMT may take a long time to become clear. This further underscores the need to uncover model systems that will allow to develop well-characterized, safe “synthetic microbiota” of “laboratory prepared” FMT-like cocktails.

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THE ROLES OF PATHOGENIC AND COMMENSAL BACTERIA IN THE INTESTINAL AND BEHAVIOURAL MANIFESTATIONS OF FUNCTIONAL GI DISORDERS

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SUMMARY

This paper reviews clinical and animal-based studies linking pathogenic microbes to the onset of functional GI disorders, and commensal microbes to the maintenance of these chronic disorders. The review will focus on post-infectious irritable bowel syndrome (PI-IBS). The link between acute gastroenteritis and PI-IBS is now well established on the basis of clinical studies. Risk factors include the severity of acute infection, pre-existing psychiatric morbidity and age <60. Host genetic determinants point to susceptibility loci in bacterial recognition, cytokine secretion and intestinal barrier function. Low-grade colonic inflammation is evident PI-IBS as well as a subset of IBS patients without prior gastroenteritis. Risk factors for IBS include infection, stress and antibiotic usage and are known to disrupt the intestinal microbiota. Recent work has shown that destabilization of the microbiota alters gut physiology, brain chemistry and behaviour in animals. Thus a vicious cycle is established in which the initial perturbation of the microbiota results in altered colonic function and the resulting change in the colonic environment selects for different bacteria. This microbial centred model of IBS accommodates key features of IBS including chronicity, variability in symptom expression, and psychiatric co-morbidity. Growing evidence of the therapeutic benefit of microbiome-directed therapies, including pre- and probiotics and as well as selected antibiotics, for this common condition, also support this model.

FUNCTIONAL GI DISORDERS

Functional gastrointestinal disorders are chronic abdominal symptom complexes for which there is no discernible underlying structural abnormality. The most common functional disorder is the Irritable Bowel Syndrome (IBS) that is characterized by abdominal pain or discomfort and altered bowel habit and is generally considered to reflect dysfunction of the gut-brain axis (*Camilleri* and *Di Lorenzo*, 2012). Up to 80% of patients exhibit behavioural changes that include anxiety, depression or somatization. There are no biomarkers and despite the availability of diagnostic criteria, the diagnosis is often made by exclusion of other pathologies. In the absence of an underlying cause, there is no cure and treatment is invariably symptom-based and of limited

efficacy. Together, these factors account for the very high socio-economic burden of IBS (*Maxion-Bergemann* et al., 2006).

It is now well established that acute enteric infection precedes the onset of chronic symptoms in a subset of IBS patients. It is estimated that between 5 and 32% of patients with acute gastroenteritis develop post-infectious IBS (PI-IBS). Bacterial infection with *Campylobacter, Shigella, Salmonella* or *Escherichia coli,* parasitic or viral infections have been associated with the development of PI-IBS. Risk factors include the existence of psychological disorders at the time of infection, the severity of infection and host genetic factors (for review, see: *Thabane* and *Marshall*, 2009). The latter includes polymorphisms in genes that encode bacterial recognition, cytokine secretion and the integrity of the intestinal epithelium (*Villani* et al., 2006). There is evidence of low-grade inflammation (without tissue destruction) in the colonic mucosa of PI-IBS patients and this may arise from inefficient down-regulation of the acute inflammatory response to the acute gastroenteritis (*Gwee* et al., 2003). Studies in an animal model of acute parasitic infection provide proof of concept that transient infection can induce persistent gut dysfunction that can be reversed by anti-inflammatory therapy (*Barbara* et al., 2001). A recent study showed that PI-IBS may last for at least 8 years post-infection but the factors that maintain gut dysfunction in the long term are poorly understood (*Marshall* et al., 2010). Attention now focuses on changes in the intestinal microbiota as a driver of immune activation, low-grade inflammation and symptom generation in IBS.

Factors known to trigger the onset of IBS include infection, stress and antibiotic usage (*Villareal* et al., 2012). Each of these factors has been shown to induce changes in the microbial composition of the gut. Antibiotics induce a transient disruption of the intestinal microbiota, but in some cases the effect may be long lasting (*Jernberg* et al., 2010). Stress has also been shown to disrupt the microbial community of the gut in animal models (*Bailley* et al., 2010, 2011). Acute gastroenteritis produces changes in the intestinal microbiota in humans (*Nelson* et al., 2010).

Disruption of the intestinal microbiota results in changes in gut function reminiscent of that found in IBS. For example, antibiotic-induced perturbation of the intestinal microbiota in mice resulted in a small increase in inflammatory activity in the gut mucosa and an increased response to visceral distension, interpreted to reflect hyperalgesia (*Verdu* et al., 2006). Similarly, disruption of the intestinal microbiota by antibiotics results in changes in gut transit mediated by Toll-like receptor-4 TLR-4 (*Anitha* et al., 2012). Thus, disruption of the intestinal microbiota changes the physiology and the physic-chemical properties of the colon; these changes in turn generate different selection pressures on the microbial community, resulting in further destabilization of the microbiota. As illustrated in Figure 1, the resulting vicious cycle produces inter-related changes in the microbial composition of the gut and in gut physiology, resulting in chronic dysfunction and symptom generation (*Collins* and *Bercik*, 2009). This model is supported by clinical observations showing (a) variation in symptom expression over time in IBS patients (*Mearin* et al., 2004) and (b) temporal instability of the intestinal microbiome in IBS patients (*Maukonen* et al., 2005; *Matto* et al., 2006). Interestingly, a recent study showed that duodenal instillation of faecal bacteria from healthy subjects delayed the development of

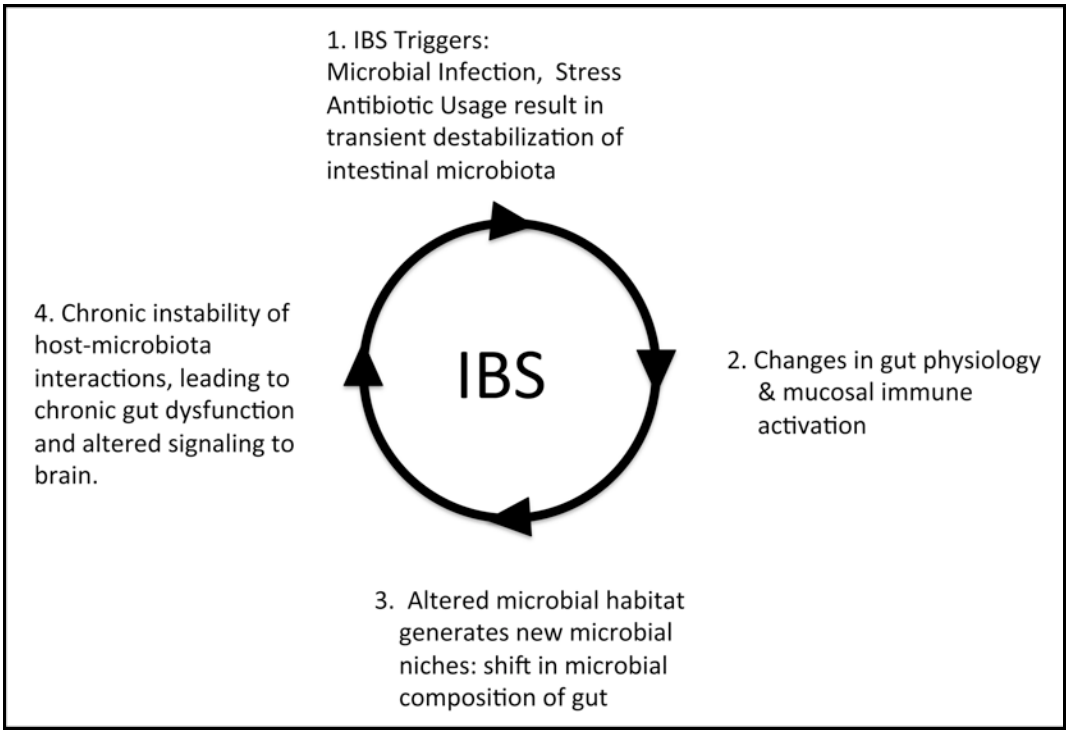

Figure 1: Schematic representation of a microbial centred model of IBS. Pathogenic microbes and other triggers cause an initial destabilization of the microbiota that creates a vicious and self sustaining cycle of host-microbial imbalance, resulting in chronic gut dysfunction and altered behaviour – IBS.

IBS symptoms in patients recovering from Giardia infection (*Morken* et al., 2009), supporting the notion that infection-induced destabilization of the microbiota plays a critical role in the expression of PI-IBS.

Up to 80% of IBS patients exhibit psychiatric co-morbidities that include anxiety and depression (*North* et al., 2007). Recent studies have shown that the intestinal microbiota influences brain chemistry and behaviour. Germ-free mice show less anxiety than colonized mice (*Neufeld* et al., 2011) and antibiotic-induced disruption of the intestinal microbiota resulted in changes in brain chemistry and behaviour (*Bercik* et al., 2011). Taken together these observations support the integration of the intestinal microbiome into the gut-brain axis, thereby providing a basis for considering the role of commensal bacteria in both the gastrointestinal and behavioural manifestations of IBS (*Collins* et al., 2012). The applicability of these animal-based studies to man is supported by the recent demonstration that probiotic bacteria alter brain activity in healthy human subjects (*Tillisch* et al., 2013).

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WHY BACTERIA MATTER: INSIGHTS FROM THE HYDRA HOLOBIONT

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SUMMARY

Animals, ranging from basal metazoans to primates, are engaged in symbiotic relationships with complex microbial ecosystems. These resident microbes influence fitness and thus ecologically important traits of their hosts, ultimately forming a meta-organism consisting of a multicellular host and a community of associated microorganisms. The evolutionary dynamics within such a meta-organism and the involved molecular interactions are rather complex and often difficult to investigate experimentally. Untangling the complex interactions requires simple animal models with only a few specific symbiotic partners. Here we show that organisms on at the base of the evolutionary scale such as the freshwater polyp Hydra may be key to dissecting the fundamental principles that underlie all host-microbe interactions.

FROM KARL AUGUST MÖBIUS TO THE HOLOGENOME THEORY OF EVOLUTION

In 1877, Karl Möbius, Professor of Zoology at Kiel University, coined the term “biocoenosis” for a community of living beings belonging to different species and associated by way of interspecies interdependence. In one of the first studies, later to become a classic, to be conducted in the emerging science of ecology, Möbius was seeking to determine why some oyster beds in the Atlantic were becoming exhausted, while the oyster beds in the British river estuaries and the Schleswig-Holstein oyster beds were very rich (*Möbius*, 1877). He related this phenomenon to the other species present, rather than to the oysters in the beds themselves. Möbius thus was the first to recognise that an ecological system must be taken as a whole and coined the term "biocoenosis" for a living community. About a hundred years later it became obvious that not only ecological systems but also complex “environmental” diseases can only be understood if the relationships between the interacting infectious agents present at a given time in a given territory are recognized. By analogy with “biocoenosis”, the understanding of a disease as a complex dynamic phenomenon was conceptualized with the word “pathocoenosis” (*Grmek*, 1969).

Today we realize that all epithelia in animals are colonized by microbial communities and that, therefore, any multicellular organism must be considered a meta-organism comprised of the macroscopic host and synergistic interdependence with bacteria, archaea, fungi, and numerous other microbial and eukaryotic species. The «meta-organism» concept (*Bosch* and *McFall-Ngai*, 2011) considers the dynamic

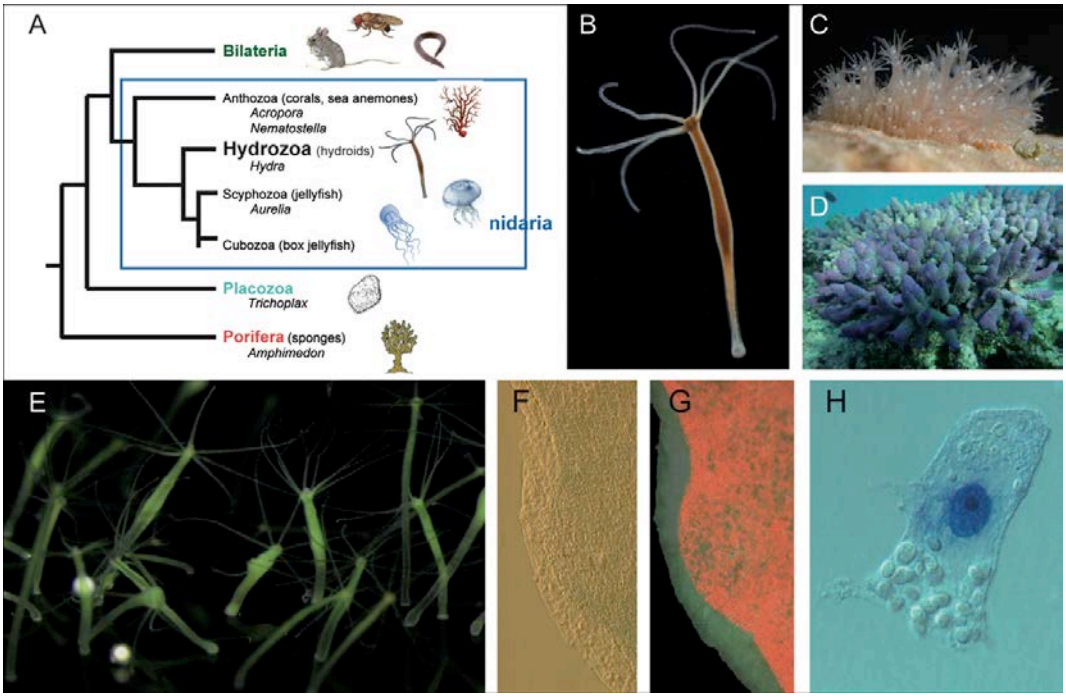

Figure 1: Cnidaria are a sister group of all Bilateria.
A: Phylogeny of basal metazoan animals.
B: *Hydra oligactis* (taken from Fraune and Bosch, 2007).
C: *Hydractinia milleri* (printed with permission from Gary McDonald).
D: Blue coral (taken from http://www.jcu.edu.au/cgc/CoralGenomicsHP.html).
E: *Hydra viridis* with Chlorella symbionts.
F: Phase-contrast micrograph of *Hydra viridis*.
G: Fluorescence microscopy of the same area shown in F. Chlorella algae appears red, Hydra tissue green.
H: Phase contrast micrograph of a single macerated endodermal epithelial cell containing symbiotic algae in the basal part below the nucleus (stained blue) (F-H taken from *Habetha* et al 2003).

communities of bacteria on epithelial surfaces as an integral part of the functionality of the respective organism itself. Today there is also an increasing appreciation that microbes are an essential part of the animal phenotype influencing fitness and thus ecologically-important traits of their hosts (*O'Hara* and *Shanahan*, 2006; *McFall-Ngai*, 2007; *Fraune* and *Bosch*, 2010). Disease onset is seen as a complex set of interactions among a variety of associated partners that affect the fitness of the collective holobiont (*Rosenstiel* et al., 2009). Discovering that individuals are not solitary, homogenous entities but consist of complex communities of many species that likely evolved during a billion years of coexistence led to the hologenome theory of evolution (*Rosenberg* et al., 2007, 2009; *Zilber-Rosenberg* and *Rosenberg*, 2008) which considers the holobiont with its hologenome as the unit of selection in evolution. Thus, modern symbiosis research has become an emerging cross-disciplinary field focused on understanding the general principles by which these complex host-microbe communities function and evolve.

What is the complexity in species number and structural organization of these associations? What is the physiological role of temporal differences of associated microbiota during life cycles? Which selective forces drive the evolution of these interactions, i.e. how do the associated organisms influence each other's fitness? Which forces shape the colonizing microbial composition? Finally, what are the consequences of the associations on molecular pathways and the reactive genomes? Here we show that for addressing these questions and untangling the complex interactions that influence the host's health and development, members of the ancient animal phylum Cnidaria may serve as simple but highly informative models.

THE HYDRA HOLOBIONT

Hydra is member of the animal phylum Cnidaria which are not only among the earliest known phyletic lineages known to contain stem cells (Figure 1A) but also possess most of the gene families found in bilaterians and have retained many ancestral genes that have been lost in *Drosophila* and *C. elegans* (*Kortschak* et al., 2003; *Miller* et al., 2005; *Technau* et al., 2005; *Putnam* et al., 2007; *Hemmrich* et al., 2012). Similar to other animals, Cnidaria are complex holobionts consisting of the animal and its associated endogenous microbiota. Inter-species interactions in several Cnidaria species (Figure 1A-1D) between symbiotic algae and host cells have been the subject of research since decades since they not only provide insights into the basic "tool kit" necessary to establish symbiotic interactions, but are also of relevance in understanding the resulting evolutionary selection processes (e.g. *Muscatine* and *Lenhoff*, 1963; *Pool*, 1979; *Thorington* and *Margulis*, 1981; *O'Brien*, 1982; for review see: *Bosch*, 2012a). In the meantime it is becoming evident that in Cnidaria such as green *Hydra viridis* **(**Figure 1E-1H**)** or many coral species, a long term persistence of mutualistic associations is prevalent not only in two-party interactions of polyp and symbiotic algae, but also in more complex systems comprising three or more associates including bacteria and viruses (*Bosch*, 2012a, 2012b). Thus, beside photosynthetic algae (Figure 1G-1H), bacteria are another important component of the cnidarian holobiont. In Hydra, the 36 identified bacterial phylotypes represent three different bacterial divisions and are dominated by Proteobacteria and Bacteroidetes (*Fraune* and *Bosch*, 2007, 2010). Disturbance or shifts in any of these partners can compromise the health of the whole animal (*Fraune* et al., 2009). Loss of symbiotic algae from coral tissues, for example, can lead to coral bleaching and death. Since healthy individuals of the same coral species from different location are colonized by similar bacterial communities (*Rohwer* et al., 2002) but diseased or bleached corals contain changed bacterial communities that differ greatly from healthy ones (*Ritchie*, 2006; *Rosenberg* et al., 2007), it seems that similar to complex "environmental" diseases in human, understanding diseases within corals requires an in-depth knowledge of the basic biology of each holobiont member.

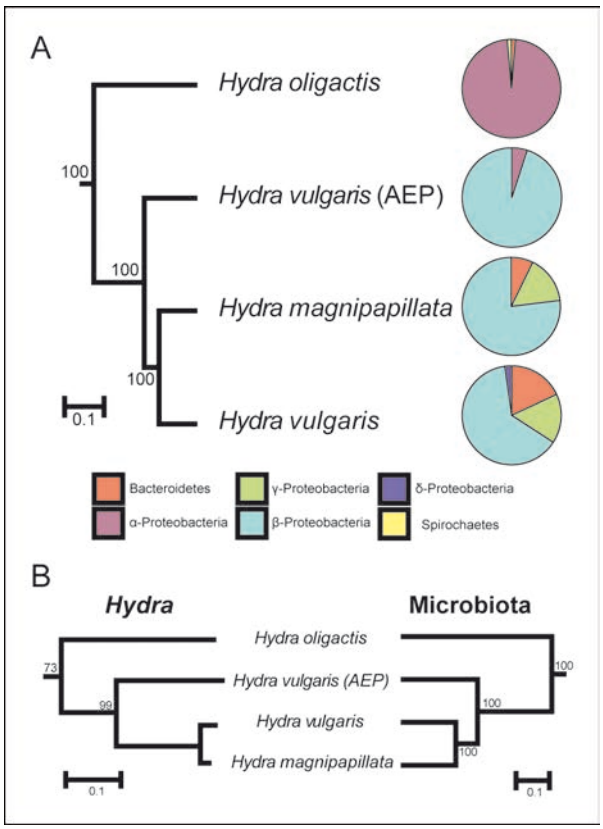

Figure 2: Hydra polyps are colonized by species-specific microbiota. A: Bacterial communities identified from four different Hydra species. B: Comparison of the phylogenetic tree from Hydra and the environmental cluster tree of the corresponding microbiota.

THE HOST ACTIVELY SHAPES THE COLONIZING MICROBIOTA

For decades a number of Hydra species have been cultivated under standard conditions at constant temperature and identical food. It came as a complete surprise, therefore, that examining the microbiota in different Hydra species kept in the laboratory for more than 20 years under controlled conditions revealed an epithelium colonized by a complex community of microbes, and that individuals from different species differed greatly in their microbiota. Even more astonishing was the finding that individuals living in the wild were colonized by a group of microbes that is similar to that in polyps grown in the lab, pointing to the maintenance of specific microbial communities over long periods of time. Bacteria in Hydra are specific for any given species (Figure 2A) (*Fraune* and *Bosch*, 2007; *Fraune* et al., 2010). Closely related Hydra species as *Hydra vulgaris* and *Hydra magnipapillata* are associated with a very similar microbial community. In contrast, *Hydra oligactis,* the most basal Hydra species analysed so far (*Hemmrich* et al., 2007), is associated with the most distinct microbial community compared to the other Hydra species. In line with this, comparing the phylogenetic tree of the Hydra species with the according cluster tree of associated bacterial communities reveals a

very similar microbial community. In contrast, *Hydra oligactis,* the most basal Hydra species analysed so far (*Hemmrich* et al., 2007), is associated with the most distinct microbial community compared to the other Hydra species. In line with this, comparing the phylogenetic tree of the Hydra species with the according cluster tree of associated bacterial communities reveals a high degree of congruency (Figure 2B). This strongly indicates that distinct selective pressures are imposed on and within the Hydra epithelium. The forces that shape the colonizing microbial composition are the focus of much current investigation (*Bevins* and *Salzman*, 2011).

HOW DOES THE HOST CONTROL THE MICROBIOTA IN THE CONTEXT OF SPECIFIC DEVELOPMENTAL OR ENVIRONMENTAL CONDITIONS?

In the same way that microbial communities are expected to change in different parts of a body, they are also dynamic in time. For a first understanding of the temporal dynamics in Hydra-microbe interactions we investigated the establishment of the microbiota during oogenesis and embryogenesis. Early embryonic stages in Hydra are colonized by a limited number of microbes (*Fraune* et al., 2010). During embryogenesis the number of bacterial colonizers changes in number and composition. For example, *Curvibacter*-related re present only in late developmental stages while they appear to be absent in the early embryo. Thus, early developmental stages have a microbiota that is clearly distinct from later developmental stages. Interestingly, the differential colonization is reflected in differences in antimicrobial activity. Hydra embryos are protected by a maternally produced antimicrobial peptide (AMP) of the periculin peptide family, which controls the establishment of the microbiota during embryogenesis. Beginning with the gastrula stage, Hydra embryos express a set of periculin peptides (periculin 2a and 2b), which replaces the maternal produced periculin peptides 1a and 1b. This shift in the expression within the periculin peptide family represents a shift from maternal to zygotic protection of the embryo (*Fraune* et al., 2011). In adult Hydra polyps, additional AMPs including hydramacin (*Bosch* et al., 2009) and arminin (*Augustin* et al., 2009) contribute to the host-derived control of bacterial colonization.

After hatching from the "cutical" stage the Hydra polyps get colonized by its specific bacterial community. The processes controlling community membership and influencing the establishment of the microbial ecosystem during development are poorly understood. Therefore, the microbial communities in polyps at various time points after hatching was profiled (*Franzenburg* et al., 2013). Distinct features included high diversity of community profiles in the first week, followed by progressive emergence of a stable adult-like pattern characterized by low species diversity and the preponderance of the Betaproteobacterium Curvibacter.

In adult Hydra polyps, additional AMPs including hydramacin (*Bosch* et al., 2009) and arminin (*Augustin* et al., 2009) and the bacterial signalling via MyD88 (*Franzenburg* et al., 2012) contribute to the host-derived control of bacterial colonization.

ANTIMICROBIAL PEPTIDES - KEY FACTORS FOR HOST-BACTERIA CO-EVOLUTION

Antimicrobial peptides (AMPs) are known as prominent effector molecules which get often secreted after external stimuli. Do they have, in addition to their killing activity against pathogens, key regulatory functions in host-microbe homeostasis as the driving force that leads to changes in microbiota composition? To investigate whether the ectotopic expression of an AMP may affect the number and composition of the colonizing microbiota at the ectodermal epithelial surface, we generated transgenic Hydra expressing periculin1a in ectoderm epithelial cells (*Fraune* et al., 2010). Comparing the bacterial load of these transgenic polyps with that of wild-type control polyps revealed not only a significantly lower bacterial load in transgenic polyps overexpressing periculin1a but also, unexpectedly, drastic changes in the bacterial community structure. Analysing the identity of the colonizing bacteria showed that the dominant β-Proteobacteria decreased in number, whereas α-Proteobacteria were more prevalent. Thus, overexpression of periculin causes not only a decrease in the number of associated bacteria but also a changed bacterial composition. With the transgenic polyps overexpressing periculin we apparently have created a new holobiont that is different from all investigated Hydra species. From these results we assume that specific associations between hosts and bacteria are a result of bacterial adaptation to different repertoires on AMPs in different host species. Evolutionary changes in the AMP repertoire of host species, therefore, are expected to lead to changes in the composition of the associated bacterial community. Future efforts will be directed towards analysing the performance of this new phenotype under different environmental conditions. Interestingly, patients with Crohn's disease often have strongly reduced α-defensin expression and drastically altered endogeneous microbiota (*Wehkamp* et al., 2005). Moreover, mice expressing human alpha-defensin-5 (DEFA5) and mice lacking an enzyme required for the processing of mouse alpha-defensins show significant changes in intestinal microbiota composition (*Salzman* et al., 2010). These findings support the view that epithelial-derived AMP may represent an important regulatory mechanism shaping the composition of epithelial microbiota.

WHAT ARE THE MICROBES FOR?

The intimacy of the interaction between host and microbiota, as well as the high evolutionary pressure to maintain a specific microbiota, points to the significance of the interkingdom association and implies that hosts deprived of their microbiota should be at a disadvantage. To investigate the effect of absence of microbiota in Hydra we have produced gnotobiotic Hydra polyps that are devoid of any bacteria. While morphologically no differences could be observed to control polyps, we are currently finding evidence that Hydra lacking bacteria suffer from fungal infections unknown in normally cultured polyps (*Franzenburg*, *Fraune* and *Bosch*, unpublished). Thus, do beneficial microbes associated with Hydra produce anti-fungal compounds?

Future efforts are directed towards isolating the active substances from these bacteria that eventually may lead to the development of novel antimycotics.

Microbes also provide signals for multiple developmental steps. One of the most pervasive examples of microbial impact in animal development is in the induction of settlement and metamorphosis of many marine invertebrate larvae (*Hadfield*, 2011). This transition is an absolute requirement for completion of the animal's life cycle, and is dependent upon induction by exogenous morphogenetic cues, many of which are produced by bacteria associated with a particular environmental surface. Hydractinia, for example, (Figure 1C) a marine colonial Cnidaria frequently found in the North Sea, commonly covers shells inhabited by hermit crabs. Fertile colonies, male and female, produce eggs and sperm, respectively, and within less than three days the fertilized egg develops into a mature planula larva. "Mature" larva means a larva that is able to metamorphose into a polyp, but under sterile laboratory conditions it will never do. It will rather die, as it is unable to take up food (*Leitz* and *Wagner*, 1993; *Walther* et al., 1996; *Frank* et al., 2001). To continue its development, it needs an external trigger that appears to be provided in the natural habitat by certain sedentary bacteria of the genus Alteromonas. A lipophilic substance produced by these bacteria is thought to act as this trigger (*Leitz* and *Wagner*, 1993). The mechanisms by which Hydractinia sense bacteria-derived environmental cues to form colonies and to reproduce may provide crucial insights into the genetic and developmental foundations of life cycles, but little is known about their natural history or biochemistry. Observations in a number of other invertebrates and vertebrates strongly support the view that microbes should be considered partners in animal development. Bacterial contributions are indispensable, for example, in shaping the immune system and development of organs such as the vertebrate intestine or the squid light organ (reviewed in *Fraune* and *Bosch*, 2010). Animal development has traditionally been viewed as an autonomous process directed by the genome. It seems that we have to rethink development at least in part, as an orchestration of both animal-encoded ontogeny and inter-kingdom communication.

THE HOLOBIONT IN A CONSTANTLY CHANGING ENVIRONMENT

The association between host and microbes is strongly affected by the environment. To determine the impact of different environmental conditions on the bacterial community in Hydra, we cultured polyps, which were taken from the wild, for two months under standard laboratory conditions. Thereafter, we analysed the associated bacteria in comparison to the bacteria from polyps taken directly from the wild. Culturing of polyps from the wild under laboratory conditions involves a change in culture temperature, culture medium and food source. These changes have significant effects on the composition of the bacterial community. For example, while one bacterial phylotype belonging to the α-Proteobacteria could be identified as the most dominant species in long term culture, in polyps from the wild and two month after the shift to the laboratory this bacterium was present only in relative low abundance (*Fraune* and *Bosch*, 2007). Other bacterial species completely disappeared from the tissue due to the change in culturing conditions. Thus,

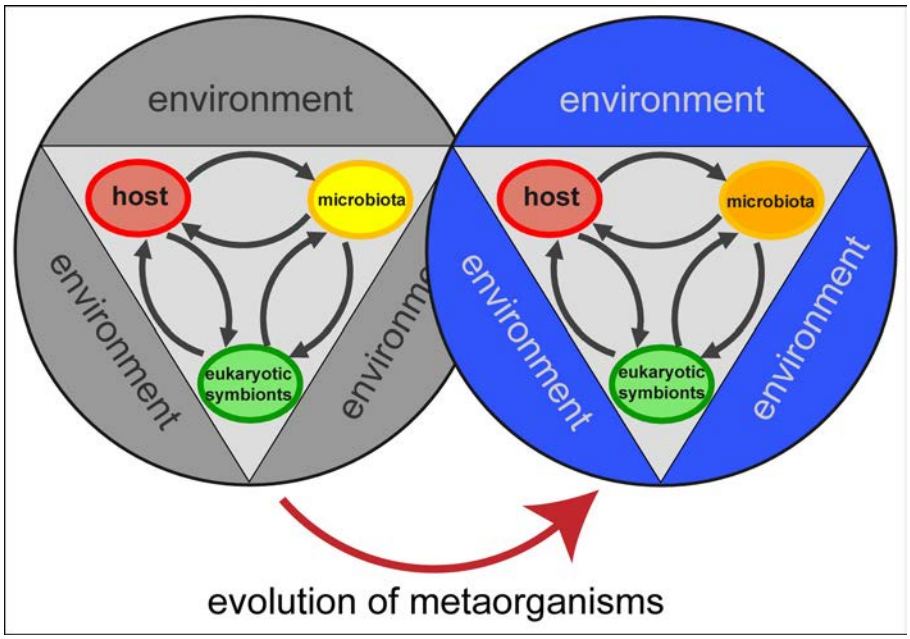

Figure 3: The meta-organism under changing environmental conditions

Hydra is not only associated with species specific bacteria but also responds to changes in the environment with changes in the bacterial community. In sum, the holobiont appears to be a dynamic system being characterized by functional redundancy and fast adaptations to altered environmental conditions.

Based on the holobiont concept, Rosenberg and colleagues in 2007 proposed that corals are able to adapt rapidly to changing environmental conditions by altering their associated microbiota (Figure 3) (*Rosenberg* et al., 2007). Depending on the variety of different niches provided by the host, which can change with developmental stage, diet or other environmental factors, a more or less diverse microbial community can be established within a given host species. Since this, for example, may provide corals with resistance against certain pathogens enabling them to adapt much faster to novel environmental conditions than by mutation and selection, host-microbe interactions may be considered as significant drivers of animal evolution and diversification. This hypothesis is supported by at least three observations: (i) corals are associated with diverse microbiota (*Rohwer* et al., 2002; *Bourne* et al., 2008); (ii) the associated microbiota change in response to environmental stress (*Ritchie* and *Smith*, 1995; Pantos et al., 2003) or seasons (*Koren* and *Rosenberg*, 2006); and (iii) corals are able to develop resistance against pathogens although they lack adaptive immune response (*Reshef* et al., 2006).

CONCLUDING REMARKS

The beneficial microbiota is a complex and multifunction ecosystem that is essential to the development, protection, and overall health of its host. Thus, the

microbiota appears to function as an extra organ, to which the host has outsourced numerous crucial metabolic, nutritional, and protective functions. Studies from Cnidaria to primates indicate that the host's role far outweighs other environmental factors in molding the composition of the microbiota. Antimicrobial peptides appear to be key factors for host-bacteria co-evolution and the driving force that leads to changes in microbiota composition. Finally, and maybe most important, the dynamic relationship between symbiotic microorganisms and environmental conditions results in the selection of the most advantageous holobiont. In corals, changing their microbial partners may allow them to adapt to changing environmental conditions much more rapidly than via mutation and selection (Figure 3).

Taken together, studying host-microbe interactions in basal metazoans is a challenging and exciting field of symbiosis research. Cnidaria not only offer valuable models for exploring the basis of interkingdom-communication and the role of bacterial signalling in animal development. Findings derived from the in vivo context of the Cnidaria models may also provide one of the simplest possible systems to address questions of how a stable host-microbe community is established and remains in balance over time. The uncovered basic molecular machinery can be transliterated to more complex organisms, providing conceptual insights into the complexity of host-microbe interactions. Symbiosis research in Cnidaria, therefore, is an emerging field in which scientists from many disciplines can make fundamental discoveries and rapidly advance scientific understanding of a strictly microbe-dependent life style and its evolutionary consequences while combining laboratory and field studies.

ACKNOWLEDGMENTS

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PERSISTING CONSEQUENCE OF INTESTINAL INFECTION: SUMMARY OF THE SEMINAR

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INTRODUCTION

Infectious diarrhoea is a global public health problem with high mortality and morbidity, particularly among children of the developing world. Each year, approximately 750,000 children under five years old die from severe, dehydrating diarrhoea and dysentery worldwide, and millions more are hospitalized, mostly in low-resource countries (*Liu* et al., 2012). In addition, many more children suffer from diarrheal disease-associated malnutrition and its adverse consequences on physical and cognitive development, which perpetuates the cycle of poverty.

While the brunt of the morbidity and mortality burden due to these infections are in the developing world, acute infectious diarrhoea is also a frequent cause for outpatient visits and hospitalization throughout the developed world and is a significant health problem. For example, Scallan and colleagues published recently updated estimates for foodborne illness in the United States (*Scallan* et al., 2011a, 2011b). Based on empirical modelling of active, passive and outbreak surveillance data it was estimated that each year 31 major pathogens acquired in the United States caused 9.4 million episodes of diarrhoeal illness, 55,961 hospitalizations, and 1,351 deaths. In addition, it is estimated that unspecified agents, resulting in 71,878 hospitalizations and 1,686 deaths, caused approximately 38.4 million episodes of domestically acquired foodborne illnesses.

While the acute consequences would appear to be of global significance and drive science and public health efforts to mitigate the problem, there is growing evidence linking such infections with a myriad of chronic health consequences including neurological, haematological and rheumatological systems (*Lindsay*, 1997). The accounting of these chronic health consequences beyond that of acute disease needs to be understood and considered in the global burden of disease assessment to inform policy and decision making around food safety and sanitation policy globally, and emphasize the reduction of enteric infection among those at high risk (e.g. travellers' and deployed military and children living in resource-poor environments) through primary and possibly secondary prevention strategies.

The difficulty of understanding how such infections may cause chronic health problems cannot be overstated, given the range of pathogens (viruses, bacteria, parasites), the genetic and acquired host factors, the often repeated infections in a person's life, and the interactions between complex neuroendocrine, immunological, and microbiological systems, many which are not well understood. But what is clear is that acute infections can colonize, invade and exert their effects locally and systemically at the individual level and chronic pathological changes to these organ systems have been noted. Thus,

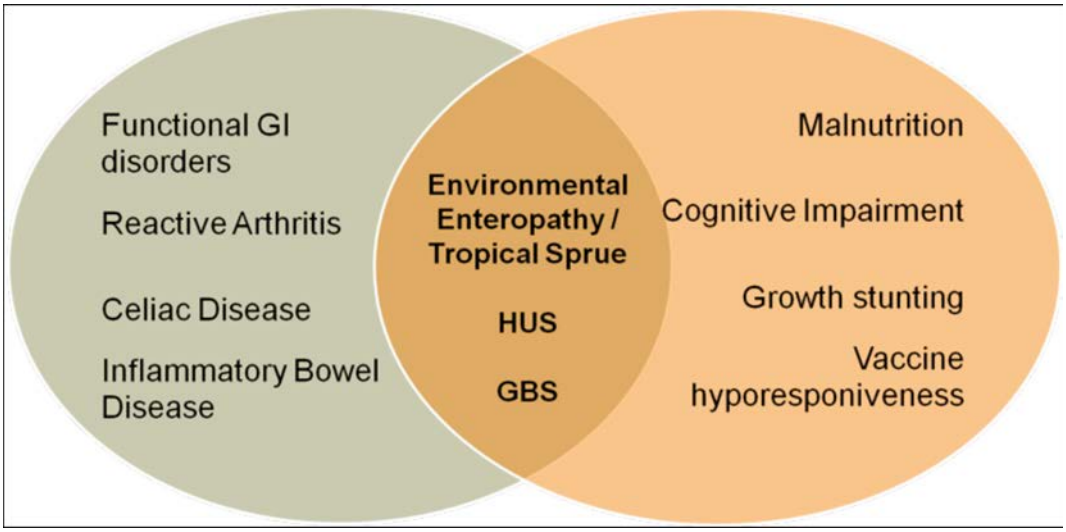

Figure 1: Persisting consequences of intestinal infection in developing (oval on right) and/or developed world (oval on left).
GI – gastrointestinal; HUS – haemolytic uremic syndrome; GBS – Guillain Barré Syndrome.

the aim of this conference was to assess our position in terms of epidemiological and pathological-aetiological understanding of the phenomenon, identify gaps in knowledge, and describe future directions related to the challenge of persisting consequences of intestinal infection.

EPIDEMIOLOGY: DEFINING THE PROBLEM

Epidemiological research is fundamental and complementary to our understanding of disease and development of primary, secondary and tertiary interventions. To put the current evidence in context, epidemiological research can identify knowledge gaps and define research priorities for increasing understanding in the areas of disease attribution, burden of disease, clinical characterization and management. A number of frameworks designed to elucidate the epidemiologic determination of causation have been advanced over the years (*Parascandola*, 2011). Koch's original postulates proved effective at establishing disease-pathogen relationships but fall short with more complex associations (*Evans*, 1976; *Marshall* et al., 1985). In recent years, Bradford Hill's criteria have been more commonly used to describe complex relationships and their epidemiology (*Bird*, 2011). Hill's criteria include strength of association, consistency of effect, specificity of effect, temporality, biological gradient or dose response, and biological plausibility to form the basis of an argument for causation and have been used successfully to establish the pathogenic role of *H. pylori*, HIV and toxins (*Szklo* and *Nieto*, 2007).

Emerging from the literature is an understanding that there are persistent consequences which are both common and unique to populations when stratified by geo-economic strata. In Figure 1 the overlapping ovals shows those issues that are primarily problems of the developed world (oval on left), while individuals in resource-poor settings suffer more from the problems

shown on the right. In the overlapping area is shown those problems currently understood to be more common to both populations. While such a divide is convenient and may be due to differences such as genetics, diet, and other unique environmental influences, it may also artificially de-emphasize possible similar pathogenic mechanisms and disease processes which may have different manifestations in a different human-environmental setting. Be that as it may, there is an emergence of evidence which describes disparate persisting consequences of acute enteric infections among those who have access to clean food water and sanitation, and those who do not.

POST-INFECTIOUS CONSEQUENCES: A DEVELOPED WORLD PERSPECTIVE

Despite food safety regulations, public education and advanced agricultural systems, occurrence of foodborne illness and other enteric infections in the developed world are not infrequent. In this seminar, Porter and colleagues (page 41) describe the utilization, challenges and opportunities of the US Department of Defense (DoD) medical encounter databases and serum repository in understanding of the problem of chronic consequences of enteric diseases. Historically, well-designed epidemiologic studies have been at the fore of linking exposures with outcomes and have enabled estimates of outcome risk, identification of host- and pathogen-specific risk factors, and understanding the timing from exposure to outcome. Studies of the post-infectious sequelae of enteric diseases are no different as highlighted by several systematic reviews on the epidemiologic evidence where functional gastrointestinal disorders appear to be most substantiated in addition to reactive arthritis (*Halvorson* et al., 2006; *Thabane* et al., 2007; *Deising* et al., 2013; *Pike* et al., 2013; *Poropatich* et al., 2010; *Porter* et al., 2013).

While these studies have enhanced our appreciation of the acute/chronic disease link, they have certain limitations. Epidemiological studies do poorly in informing disease mechanisms, are fraught with challenges in controlling for unmeasured factors and generally are only able to account for a fraction of explainable risk (though there are notable exceptions where the attributable fraction is stronger such as in the case of some strains of *Campylobacter* associated with GBS), and misclassification of both exposures and outcomes generally will bias associative estimates towards the null. Relatively rare events are also harder to study in rigorous cohort study designs without large and expensive studies. Despite these limitations, continued epidemiological studies from a diversity of populations and designs are needed to validate initial findings as well as explore new associations between acute enteric infections and the growing spectrum of illnesses.

An example of one such chronic health illness, coeliac disease, is an illustrative example of the complexity in which the immune system, the environment, and infection may interact to cause disease. In this seminar Murray (page 71) describes some unique observations on the pathogenesis and triggers of coeliac disease (CD), an increasingly common chronic disease affecting primarily the upper small intestine associated with significant morbidity and mortality in much of the developed world. While there is certainly a genetic predisposition to coeliac disease which is required, not everyone

with this predisposition will develop disease, and the onset of coeliac disease can occur at any time in someone's life suggesting an environmental triggering event. It appears that infection-colonization events in early life may have an impact on risk given the findings of higher risk of CD in individuals born via caesarean section (*Decker* et al., 2010; *Marild* et al., 2012), and weaning in the winter months (*Ivarsson* et al., 2003). These events can alter the nature of the microbiota. It has been clearly shown that microbiota are important for immunological maturation of the host (*Lanning* and *Knight*, 2005; *Sjogren* et al., 2009; *Chung* et al., 2012), but how the early constitution of an individual's microbiota can have affects such as susceptibility to the loss of tolerance a grain protein remains a mystery and opportunity to study.

Emerging evidence is also suggesting that CD may be triggered after an acute enteric infection in some individuals. Anecdotal reports and case series suggesting an association have been described (*Landzberg* and *Connor*, 2005; *Ginsburg* and *Bayless*, 2008; *Chae* et al., 2010). It has also been described that exposure to three or more infectious gastroenteritis events in young children at or around the time of introduction of follow-on formula was associated with a substantial increased risk of childhood diagnosis of coeliac disease (*Falth-Magnusson* et al., 1996). More recently, a case of a healthy subject who developed sudden irritable bowel syndrome (IBS)-like symptoms after a confirmed episode of *Campylobacter jejuni* enteritis was subsequently diagnosed with new onset CD (*Verdu* et al., 2007). Studies among the previously described DoD medical encounter system database have also recently been reported which support association between bacterial acute enteric infection (in particular *Campylobacter*) and the onset of CD. (*Riddle* et al., 2012). One explanatory hypothesis which needs to be confirmed is that *Campylobacter jejuni*, which has been shown experimentally to permit the translocation of normal non-invasive microflora (*Chen* et al., 2006; *Kalischuk* et al., 2009; *Kalischuk* and *Buret*, 2010), could trigger aberrant immune responses/loss of tolerance to co-transported luminal antigens, including gluten peptides, across the intestinal barrier. In certain susceptible individuals primed towards a mucosal immune response towards such antigens this could result in loss of tolerance to these antigens due to an inappropriate inflammatory response (*Jabri* et al., 2005; *Jabri* and *Sollid*, 2009). As demonstrated in animal models of gluten sensitivity (*Verdu* et al., 2008; *Natividad* et al., 2009), gastrointestinal infection may trigger or facilitate the onset of clinical CD, either by increasing intestinal permeability or enhancing uptake and dysfunctional anti-gliadin immune response in the genetically susceptible host (*DeMeo* et al., 2002; *Fasano* and *Shea-Donohue*, 2005).

POST-INFECTIOUS CONSEQUENCES: A DEVELOPING WORLD PERSPECTIVE

Human enteric infection in the developing world is different from that of the developed world on a number of features including the age of onset of infection (earlier), force of infection (multiple repeated exposures), variety of pathogens (more diverse), nutritional status of the host, as well as a number of other factors including co-infection, diet and genetics. As such, approaching

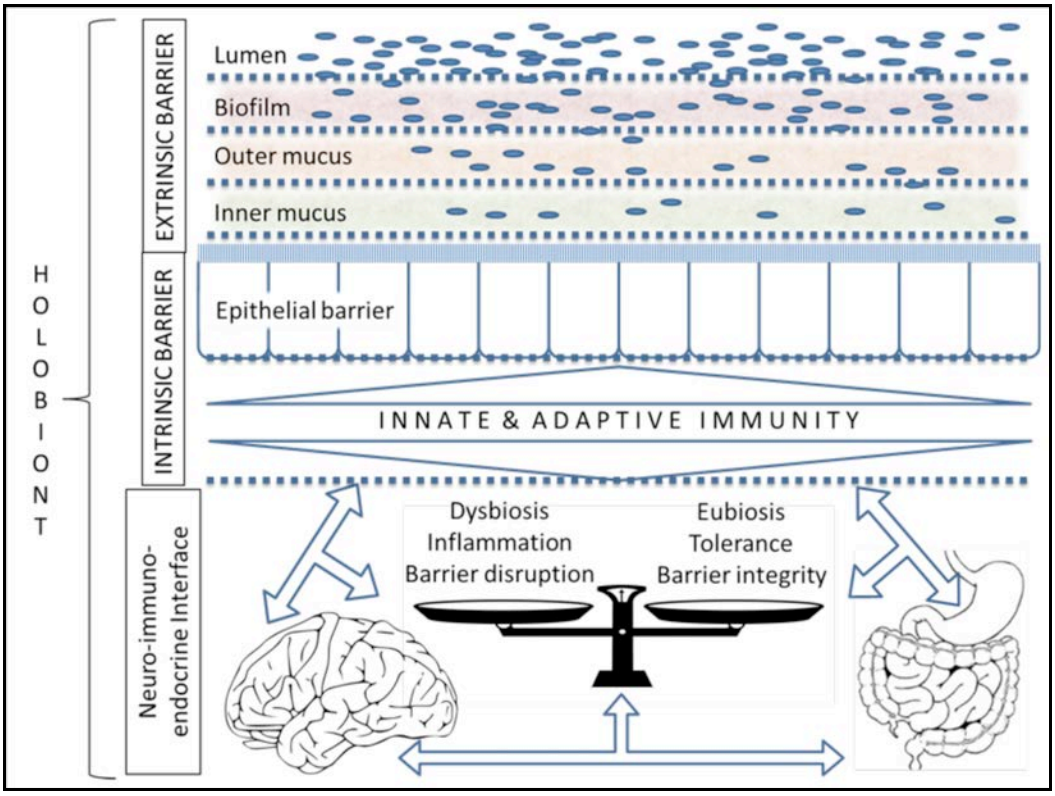

Figure 2. Framework to understand the patho-aetiology of persisting consequences of acute enteric infections.

an understanding of the link between infections and chronic illnesses in this population is challenged as the "one-infection" leads to "one outcome" approach is difficult to tease out. A unique study entitled the "Malnutrition and Enteric Infections network" ("MAL-ED") exploring the aetiology, risk factors and interactions of enteric infections on child health was presented with a unique design constructed for this environment (Malnutrition & Enteric Infections Network MAL-ED: The Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development. Project website (accessed Dec 10, 2013) http://mal-ed.fnih.org/). While results presented were preliminary and did not include cognitive and nutritional metrics, the results are shedding light on new insights on the often described "vicious cycle" of enteric infections leading to enteropathy leading to malnutrition and effects on growth, cognition and immunity, which themselves contribute to increased susceptibility to enteric infections (*Guerrant* et al., 2008; *Moore* et al., 2010). The results presented by McCormick and colleagues (page 23) expand on this conceptual model but also challenge our understanding of the significance of the repeated asymptomatic episodes of infections with disease-causing potential on growth and nutrition outcomes. It would appear that infection with pathogens which may or may not be associated with symptoms is the driving factor for the chronic consequences in these populations.

Expanding on the findings of MAL-ED, Petri and colleagues (page 61) described how recurrent infections, and in particular some combinations of protozoal and bacterial infections, can result in a subclinical condition of environmental enteropathy (EE) characterized by disruption of intestinal architecture and chronic inflammation. Such changes would appear to have direct effects on nutrition, the individual's ability to respond to additional infections, and evidence also suggests that such a condition may negate the valuable contributions of some orally-administered vaccines through evidence of hyporesponsiveness (see: Old Herborn University Seminar Monograph 24; Development of strategies to overcome barriers to effective mucosal immunization of infants in developing countries).

PATHOGENIC MECHANISMS: A GROWING FRAMEWORK OF COMPLEXITY

While epidemiological studies are increasing our confidence that there are real associations between enteric infections and chronic health effects, these data are limiting and only tell us that there is a problem and, given the magnitude, ought to do something about it. It is critical is to understand what is occurring in order to explain these observed associations. Based on presentations and discussions at this Old Herborn University Seminar, a framework has emerged by which to understand the problem (Figure 2). At the gut surface there is an increasing density and complexity gradient of microbial flora (both bacteria and viruses) that are found in the lumen of the GI tract in the oral to aboral direction. Within the lumen are also nutrients (taken in by the individual and produced by the flora), chemicals, antibiotics and non-digestible matter which shape and contribute in part to the diversity of this microbial milieu. There is also a microbiome gradient which exists from the lumen to the surface of the enterocyte where there is a decreasing density as you get closer to the cell surface. It is within this interface, often referred to as the extrinsic barrier which acts as a two-way filter, where many important processes take place including allowing nutrients to be absorbed and keeping harmful bacteria at bay. Underlying this extrinsic barrier is what is often referred to as the intrinsic barrier which is made up of a layer of host cells. While the figure is a simplification, this layer is not homogenous and includes a number of cell types which are derived from stem cells and include absorptive enterocytes, mucus-secreting goblet cells, entero-endocrine cells and Paneth cells. This cell layer is highly dynamic and includes complete turnover every few days and relies on a delicate balance between cell proliferation and cell death. This cell layer has a diverse set of functions which include production of antibacterial peptides, sampling of luminal contents, and absorbing important nutrients and fluid into the host circulatory system. This layer is at an interface between the luminal extrinsic barrier and the host innate and adaptive immune system which largely takes in the sub-epithelial level where neuro-endocrine, circulatory and cellular and humoral immune cells systems interface. While the basic systems involved are delineated in such simple terms, each of these systems are complex, and it is likely that it is the interaction of each of these systems which needs to be understood - like a puzzle which is made up of hundreds of other puzzles.

Several presenters revealed insight

into pathogenic mechanisms which have advanced our understanding of a piece of the puzzle. Buret and colleagues (page 87) reported on recent findings from an animal model suggesting that enteropathogen-induced disruptions of the commensal microbiota have a part play in triggering the sequence of events that result in various intestinal and extra-intestinal chronic health outcomes. Data from animal models of campylobacteriosis and giardiasis suggest that infection by enteric pathogens may promote the transcellular and paracellular translocation of non-invasive commensal bacteria, as well as disruptions of the composition and structure of microbiota biofilms. Such actions then lead to the activation of the host autoimmune reactions that are implicated in the production of post-infectious complications. Beyond the well-established need to better understand host-pathogen cross-talks, as well as interactions between the host and its microbiota, these observations lay the foundations for future research into enteropathogen-microbiota interactions and highlight that the mechanism of intestinal dysbiosis (a process whereby an enteric pathogen disrupts the normal commensal flora such that an imbalance among microbial populations occur on a body surface, often with deleterious effects on health) is a central piece of the puzzle to solve. The availability of animal models like these is quite useful to help better understand gut homeostasis.

Building on this theme (and adding an additional layer of complexity), Collins (page 107) describes the phenomenon of acute enteric infections and functional gastrointestinal disorders as an important model to understand the relationship between the gut-brain axis, the microbiome and acute infection. In simple terms, infection, stress and antibiotics (alone or in combination) can have a disrupting effect on the balance within the gut epithelial barrier and microbiome. This disturbance in susceptible individuals can result in abnormal immune activation which may further alter the balance of the microbiome in the gut (dysbiosis). This dysbiosis may have direct effects on the neuroendocrine system with central effects on the brain (perception/pain), as well as local effects on the enteric system (e.g. motility). While studies have shown that risk for functional disorders are associated with psychiatric co-morbidities, the opposite has been described in animal models where microbiome changes can change behaviour and brain neurochemistry (*Bercik* et al., 2011). Finally, it must not be forgotten that stress itself can have an impact on the gut epithelial barrier and increase risk of infections including enteric infections, which in combination with stress appear to increase the risk of functional disorders (*Lyte* et al., 2011; *O'Malley* et al., 2011). Thus, in our framework of understanding, it is important to consider the impact of the important two way cross-talk between the brain and the gut (see: Old Herborn University Seminar Monograph 26; The gut microbiome and the nervous system), and how these interact with the microbiome to result in dysbiosis and disease associated with nervous system disturbances.

Finally, Chang and colleagues (page 13) described evidence from a *Campylobacter* model in rats whereby infection, potentially through molecular mimicry, can have an effect on gastric motility, which in itself may lead to a type of dysbiosis where the GI system's ability to maintain healthy levels of bacteria in the small intestine can be disrupted. The findings of this model suggest that there may be multiple mechanisms by where a single pathogen my cause a chronic health consequence.

THE FUTURE: UNDERSTANDING COMPLEXITY AND EXPLORING NEW FRONTIERS

As described, each of the systems that may influence the determination of persistent infectious consequences is independently complex, and together the complexity is compounded. Clearly, better tools and model systems are needed to piece together the puzzles. One such tool which may have potential is in the area of intestinal stem cell research described by van der Flier and colleagues (page 1). The stem cell intestinal epithelium model represents a unique opportunity to study adult stem cell biology and lineage specification. The combination of a rapid self-renewing tissue, evident compartmentalization of proliferating and differentiated cell types and a relatively simple, repetitive tissue architecture, is ideal for the visualization and identification of stem cell types, cell fate specification and cellular behaviour. In the past, genetic studies in mice have created a wealth of new insights on the biology of the intestinal epithelium. The recently established long term culture conditions of intestinal epithelium, especially mini-organs from human origin, may boost the research field for the next generation by providing a variety of possibilities for research and therapeutic applications of intestinal biology. The system in its current construct lacks the enteric neuroendocrine and microbiome integration, but additions of such components may be feasible and add to the utility.

New data challenge the way we understand the relationship between infection and illness. It is apparent that even though one can recover a pathogen from an individual it is often noted that that pathogen is not causing illness, which begs the question of is it a pathogen? Clearly understanding how asymptomatic infections with pathogenic organisms affect the holobiome is an important gap. In addition, with the increasing number of chronic health problems in which epidemiological associations are being found, we are challenged by understanding how big the problem is or may well be. Microbiome changes are now being associated with obesity and liver disease in developed world populations (*Henao-Mejia* et al., 2013; *Karlsson* et al., 2013; *Zhao*, 2013). Natural questions which follow include what are the factors behind these changes and could infection be one of them? Epidemiological studies and perhaps animal models could be directed at looking for such associations. A study of certain value would be to conduct a developed world post-infectious microbiome cohort studies among populations at high risk for acute enteric infection (e.g. travellers). Evaluating baseline microbiome as well as changes that may occur with travel, travel-related infections and antibiotics, and the persistence of such changes which may be linked to well defined post-infectious functional gastrointestinal disorders may offer new insights.

Finally, we need a framework which approaches the problem not only by considering the contributions of pathogens, or the host or the commensal flora, but rather as an integrated system. The concept and importance of the holobiont has emerged over the last two decades. Bacterial cells outnumber human cells by a factor of 10 to 1, and the collective genes of these bacteria outnumber our genes by 150 to 1 (*Qin* et al., 2010). We know that these bacteria are critical to our survival and include important functions such as food break down, biotransformation of nutrients, development and function of a

Box 1: Remaining gaps in our understanding and questions for future directions.

- How does the viriome interact and what of the effects of viral enteric infection on the viriome and bacteriome and vice versa?
- How do antibiotic challenges to the system interact?
- What is the relationship of enteropathy with nutrition? Can complex nutrient effects be isolated and rebalanced through nutrition?
- What is the impact of environmental chemicals on the microbiome and host factors associated with disease?
- How do neuro-immune interactions work?
- What is the nature of inflammation in various disease models? Why is inflammation sometimes healthful and sometimes deleterious?
- How do we facilitate inflammation resolution (not stopping, but helping resolution)?
- What could generational/adoptive studies teach us about the association between acute enteric infection and chronic health consequences?
- What are the short-term opportunities to treat these diseases? For example what role might faecal microbiota transplant play? Could well-characterized synthetic faecal microbiome products be useful? What might the value of microbiome feeding and topical anti-inflammatories be?
- Can continued epidemiological studies from a diversity of populations and designs validate initial findings as well as explore new associations between acute enteric infections and the growing spectrum of illnesses?
- How does *Campylobacter jejuni* trigger aberrant immune responses/loss of tolerance to co-transported luminal antigens across the intestinal barrier?
- How does the early constitution of an individual's microbiota have affects such as susceptibility to the loss of tolerance a grain protein?
- How do enteropathogen-microbiota interactions trigger intestinal dysbiosis (a process whereby an enteric pathogen disrupts the normal commensal flora such that an imbalance among microbial populations occur on a body surface, often with deleterious effects on health)?
- Could microbial toxins from enteric pathogens like ETEC and *Campylobacter* contribute to microbiome disruption and its consequent effects?

normal immune system, angiogenesis, and regulation of fat accumulation (*Singh* et al., 2013). In addition to these functions, the microbiota compete for space with pathogens and can exert other local effects to protect the host from infections. The microbiota is also adaptable which provides an advantage to the host which may need to rely on the flexibility of the microbiota structure to adapt to changes of diet or climate or infection. Bosch and colleagues (page 113) describe an elegant system in Hydra, where the microbiota is a complex and multifunctional ecosystem that is essential to the development, protection, and overall health of its host. Furthermore the dynamic relationship between symbiotic microorganisms and environmental conditions results in the selection of the most advantageous holobiont. Systems like these may provide tools to further study and explore and potentially lead to insights to prevent the unintended consequences of holobiome disturbances.

CONCEPTS AND QUESTIONS FOR FURTHER CONSIDERATION

Continued epidemiological studies from a diversity of populations and designs are needed to validate initial findings as well as explore new associations between acute enteric infections and the growing spectrum of illnesses. Studies are also needed to better understand the mechanism by which an enteropathogen such as *Campylobacter jejuni* which induces disruptions of the commensal microbiota can affect the sequence of events that result in various intestinal and extra-intestinal chronic health outcomes. Future research into enteropathogen-microbiota interactions should highlight dysbiosis as a central piece of the puzzle to solve. There may be multiple mechanisms by which a single pathogen my cause a chronic health consequence. Understanding how asymptomatic infection with pathogenic organisms affects the holobiome is an important gap. How does the viriome interact and what of the effects of viral enteric infection on the viriome and bacteriome and vice versa? Non-infectious events can also contribute to the problem of dysbiosis, as stress and antibiotics (alone or in combination) can have a disrupting effect on the balance within the gut epithelial barrier and microbiome

Clearly better tools and model systems are needed to help piece together the puzzles with which we are confronted (Box 1). Although these questions are challenging, the benefits to human life which can be achieved make the undertaking well worth the effort.

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