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**GUT MICROBIAL METABOLITE INDOLE: A STIMULATOR OF  
ENTEROENDOCRINE CELL DIFFERENTIATION VIA ACTIVATION OF ARYL  
HYDROCARBON RECEPTOR**

A thesis submitted to  
Marshall University  
in partial fulfillment of  
the requirements for the degree of  
Master of Science  
in  
Biomedical Research  
by

James Hart

Approved by

Dr. Alip Borthakur, Committee Chairperson

Dr. James Denvir

Dr. Richard Egleton

Marshall University  
August 2023

## Approval of Thesis

We, the faculty supervising the work of James Hart, affirm that the thesis, *Gut Microbial Metabolite Indole: A Stimulator of Enteroendocrine Cell Differentiation via Activation of Aryl Hydrocarbon Receptor*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences Department and the Joan C. Edwards School of Medicine. The work also conforms to the requirements and formatting guidelines of Marshall University. With our signatures, we approve the manuscript for publication.



07/06/2023

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## **Abstract**

Enteroendocrine cells (EECs) regulate energy balance and glucose homeostasis by releasing hormones in response to food intake. Dysregulated EEC differentiation is observed in obesity, while gut microbiota metabolites influence this process. Here, we investigated the role of indole, a biologically active gut microbial metabolite, in EEC differentiation through aryl hydrocarbon receptor (AhR) activation. Human intestinal organoids derived from jejunal mucosal biopsies were exposed to indole. Indole treatment significantly increased mRNA levels of chromogranin A, an EEC marker. The effect was reversed by an AhR antagonist, indicating AhR involvement. Indole also upregulated AhR target gene mRNA levels. These findings highlight the potential of AhR activation as a novel pathway by which gut microbial metabolites, such as indole, stimulate EEC differentiation. Understanding the mechanisms underlying EEC differentiation may offer insights into therapeutic strategies for metabolic disorders associated with impaired EEC function.



The human gastrointestinal (GI) tract is the passage by which food travels through the body. Along this tract, a single layer of epithelial cells lining the lumen plays pivotal role as a protective barrier resisting invasion of luminal microbes and other harmful substances. Further, the intestinal epithelial cells (IECs) are critically required for the absorption of the nutrients derived from food digestion. Nondigestible materials of the food transit to the large intestine (colon) wherein they are fermented and utilized by colonic microbiota. Additionally, the human intestinal epithelium is involved in a range of physiological processes beyond absorption such as metabolic balance (Meyer & Duca, 2023). For instance, the GI tract is involved in the sensing of ingested food and responding to it via secretion of various gut hormones. These gut hormones are carried via circulation to various target organs to regulate insulin secretion, gastric transit and motility, and appetite (Gribble & Reimann, 2019). In order to accomplish various functions, the intestinal epithelium is composed of four major cell types: mucus secreting goblet cells, hormone secreting enteroendocrine cells (EECs), antimicrobial secreting Paneth cells, and nutrient absorbing enterocytes (Cheng & Leblond, 1974). These cells exist within a crypt stem cell system of renewal and differentiation, where stem cells residing in crypts are differentiated to the various cell types depending upon different factors, such as the cell fate controlling developmental Wnt signaling and Notch signaling pathways (Gasnier et al., 2023). Additionally, gut microbial metabolites have been shown to influence epithelial cell differentiation from stem cells (Zhou et al., 2023). One important cell type is the enteroendocrine cell (EEC), specialized cells responsible for secreting hormones that play a vital role in the regulation of energy balance and glucose homeostasis (Kaelberer & Bohórquez, 2018). EECs can be found throughout the intestinal tract. In response to food intake, they release incretin hormones such as glucagon-like peptide 1 (GLP1) and glucose-dependent insulinotropic peptide (GIP) (Drucker, 2006). The

effects incretin hormones have on the body are numerous, including glucose and insulin homeostasis and the neurological balance of hunger and satiety (Nauck & Meier, 2018). This makes them highly relevant molecules for human metabolic health.

### **Prevalence, Pathogenesis, and Associations of Obesity**

Among the various human health conditions relevant to EEC function is obesity. With a 41.9% prevalence among adults in the United States, obesity is a very common condition (Stierman et al., 2021). The association of adult obesity with other conditions such as hypertension, type 2 diabetes, cardiovascular disease, and cancer has been very well established by the scientific community (National Heart, Lung, and Blood Institute, 2013) (Bhaskaran et al., 2014). Childhood obesity has also been more specifically correlated with psychological problems such as anxiety and depression (Morrison et al., 2015) (Halfon et al., 2013). The connection between obesity and its comorbidities is the reason behind the mortality rate increase for people with a Body Mass Index (BMI) falling in the obese range (Zheng et al., 2011).

The pathogenesis from obesity to type 2 diabetes is a commonly studied obesity complication. One study found, there are various cytokine signaling pathways that are dysregulated in obesity (Esser et al., 2014). More specifically, suppressors of cytokine signaling (SOCS) proteins have been shown to be downstream inhibitors of insulin receptor, contributing to insulin resistance and type 2 diabetes; their expression is induced by cytokines such as interleukin-1 beta, and interleukin-6 (Mooney et al., 2001), known to be elevated during obesity (Mohamed-Ali et al., 1997) (Hotamisligil et al., 1993). This example is but one of many mechanistic ways the pathogenic condition of obesity is known to lead to systemic dysregulation of key human biological processes.

## *Pathogenesis of Obesity*

As shown, obesity is a condition that is debilitating to human health, 41.6% of adults with disabilities have obesity (Centers for Disease Control and Prevention, 2016). In order to comprehensively understand the role of EECs in obesity, first a broad overview of the pathogenesis of obesity will be established, followed by a more detailed examination of the physiological changes related to the gut and EECs. Specifically, the endocrine and metabolic syndromes associated with the pathogenesis of obesity will be the focus.

Obesity is a complex multifaceted disorder characterized by excessive storage of fat and adipose tissue. From a physical perspective, the condition arises when there is an imbalance between energy intake and expenditure of the body, resulting in storage of energy in the form of fat. The pathogenesis of obesity involves a complex combination of genetic, environmental, and behavioral factors, that contribute to the initial development and the progression of the disease.

Clinically, obesity is typically defined in adults over the age of 20 years, as a person having a Body Mass Index (BMI) greater than 30 (WHO, 2023). BMI is a simple method clinicians use to estimate a person's relative body fat. It is calculated as (Davies & Lucas, 1989):

$$BMI = \frac{Weight(Kg)}{Height(m)^2}$$

The BMI scale has shown to have a strong specificity (95-99%) for identifying actually obese patients but a significantly lower sensitivity (36-49%) (Romero-Corral et al., 2008). This means while the scale seldom leaves obese patients in the healthy range, it has the potential to misdiagnose healthy patients as obese. Given the scale only accounts for weight and height, disregarding body composition, healthy individuals with great muscle, bone, or water mass, may still be diagnosed as obese making the scale imperfect. While alternative techniques for

diagnosing BMI are used in various clinical settings, such as waist-to-height ratio (Park et al., 2009), BMI is still widely accepted as the standard for diagnosis of obesity by most institutions.

### *Genetics in Obesity*

Naturally, genetics are the initial factor of consideration when understanding a person's path to obesity, as they lay the foundation for susceptibility to the disease. It has been shown through a large-population study that obesity has a moderate-to-high heritability across its varying phenotypes; with the lowest heritability being insulin at 47% and the highest being BMI at 78% (Van Dongen et al., 2013). In agreement, twin studies comparing monozygotic and dizygotic twins, have shown a powerful impact of genetics on the development of obesity. One study with a sample size of 5092 twin pairs showed an approximate 40% independent contribution to developing an obese BMI (Wardle et al., 2008).

A deeper exploration of genetics contributing to obesity uncovers a clear association of particular genotypes of energy balance related genes with BMI. For example, in a 2021 meta-analysis, five polymorphisms of the appropriately named fat mass and obesity-associated gene (FTO), have been identified significantly with an increased risk of obesity (Ali et al., 2021). The FTO gene encode a transcription factor protein which works as Deoxyribonucleic acid (DNA)/ Ribonucleic acid (RNA) methylase that depends on Iron(Fe) (II) and 2-oxoglutarate (Jia et al., 2008). Its inactivation, via genetic knock-out (KO) study, was shown to protect against obesity, although additionally stunting normal growth and lean body mass, in mice (Fischer et al., 2009).

Furthermore, some specific single gene mutations or polymorphisms have been shown to cause obesity. Specifically, inactivating mutations of either the leptin (LEP) or leptin receptor (LEPR) genes cause uncontrollable food consumption and lead to obesity (Dubern & Clement, 2012). Although obesity cases caused by monogenetic mutation are rare (5-7% of obese

children) (Farooqi & O’Rahilly, 2004), they highlight how individual genes can contribute so much to the disease of obesity, that they can be fully responsible. These various effects of genetics on the development of obesity suggest that gene polymorphisms associated with increasing appetite or decreasing energy expenditure hormonally, majorly account for the heritability of the disease. Otherwise, childhood obesity can arise in a heritable pattern via the passing down of poor eating and health habits from parents (Kansra et al., 2020).

### **Hormone Secretion in the Gut**

Resting upon this concept is the role of EECs in the development of obesity. As stated, EECs respond to the presence of food in the intestine by releasing hormones (Drucker, 2006). Hormones released by EECs work synergistically to increase satiety, promote digestion, and release of insulin (Pizarroso et al., 2021). Genetically, polymorphisms of these hormones and their associated receptors may vary in regard to their binding affinity or concentrations in blood, and subsequently the magnitude of their physiological effect. For example, the polymorphism rs17782313 near melanocortin-4 receptor (MC4R) gene has been shown to be associated with a reduced effect of the EEC released GLP-1 and leads to greater food intake (Pizarroso et al., 2021).

Aside from genetic variation, EEC released hormones including: GLP-1, cholecystokinin (CCK), peptide YY (PYY), oxyntomodulin (OXM), and GIP (Pizarroso et al., 2021) have well established roles in promoting satiety and regulating appetite, cementing the role of EECs as key players in the process.

Intravenous GLP-1 injection given to mice has been shown to strongly inhibit appetite and reduce body mass (Pizarroso et al., 2021). This effect occurs via regulation of postprandial

glucose homeostasis (Gribble & Reimann, 2021), but additionally through other direct mechanisms affecting glucose metabolism outside of the pancreas (Gribble & Reimann, 2021).

While CCK has been shown to trigger the release of hormones from the pancreas and gallbladder contractions, which is critical for digestion of food (Okonkwo et al., 2023), it is also known that the hormone is associated with increased satiety in humans at pharmacologically relevant dosages (Warrilow et al., 2023).

Similar to GLP-1, PYY is tied directly to appetite control. PYY was also shown to reduce appetite, adiposity and body weight following peripheral injection in mice (Renshaw & Batterham, 2005). Receptors of PYY can be found in the arcuate nucleus of the hypothalamus; when PYY binds its receptor in this location it directly inhibits these neurons postsynaptically in mice (Riediger et al., 2004). Conversely, these neurons are stimulated via increased calcium signaling by ghrelin (Kohno et al., 2008), a hormone released by the stomach when empty, known to significantly increase food intake when intravenously dosed to human anorexia nervosa patients (Hotta et al., 2009), and to healthy volunteers (Wren et al., 2001). Thus, increasing the inhibition of neurons in the arcuate nucleus via PYY postsynaptically is a desired outcome when treating obesity.

Furthermore, OXM has been shown to reduce food intake and weight gain when intravenously dosed to rodents as well (Dakin et al., 2004). Each of these findings suggest that an important function of EEC hormone secretion in response to the presence of food is to inhibit further food intake. Logically, a disruption or decrease in this response could lead to impairment of a person's overall energy balance, via removal of an important negative feedback loop for eating. Intriguingly, such compromises to EEC function exist in the condition of obesity.

## **Physiology of Differentiation in the Intestinal Epithelium**

As previously stated, cells of the intestine exist in stem cell niches where cells are renewed by stem cells and differentiated into absorptive or secretory cells in response to various factors and molecular gradients (Haber et al., 2017). Figure D1 diagrams this process, the various lineages, and the possible cell fates in the intestine (Sundaram & Borthakur, 2021).

Intestinal stem cells (ISCs) lie in invaginated crypt structures and exist throughout the intestinal epithelium. Villi structures exist in the small intestine providing increased surface area for absorption (Gehart & Clevers, 2018). Within these crypts intestinal stem cells are afforded some level of protection from substances in the intestinal lumen. Primarily through a signaling pathway named wingless (Wnt) signaling, stem cells are kept in their naive state and proliferation is promoted. However, when the Wnt signaling pathway is blocked by genetically knocking out T-cell factor 4 (Tcf-4) in mice, stem cells are lost in the intestine (Korinek et al., 1998).

These stem cells normally divide symmetrically and move upwards into a region called the transit amplifying compartment via competition for limited space in the crypt niche (Snippert et al., 2010). Initially, in the transit amplifying compartment ISCs make contact with a range of initial transcription factors which work to modify chromatin accessibility via epigenetic regulations such as histone modification (Verzi & Shivdasani, 2020). These modifications either enable or restrict access to enhancer regions of the genome, and subsequently increase or decrease gene transcription activity based upon the specific region the epigenetic modification was made (Atlasi & Stunnenberg, 2017). This activation or inhibition of specific gene activity begins to set the specialized lineage ISCs will follow and differentiate into.

It is well established that this system of progeny and differentiation is internally regulated by the Wnt and notch signaling pathways (Yin et al., 2014). However, various molecules from food or drugs, which enter the body from the outside are known to modify epigenetic regulation and subsequently gene expression (Niculescu, 2014). For example, when in-vitro models of the ISCs niche and differentiation, known as intestinal organoids, are treated with a drug known to inhibit the protein Exportin-1 (a protein involved in transporting cargos out of the nucleus of cells), a significant increase in the differentiation to Paneth cells was observed (Mead et al., 2022). Moreover, obesogenic diet in mice has been shown to lead to hyperproliferation of ISCs and impairs the relative quantities of absorptive enterocytes and enteroendocrine cell (EEC) subtypes (Aliluev et al., 2021).

Interestingly this study from Aliluev et al., 2021, shows these changes to ISCs proliferation and differentiation in response to high-fat and high-sugar diets, rather than a consequence of obesity. This is of great relevance as humans with obesity are known to have a decreased relative numbers of EECs (Wölnerhanssen et al., 2017). These findings, along with similar results (L. Ye et al., 2019) (Osinski et al., 2020) showing long term change in intestinal cell progeny via epigenetic regulation, provide insight into the underlying physiological changes that contribute to energy imbalance in obesity.

With proven pharmacological access to the determination of intestinal cell fate (Mead et al., 2022), correcting for these dysregulations becomes an important and feasible facet of treating obesity. However, as obesity is a chronic disease (Gossmann et al., 2021), it is crucial to take a closer examination at what steps can be taken to prevent the disease state from reoccurring. Thus enters the role of gut microbiota in regulating long term homeostasis of intestinal cell proliferation, differentiation, and function.



## **Development and Pathophysiology of the Human Gut Microbiome**

Along the human GI tract exist communities of microbes ranging from bacteria and fungi to viruses known as the microbiome (Prescott, 2017). While other microbes play important roles in human health, the present study will focus on bacteria. This choice was made as a result of the clinical relevance and quantity of bacteria in the human intestine. It is known that there are approximately 10 times as many bacteria cells residing in the average human body than there are human cells (Gill et al., 2006).

A range of bacterial types reside and reproduce healthily in the human intestine and have a large array of effects and relationships with the human body. Generally, certain types of bacteria exist in long term mutualistic relationships with the human body and play roles in the functions of food digestion, nutrient absorption, immune response, and protection from pathogens (Bäckhed et al., 2005). Here the key roles of these microbiota in human health, relating to intestinal and metabolic physiology will be outlined. We will highlight key functions and findings supporting the more specific focus of the study EEC differentiation and function as it relates to obesity.

### ***Normal Human Gut Microbiome Development***

To begin the investigation of the microbiome's role in human health, understanding the process by which a human being obtains their microbiome is essential. Acquisition of microbes for humans begins at birth and is highly dynamic for the first year of life (Bäckhed et al., 2015) (Levin et al., 2016). Initially, newborns obtain bacteria and other microbes during their delivery from the mother, via contact with the mother's vaginal microbiota. This mode of delivery can determine quite significant differences between a mother and their child's microbiome; vaginal

births show much more similar microbiome compositions than cesarean section (c-section) births (Bäckhed et al., 2015).

This finding supports the concept that the assembly of a human microbiome is initiated by passing bacteria down from mother to child. Following birth, additional microbiota may be obtained through diet and from the environment. Importantly, infants can obtain diverse, non-pathogenic bacteria from their mothers breast milk, the composition of which is determined by a mother's maternal and early life (Moossavi et al., 2019). Alternatively, infants fed formula rather than breastmilk still obtain diverse microbiota. It has been shown that infants fed exclusively formula in fact assemble a more diverse gut microbiome than exclusively breast milk fed infants (Ma et al., 2020). However, while both breastmilk and formula fed infants can develop healthy gut microbiomes, formula fed infants have higher risks of some negative health outcomes such as childhood obesity (Arenz et al., 2004) or quantity of pro-inflammatory bacteria in the gut (O'Sullivan et al., 2015).

While these factors are important in developing a healthy gut microbiome and subsequently healthy digestion and metabolic health, its composition is modifiable throughout the lifespan. Diet is essential in making this possible, as some foods such as yogurt probiotic drinks can contain around 25-30 billion colony forming units (CFUs) of bacteria. A colony forming unit is defined as “a single, viable propagule that produces a single colony (a population of the cells visible to the naked eye) on an appropriate semisolid growth medium.” (Ujváry, 2010). Given many foods contain significant amounts of bacteria, diet has been shown to modify the composition of the human intestinal microbiome very rapidly. Following consumption of certain types of food, changes in the relative quantities of bacterial strains can be observed in a single day (David et al., 2014).

### ***The Gut Microbiome is Dynamic Throughout Lifespan***

Of the relevant changes that can occur to the human gut microbiome, one of the most essential is increasing commensal/probiotic bacteria strains and decreasing pathogenic bacteria strains. Commensal bacteria are those who have either neutral or positive effects on human health and reside and reproduce without issues in the human GI tract (Murray et al., 2015). They interact with and are tolerated by the host immune system and not targeted as foreign molecules needing degradation (Xu et al., 2019).

These bacteria are vital for digestion and absorption of some food biomolecules, particularly those of which humans lack the enzymes to catabolize. A more recent case in point shows an increase of bacterial diversity, and of abundance of bacteria involved in macronutrient metabolism, was found to be associated with increased levels of fruit and vegetable derived molecules such as carotenes (found in carrots) (Frankenfeld et al., 2022). A more established effect of commensal bacteria is the improved absorption of lactose in humans, which is improved when lactic acid bacteria are present in the gut (Hove et al., 1999).

### ***Human Gut Microbiome and Obesity***

Of notable relevancy to this study, numerous changes occur to the gut microbiome that both result in and are a result of obesity. The two most common commensal bacterial phyla in the normal human and mouse intestines are Bacteroidetes and Firmicutes (making up greater than 90% of all bacterial phyla in the gut) (Eckburg et al., 2005). Changes in the ratio of Bacteroidetes to Firmicutes was one of the first known major alterations to the microbiome in obesity (50% increase in Bacteroidetes abundance and 50% increase in Firmicutes abundance) (Ley et al., 2005).

In addition to microbiome composition, microbiome function also changes with obesity. Early studies have shown an increase in circulating bacterial lipopolysaccharide (LPS) in mice fed a high fat diet, and an increase in the proportion of LPS-releasing bacteria in the gut (Cani et al., 2007). LPS is a bacterial toxin which is harmless at low grades in the human intestinal lumen, but lethal at higher doses in the blood (Farhana & Khan, 2023). When in the blood, LPS induces inflammatory cytokines and results in concentration dependent damage to cells throughout the body (it is a key player that leads to death in sepsis) (Opal et al., 1999). Cani et al. (2007) hypothesized that the increase in absorption of LPS in response to a high fat diet, is an initiator of obesity. This effect was caused by insulin resistance triggered by an LPS receptor, CD-14 (cluster of differentiation 14) (Cani et al., 2007).

LPS is also the underlying cause of a condition known as leaky gut syndrome (Camilleri, 2019), where LPS that is absorbed damages intestinal epithelial cells breaking down the intestinal epithelial barrier, keeping toxins like LPS in the intestinal lumen, (Stevens et al., 2018) and leads to further LPS entering the blood, and subsequently worsening insulin resistance and increased fat storage. This pathophysiology is known as metabolic endotoxemia (the term was coined in the previously cited study from Cani et al. in 2007. Loss of the intestinal epithelial barrier in leaky gut syndrome is also implicated as a cause of inflammatory bowel disease (S. H. Lee, 2015).

While metabolic endotoxemia is far from the only contributing cause of obesity, it is an essential factor to consider in treating the systemic issues of the disease. It is a contributing factor of which is treatable in part by consuming diets containing *Bacteroides vulgatus* strains of bacteria, which are protective against LPS and its negative effects (C. Wang et al., 2022). These bacteria may have this positive effect as a consequence of secretion of short-chain fatty acids

(SCFAs) such as acetate or propionate (Deleu et al., 2021). SCFAs are an example of a secreted bacterial metabolite, and they are created as a byproduct of carbohydrate fermentation (Miller & Wolin, 1996). Outside of their metabolic activity, bacterial metabolites often play critical roles in signaling (i.e., microbiota-host communication) (Hosseinkhani et al., 2021).

### ***Role of Bacterial Metabolites in Obesity***

Some bacterial metabolites have been implicated as key players in metabolic syndromes: “short-chain fatty acids, branched-chain amino acids, trimethylamine N-oxide” and the focus of this study, indole and its derivatives (Agus et al., 2021). The panel of bacterial derived metabolites in the human intestine, and their physiological impacts on human health is quite diverse and reaches beyond just metabolic function.

**Short-chain Fatty Acids.** For example, in the colons of rats treated with intercecal perfusion of the SCFA butyrate, increased gastric motility was observed; this was hypothesized to be a result of the butyrate’s effect on the enteric nervous system via the sarcoma (Src) signaling pathway (Soret et al., 2010). At the other end of the spectrum, SCFAs also serve as an essential source of energy for intestinal epithelial cells (IECs) and contribute to strengthening the IEC barrier (Martin-Gallausiaux et al., 2021).

**Branch-chain Amino Acids.** The branch-chain amino acids (BCAAs): isoleucine, valine, and leucine, are synthesized by plants, fungi, and bacteria (Neinast et al., 2019). Subsequently, the concentration of BCAAs can be modulated by the microbiome, and when dysregulated can contribute to the development of obesity or type-2 diabetes mellitus (N. Li et al., 2023).

**Trimethylamine N-oxide (TMAO).** TMAO is synthesized by bacteria in the gut using compounds betaine, carnitine and choline (Janeiro et al., 2018). This bacterial metabolite was

strikingly linked to obesity in a positive, dose-dependent response between BMI and circulating TMAO levels (Dehghan et al., 2020). This is relevant, as most of the TMAO in the human body is derived from the gut microbiome (Zeisel & Warrier, 2017). While TMAO is not directly absorbed in the intestine, bacteria secrete its precursor trimethylamine (TMA), which is absorbed and subsequently converted to TMAO by the liver (X. Li et al., 2021). Additionally, its concentration can possibly be regulated through diet, via a change in the amount of the TMA precursors: choline and carnitine, that are consumed (Zhang et al., 1999).

**Tryptophan Metabolism.** Central to the topic of this study, some bacterial metabolites of the amino acid tryptophan have emerged as important regulators of intestinal and metabolic health (Konopelski & Ufnal, 2018). Here the effects and metabolic pathways of tryptophan will be discussed more in-depth. Firstly, several bacteria have been shown to catabolize tryptophan in the colon (Roager & Licht, 2018). There are three major pathways of tryptophan: the serotonin generating pathway (in human cells), the kynurenine generating pathway (in human cells), and the indole/indole derivatives pathway in gut microbiota (Agus et al., 2018), all of which are affected by the activity of bacteria in the intestine.

### *Human and Bacterial Tryptophan Metabolism*

**Serotonin Pathways.** Serotonin is of great importance to both physical and mental health, due to its peripheral physiological effects as well as its role as a neurotransmitter. Its positive correlation with Proteobacteria abundance in the gut is also inversely linked to the emotional distress of depression and anxiety in human patients with irritable bowel syndrome (IBS) (Barandouzi et al., 2022). This finding and others, such as the previously cited role of GLP-1 in regulating satiety, highlight the gut microbiota to nervous system connection known as the gut-brain axis. To further our knowledge of the magnitude of the gut-brain axis' effect, it is

known that approximately 90% of the serotonin present in the human body is synthesized by the enterochromaffin cells of the gut (Yijing Chen et al., 2021).

In the peripheral circulation serotonin is mostly stored in platelets, where it becomes involved with platelet and immune functions such as thrombosis and thrombosis or neutrophil adhesion (Kanova & Kohout, 2021). While the gut microbiota do directly produce serotonin through tryptophan catabolism, they more importantly regulate expression of tryptophan hydroxylase (Tph), in enterochromaffin cells (Yano et al., 2015), which is the rate limiting enzyme in the conversion of tryptophan to serotonin (Beck et al., 1995).

Production of serotonin by the gut is essential for many physiological processes, but too much can contribute to metabolic disorder. Increased peripheral serotonin levels and Tph are observed in obesity (Kwak et al., 2012), and inhibiting Tph, and subsequently serotonin production, can protect against obesity in mice (Crane et al., 2014). Crane et al. (2014) demonstrated that this is a result of peripheral serotonin's inhibitory effect on the energy expenditure of brown adipose tissue, by reducing its thermogenic mechanisms.

**Kynurenine Pathway.** Directly related to the concentration of tryptophan available in the body is the kynurenine pathway. Through this metabolic pathway, liver cells convert tryptophan into a variety of metabolites, including kynurenine (Gao et al., 2020). This process is regulated by the initial and rate limiting enzyme Tryptophan-2,3-dioxygenase (TDO) (Platten et al., 2019). The pathway is important as it is the route of which nearly all catabolized tryptophan (that is not used for protein synthesis takes), and it affects the amount of tryptophan available for serotonin synthesis (Ha & Bhagavan, 2023). The kynurenine pathway at the level of TDO is the initial step in de novo synthesis of nicotinamide adenine dinucleotide (NAD) (Davis & Liu, 2015), a co-enzyme essential for glycolysis and other core cellular metabolic pathways (H. S.

Lee, 2019). Additionally, kynurenine and its derivatives have been determined as drivers of some neurological diseases (Davis & Liu, 2015).

**Microbial Indole Production.** Each of these human catabolic pathways of tryptophan digestion is linked to the pool of tryptophan available after protein synthesis, but more relevantly after gut microbiota catabolism to indole and its derivatives (Gao et al., 2020). Indole and derivatives are critically important signaling molecules in intestinal and metabolic health. Here we will detail indole's known roles and mechanisms as signaling molecules in the gut, revealing some unknown mechanisms and effects of indole in intestinal stem cell differentiation, and necessitating the aims of the present study.

Tryptophan microbial catabolism to indole occurs in bacteria, such as lactobacillus strains, via direct conversion of tryptophan to indole by the enzyme tryptophanase (J. H. Lee & Lee, 2010), the process performs best under carbohydrate free conditions. In respect to other indole derivatives, synthesis occurs via multistep processes, and several different enzymes, to produce end products such as indole-3-aldehyde (IAld), indole-3-acetic acid (IAA), or tryptamine (X. Ye et al., 2022). Indole derivatives have varying, but often overlapping, effects dependent upon their specific structure. For example, indole derivative tryptamine, which is created from tryptophan in the gut microbiota by decarboxylase enzymes, serves as an excitatory neurotransmitter in the brain (Williams et al., 2014). On the other hand, IAA plays roles in modulating gut permeability, but has additionally been shown to attenuate depressive behaviors when dosed to mice (Ying Chen et al., 2022).

Because bacterial conversion of tryptophan to indole is a single step reaction, it is the primary metabolite of tryptophan by gut microbiota and is produced in greater quantities than its



derivatives. A host of positive physiological responses have been observed through normal use of indole, along with a few negative impacts of its overproduction.

For an example of overproduction, in rats injected with indole to the cecum, and in concentrations simulating overproduction, negative emotional behaviors were observed (Jaglin et al., 2018). The positive effects of Indole range from reducing gut permeability, combating the earlier mentioned condition “leaky gut”, to attenuating inflammation in the liver. It has been well established, in both animals (Shimada et al., 2013) and cells (Bansal et al., 2010), that indole reduces intestinal permeability through increasing expression of tight junction proteins (such as occludin or claudins), thus reducing the gaps between intestinal epithelial cells making them less permeable to toxins such as LPS.

In great relevance to this study, indole has been shown in isolated EECs to modulate secretion of incretins like GLP-1; under acute exposures increasing GLP-1 secretion, but decreasing it in response to longer exposures (Chimerel et al., 2014). While this finding is in isolated EECs, an environment very different from the in-vivo gut, this inhibitory effect of long duration indole doses should be kept in mind. A clinical objective we seek to provide insight to is increasing total incretin secretion from the GI tract, making this a key point of consideration when attempting to modulate incretin secretion in response to indole.

### ***Indoles Bind Aryl Hydrocarbon Receptor***

Finally, indole as well as many of its derivatives are known to bind and activate the aryl hydrocarbon receptor (AhR) (Zgarbová & Vrzal, 2023). The AhR receptor was first discovered to be responsible for detecting xenobiotic toxins in the liver (Poland et al., 1976). In more recent years AhR has emerged as a key player in development and regular sensing of environmental factors. For example, AhR deficient KO mice have been seen to develop tumors in the colon at

10 weeks of age (Kawajiri et al., 2009). An effect which is suppressed by derivatives of indole (Fujii-Kuriyama & Kawajiri, 2010).

AhR itself is a ligand-gated transcription factor that activates expression of the many genes such as and similar to cytochrome P450 family 1 subfamily A member 1 (Cyp1A1) (Torti et al., 2021), of which expression is usually measured to determine activation of AhR.

Additionally, AhR has been shown to play key roles in the regulation of differentiation for a number of cell types throughout the body. Specifically, the AhR, when activated, has been shown to increase differentiation of immune, germline, adipose, bone and some epithelial cells (Mulero-Navarro & Fernandez-Salguero, 2016).

More importantly, and very recently, AhR activation has been shown to increase differentiation from stem cells to goblet cells in the mouse small intestine (Zhou et al., 2023). While most commensal microbiota reside in the colon, this finding highlights the AhR's capability to modulate differentiation of intestinal stem cells. However, the effect of AhR activation on the differentiation of intestinal stem cells to EECs has not yet been evaluated.

## **Summary and Scope**

This introduction briefly summarizes the prevalence, correlations with poor health outcomes, and the pathogenesis of obesity. Further, the basic physiology of the human GI tract's cell types and roles, stem cell niche system of proliferation and differentiation, and development/maintenance of the gut microbiome have been explored. During this process enteroendocrine cells have emerged as important regulators of hunger and insulin metabolism, serving as a negative feedback loop in response to food, through their secretion of incretins like GLP-1 and GIP to the blood. A feedback loop that is known to be impaired during obesity, contributing to the chronic nature of the disease. During exploration of the human gut

microbiome, the role of toxins and bacterial metabolites were observed harming and benefiting intestinal and metabolic health. Importantly, these processes, as well as the composition of the gut microbiome, were shown to be dysregulated in obesity. In connection the gut bacterial metabolite of tryptophan, indole, was shown to have a variety of positive effects on intestinal and metabolic health. Most importantly indole is known to bind and activate the ligand-gated transcription factor AhR, which was shown to play important roles in human health across the body, but more specifically in the regulation of differentiation in intestinal stem cells. Yet, indoles effect on the differentiation of intestinal stem cells to EECs has not been studied. This necessitates the aims of this study, where we show indoles positive effect on EEC differentiation via activation of the AhR.

## Hypothesis and Specific Aims

### Hypothesis

Activation of aryl hydrocarbon receptor (AhR) by gut microbial metabolite indole stimulates enteroendocrine cell (EEC) differentiation.

### Aims

#### *Specific Aim 1*

To investigate the effect of indole, a gut microbial metabolite, on the activation of the aryl hydrocarbon receptor (AhR) pathway in human intestinal organoids:

- A. Measure the messenger ribonucleic acid (mRNA) levels of Cyp1A1, a target gene of AhR, in response to indole treatment and an AhR antagonist, CH-223191, using quantitative reverse transcription polymerase chain reaction (QRT-PCR).

#### *Specific Aim 2*

To assess the impact of indole induced AhR activation on enteroendocrine cell (EEC) differentiation in human intestinal organoids:

- A. Measure the mRNA levels of Chromogranin A, a marker of EECs, upon indole treatment using qPCR.
- B. Confirm an expression difference by evaluating the protein levels of Chromogranin A through western blot analysis following indole treatment.

## **Experimental Procedures and Methodology**

### **Overview of Methodology**

Histologically confirmed normal small intestine samples were obtained from endoscopic procedures conducted under an approved IRB protocol. These samples were used to create human intestinal organoids in the laboratory. To determine the differentiation of enteroendocrine cells (EECs), the organoids were treated with 0.2 mM indole for a duration of 24 hours. The expression of chromogranin A, a marker commonly found in EECs, was used to identify their presence and quantity. Two different human intestinal organoid models, namely MU-039 and MU-040, derived from separate donors, were utilized in this study. All protocols involved in the use of these organoids were adapted from published procedures (Haynes et al., 2022) (Fujii et al., 2015). The levels of mRNA for the aryl hydrocarbon receptor (AhR) and its immediate target gene, Cyp1A1, as well as the expression of the EEC marker chromogranin A, were quantified using QRT-PCR. The activation of AhR was confirmed by observing the induction of Cyp1A1 via PCR.

### **Human Intestinal Organoid Culture**

#### ***Factor Producer Cells***

L-cells derived from mice and producing an excessive amount of Wnt-3a protein (acquired from Hans Clevers at the Hubrecht Institute in the Netherlands) as well as human embryonic kidney 293T cells with a stable overexpression of HA-R-Spon-din1-Fc (Rspo1; obtained from Trevigen) were cultivated in specialized medium: Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12; purchased from Gibco). This medium was supplemented with 10% fetal bovine serum (heat inactivated, Gibco), 10 mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), and 100 U/mL penicillin-streptomycin (Pen-Strep; Gibco). To ensure

the survival of these cells, Zeocin (purchased from Gibco) was added as a selection agent, at a concentration of 125 mg/mL for L-Wnt-3a cells and 300 mg/mL for 293T-Rspo1 cells. These cell lines were cultured at 37°C in a humidified incubator with an atmosphere containing 6% CO<sub>2</sub>.

### ***Conditioned Media***

Conditioned media was prepared following established procedures outlined in published protocols, with minor adjustments (Fujii et al., 2015). In summary, to generate Wnt-3a conditioned medium, L-Wnt-3a cells were seeded in a complete growth medium and cultivated until reaching an approximate cell confluence of 90% within a span of two days. The growth medium was then exchanged with fresh complete growth medium. After a lapse of five days, the conditioned medium was amassed, subjected to centrifugation at 450 x g for six minutes to discard cellular remnants, and subsequently, the resulting supernatant was sieved through a 0.22-micrometer filtration unit. In the case of Rspo1 conditioned medium preparation, 293T-Rspo1 cells were seeded in a complete growth medium and nurtured for a duration of four to five days until attaining confluency. The medium was then replaced with fresh growth medium sans serum. Following an incubation period of seven days, the conditioned medium was harvested, subjected to centrifugation at 450 x g for six minutes to eliminate cellular debris, and the ensuing supernatant was strained through a 0.22-micrometer filtration unit.

### ***Human Tissue Collection & Processing***

Human normal intestinal tissue was obtained with informed consent from participants without intestinal disorders, approved by the Marshall University Institutional Review Board (protocol #964144-18). Jejunal biopsy specimens were collected during routine diagnostic endoscopic procedures, stored in Dulbecco's Phosphate Buffered Saline (DPBS) on ice, and

processed on the same day. Tissue fragments were washed in DPBS, incubated with Gentle Cell Dissociation Reagent (STEMCELL) containing 0.5 mM EDTA at 4°C for 30-40 minutes. After centrifugation at  $250 \times g$  for 2 minutes (4°C), the fragments were resuspended in ice-cold Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 1% BSA. Intestinal crypts were obtained by pipetting up and down 25 times, followed by filtration through a 100- $\mu$ m cell strainer (Corning). The cells were collected by centrifugation at  $200 \times g$  for 4 minutes (4°C), resuspended in growth factor-reduced Matrigel (Corning), and seeded at 50  $\mu$ L of the Matrigel-crypt suspension per well in 24-well cell culture plates. After a 10-minute incubation at 37°C, the Matrigel and embedded cells were overlaid with 600  $\mu$ L per well of Human IntestiCult Organoid Growth Medium (STEMCELL) supplemented with 100 U/mL Pen-Strep. For initial plating, 10  $\mu$ M Y-27632 (Tocris) and 5  $\mu$ M CHIR 99021 (Tocris) were included in the medium for 2-3 days. The medium was changed every 2-3 days, and cultures were passaged every 7-12 days until established. For an overview of this process see Figure D2.

### ***Normal Organoid Culturing***

After the establishment of the models, which involved maintaining and expanding them for more than 30 days, the 3D organoid cultures were sustained using the same media composition as described by Fujii et al. (Fujii et al., 2015) The organoid growth medium (OGM) consisted of Advanced DMEM/F-12 supplemented with 10 mM HEPES, 2 mM GlutaMAX, 100 U/mL Pen-Strep, 40% Wnt-3a conditioned medium, 10% Rspo1 conditioned medium, 1X B-27 Supplement (Gibco), 1.25 mM N-acetyl-L-cysteine (Sigma), 10 nM Gastrin I (Sigma), 100 ng/mL mouse Noggin (Peprotech), 50 ng/mL mouse EGF (Gibco), 500 nM A83-01 (Tocris), and 10  $\mu$ M SB202190 (Sigma). Upon passaging, the medium also included 10  $\mu$ M Y-27632 and 2.5

mM CHIR 99021 for the initial 2-3 days. The organoid cultures were grown in 50 mL Matrigel domes overlaid with 600  $\mu$ L per well of OGM in 24-well cell culture plates. The medium was changed every 2-3 days, and the cultures were split once a week. The organoid cultures were maintained at 37°C in a humidified incubator with 6% CO<sub>2</sub> and were cultured for a maximum of 12 passages. All organoid models were tested and confirmed to be free of mycoplasma contamination. For all experiments, normal jejunal organoid models derived from two independent donors were utilized.

### ***Organoid Protein Extraction***

Cultures were washed three times using cold DPBS. Next, the Matrigel domes containing the organoids were gently scraped in ice-cold Cell Recovery Solution (Corning) and transferred to pre-chilled centrifuge tubes. The samples were then incubated on ice for a duration of 45 minutes, with the tubes being gently rocked every 15 minutes. After the incubation period, the released cells were collected through centrifugation at 200 x g for 5 minutes (at 4°C) and subsequently washed with ice-cold PBS. Cell pellets were resuspended in ice-cold RIPA Lysis and Extraction Buffer (Thermo-Fischer), supplemented with protease inhibitors (Thermo-Fischer), and transferred to pre-chilled microcentrifuge tubes. Following, all the samples were incubated on ice for 45 minutes, with periodic pipetting up and down every 15 minutes to ensure homogenization. To remove debris, lysates were centrifuged at 16,000 x g for 20 minutes (at 4°C), and protein concentration was determined using the DC Protein Assay kit (Bio-Rad) and a SpectraMax i3x Microplate Reader (Molecular Devices).

### **Indole Treatments**

Indole (CAS-No. 120-72-9) was added to the media of human intestinal organoids at a concentration of 0.2 mM for 24 hours before being removed, and the organoids harvested for



analysis. Prior to, the density of all organoids was controlled and confirmed microscopically and through cell counting using a hemocytometer.

### **RNA Isolation and Real Time - Polymerase Chain Reaction (RT-PCR)**

After the media was removed and organoids were washed with PBS, the process included isolating total RNA from human intestinal organoid samples that were subjected to indole treatment, as well as control samples. This was accomplished by utilizing RNeasy kits (Qiagen). The isolated RNA was then subjected to reverse transcription and amplification, employing a Brilliant SYBR Green qRT-PCR Master Mix kit (Agilent Technology). The sequences of all RNA primers used are shown below. Finally, the resulting RNA samples were measured using a Bio-Rad CFX Opus 96 Real-Time PCR System.

### **PCR Primer Sequences**

Primer pair	Sequence (5'-3')
Chromogranin A - Forward:	CAGCCCCACAACCTTAAACATTG
Chromogranin A - Reverse	GCATGGAGGAAGGGAAACTTCTAG
CYP1A1 – Forward	TGGAGACCTTCCGACACTCT
CYP1A1 -Reverse	ACAAAGACACAACGCCCTT
$\beta$ 2-Microglobulin (B2M) – Forward	CTCCGTGGCCTTAGCTGTG
$\beta$ 2-Microglobulin (B2M) -Reverse	TTTGGAGTACGCTGGATAGCCT

### **Western Blotting**

Protein lysates (30  $\mu$ g/sample) from control and treated organoids were solubilized in SDS-gel loading buffer, boiled for 7 minutes, and subjected to electrophoresis on a 4-20% SDS-polyacrylamide gradient gel. These proteins were then transferred onto a PVDF membrane. After blocking with 3% BSA in 1X-Tris Buffered Saline (TBS) for 1 hour, the membrane was

incubated overnight at 4°C with the appropriate primary antibody in 1%BSA-1XTBS-0.1% Tween-20. The membrane was washed five times with 1X-TBS and 0.1% Tween-20 for 5 minutes each and probed with HRP-conjugated goat anti-rabbit/mouse antibody (1:10,000 dilution) for 1 hour. Finally, enhanced chemiluminescence (ECL) detection (Bio-Rad, Hercules, CA) was performed and the blots were imaged.

### **Bacterial culture and preparation of conditioned culture supernatant**

*Lactobacillus acidophilus* (ATCC strain #4346) was cultured in Mann-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) at 37°C for 24 hours, both with and without 5 mM tryptophan, without agitation. Following incubation, the overnight culture was subjected to centrifugation at 3,000 x g for 10 minutes at 4°C. The resulting supernatant, which was obtained after filtration through a 0.22- $\mu$ m filter (Millex, Millipore, Billerica, MA) to ensure sterility and eliminate all bacterial cells, was designated as conditioned culture supernatant (CS). To treat the cell monolayers, the CS was diluted 1:10 using DMEM/F-12 media (Invitrogen, Carlsbad, CA), with 1:10 diluted MRS serving as the control medium. Organoids were treated for 24 h with MRS, CS with or without tryptophan, followed by isolation of RNA or preparation of cell lysate as described above for indole treated organoids.

### **Statistical Analysis**

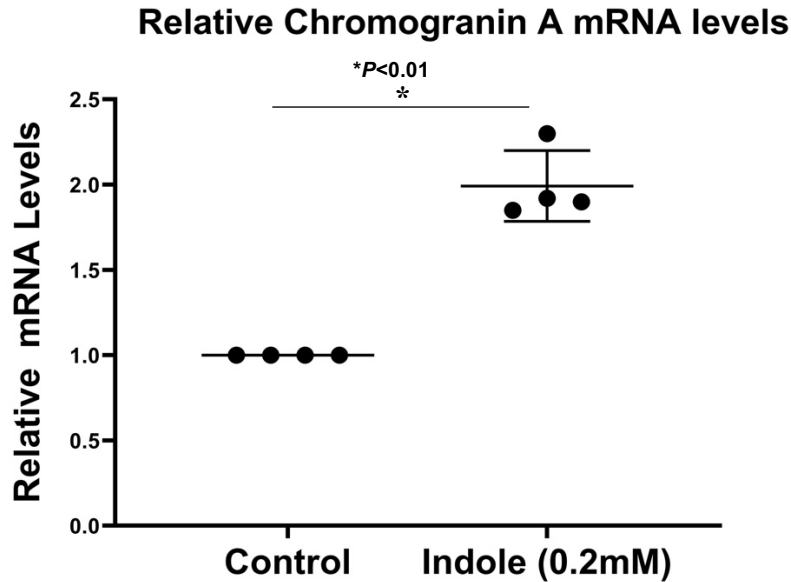
The results were expressed as individual fold change means  $\pm$  SEM (standard error of the mean) of 3-4 independent experiments relative to the mRNA or protein expression of their respective controls. Statistical analysis was conducted using a one sample t-test directly on fold change values (relative to controls) for mRNA and protein data. Differences between the control and treated groups were considered significant at a level of  $P < 0.05$ .

Because it is impossible to control for the exact differentiation states of human intestinal organoids across experiments, control expression variance is unknown, necessitating its estimation in a one sample t-test. The theoretical population mean for expression fold change is 1 in this case for both protein and mRNA, as control groups represent normal expression (regardless of their exact physiological or differentiation states). Thus, all control protein and mRNA expression values are displayed as 1, representing the theoretical population mean.

## Results

**Figure 1**

*Indole Increases Chromogranin A mRNA in Human Intestinal Organoids*

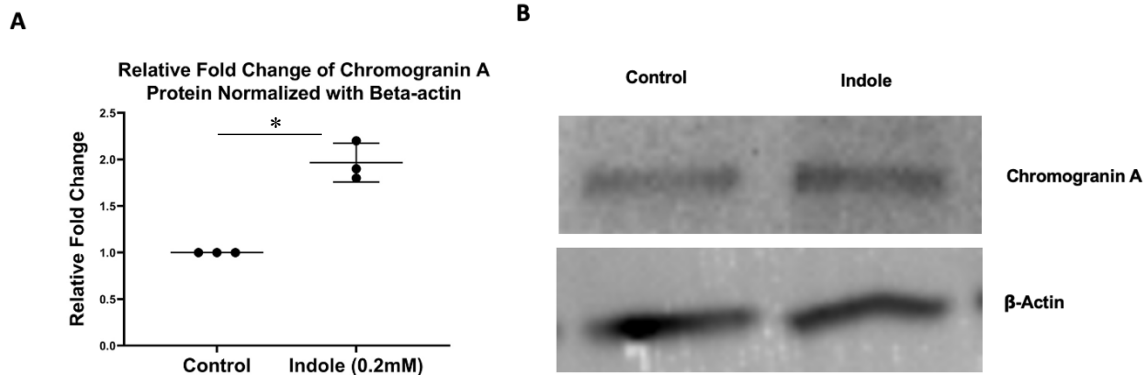


*Note.* Normalized mRNA levels show a significant increase in the genetic expression of Chromogranin A, a marker of EECs, in human intestinal organoids in response to Indole treatment (0.2mM) for 24 hours. This implicates a possible greater number of EECs in human organoid samples treated with Indole compared to those which received only normal media.

Before assessing indoles role as a modulator of EEC differentiation, a reliable response concentration had to be established in our intestinal organoid models. Because the expression of mucin-2 (Muc-2), a molecular marker of goblet cells, is known to be increased in response to indole (Zhou et al., 2023), it was used as a marker for determining the minimum response conditions for indole to have an effect on differentiation in our human intestinal organoid models (0.2 mM) (Figure D3).

## Figure 2

### *Indole Increases Chromogranin Protein Expression in Human Intestinal Organoids*



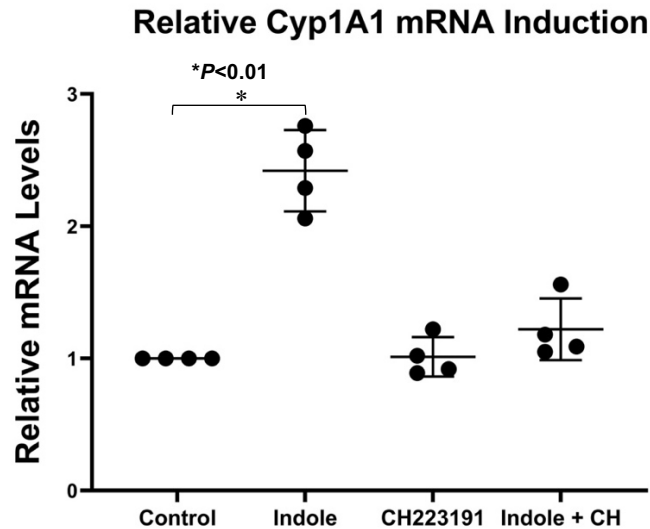
*Note.* Treatment of 0.2 mM Indole for 24 hours increases the expression of chromogranin A protein expression, observed via western blot (\* $p < 0.02$ ). Confirming translation of increased chromogranin A mRNA to molecular structure. Western Blot protein levels are plotted as the relative fold changes of individual experiments relative to their controls (A). Western Blot images are also shown (B).

### **Indole Increases Differentiation to Enteroendocrine Cells in Human Intestinal Organoids**

To establish a relationship between the effect of indole on human intestinal organoids, indole was added to the culture media of the treatment group of organoids for 24 hours at a concentration of 0.2 mM, whereas media was prepared as normal for controls. As the protein chromogranin A is marker of EECs, its mRNA expression was first observed through RT-PCR (figure 1). A significant increase in the expression of chromogranin A was observed, indicating a possible increase in the differentiation from intestinal stem cells to EECs. Further confirming this

**Figure 3**

*Indole activates Aryl Hydrocarbon Receptor in Human Intestinal Organoids*



*Note.* PCR mRNA levels indicate indole significantly increases expression of Cyp1A1, a primary target gene of AhR, indicating indole induces activation of AhR. Cyp1A1 induction is subsequently blocked by AhR antagonist CH223191, in the presence of indole and without. Showing no significant difference in expression compared to controls ( $p > 0.05$ ).

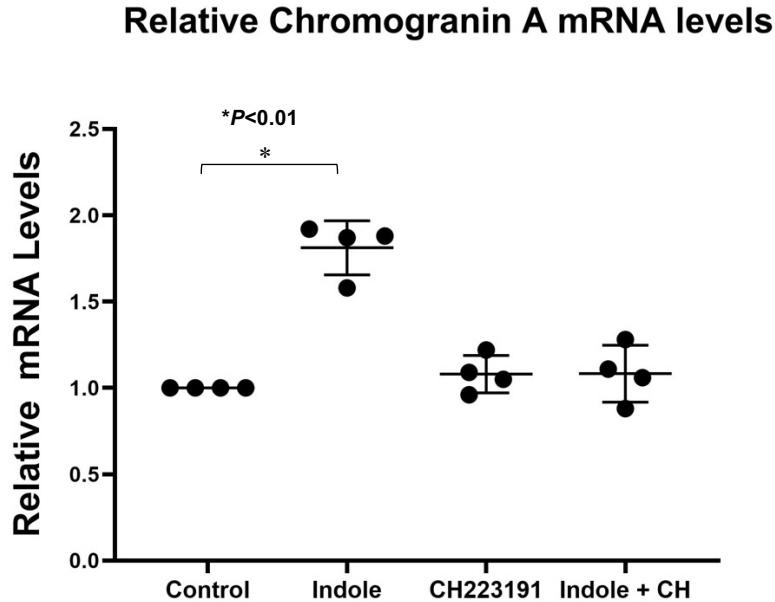
increased differentiation of EECs, chromogranin A protein was also shown to be increased in organoids treated with indole (figure 2). This confirms the increased chromogranin A transcription is followed through with translation to actual protein in differentiated EECs. These combined findings suggest that indole has the capacity for elevating the generating more EECs in human intestinal organoids as compared to normal controls.

### **AhR-Antagonist Blocks Indole-Induced Increase in Cyp1A1 and Chromogranin A**

Upon establishing indoles capacity for increasing EEC differentiation, the proposed receptor, AhR, and possible mechanism by which indole carries out this effect was explored.

**Figure 4**

*Indole Increases Chromogranin A mRNA expression via AhR Activation*



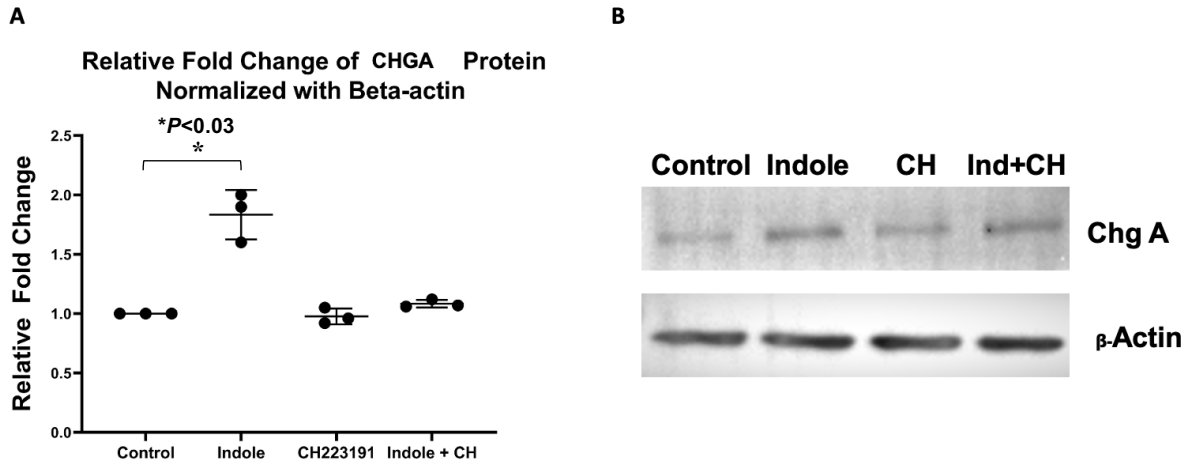
*Note.* Chromogranin A mRNA expression is increased by indole treatment in an AhR dependent manner. Indole has the effect of increasing chromogranin A levels in human intestinal organoids. This effect is eliminated under the presence of the AhR antagonist CH223191, indicating indole increases chromogranin A expression via activation of AhR. Compared to controls, there is no significant difference in organoids treated with CH223191 ( $p>0.05$ ).

Firstly, Cyp1A1, is a primary target gene of the ligand activated transcription factor AhR, and thus its mRNA expression can be used as a measure of AhR activity (Hu et al., 2007). When Cyp1A1 mRNA induction was observed via RT-PCR, there was a significant increase from control in human intestinal organoids treated with indole. This effect was obliterated by AhR antagonist CH223191, when added to organoids media in addition indole (figure 3).

Furthermore,

**Figure 5**

*Indole Increases Chromogranin A Protein expression via AhR Activation*



*Note.* Indole treatment significantly increases Chromogranin A protein levels in human intestinal organoids. This effect is eliminated under the presence of the AhR antagonist CH223191, implying indole increases chromogranin A expression via activation of AhR. Western Blot protein levels are plotted as the relative fold changes of individual experiments relative to their controls (A). Example western blot images are shown (B). Compared to controls there is no significant difference in either group treated with CH223191 ( $p > 0.05$ ).

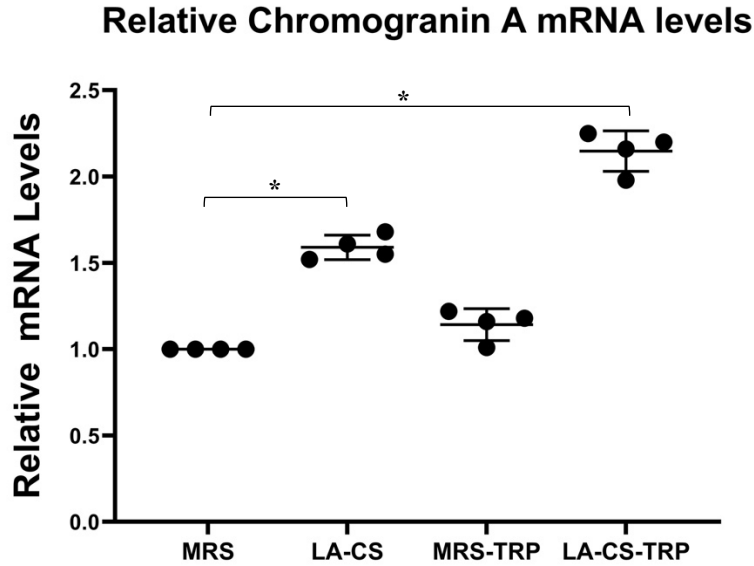
under identical conditions chromogranin A mRNA expression was also significantly increased in indole treated organoids, and this effect was blocked by CH223191 (figure 4).

These findings clearly demonstrate that AhR is closely involved in mediating the positive effect indole has on the differentiation of EECs, as the process requires AhR activation. To further confirm AhR's involvement in increasing EEC differentiation, the protein content of



**Figure 6**

*Tryptophan Metabolites from Lactobacilli Culture Supernatant Activate AhR*



*Note.* Lactobacillus acidophilus (LA) cultures supernatant (CS), when added to organoid media (MRS), increases the expression of Chromogranin A mRNA in human intestinal organoids (\* $P < 0.001$  MRS vs LA-CS). An effect that is amplified by the addition of tryptophan (TRP) to LA culture media (\* $P < 0.001$  MRS vs LA-CS-TRP). When organoids are only treated with normal media and TRP, no significant chromogranin A expression change was observed ( $P > 0.05$  MRS vs MRS-TRP).

chromogranin A was also measured with and without the AhR antagonist (figure 5). As shown the chromogranin A protein level increase in human intestinal organoids triggered by indole, was also inhibited when CH223191 was added in addition to indole to organoid media. This confirms indoles effects on EEC differentiation as AhR dependent.

### **Tryptophan Metabolites in *L. acidophilus* Culture Supernatant Increase Chromogranin A**

While it is known indole is excreted from gut microbiota through digestion of tryptophan, the effect of tryptophan may also influence excretion of other factors that may impact the differentiation of EECs in the intestine. Thus, to observe the previously shown impact of indole on EEC differentiation in more physiologically relevant conditions, human intestinal organoids were treated with *Lactobacillus acidophilus* (LA) conditioned culture supernatant (figure 6). The media of control organoids was supplemented with unconditioned LA media (which never came in contact with LA). Organoids treated with LA conditioned media that did not receive a tryptophan supplement showed a modest but significant increase in chromogranin A mRNA levels. However, when LA conditioned media in the presence of tryptophan, this media had an additionally stronger increase in chromogranin A mRNA in human intestinal organoids. Finally, to rule out the possibility of tryptophan directly causing this chromogranin A expression increase organoids were treated with unconditioned LA media in addition to the tryptophan supplement, showing no significant change in chromogranin A mRNA levels compared to controls.

## **Discussion & Conclusions**

The above results and introductory information have outlined the role of the human gut microbiome and the importance of EECs in maintaining a healthy energy balance. The hormones released from EECs are essential in regulating insulin interactions and forming a satiety feedback loop in response to food. Pertinently, EECs are known to be reduced in their number and their hormone secretion silenced during obesity (Osinski et al., 2020) (L. Ye et al., 2019) (Wölnerhanssen et al., 2017). This necessitates the development of methods for increasing EECs in terms of their function and number.

Initially, the exploration of bacterial metabolites in the gut highlights the bacterial tryptophan metabolite indole as a key player in intestinal and metabolic health. Beyond its role in maintaining the intestinal epithelial barrier, its closely involved with intestinal stem cell fate. Thus, we investigated the effects of indole on the differentiation of EECs.

Human intestinal organoids are an experimental cell-based model of the human intestine. They conserve a similar physiology of cell proliferation and differentiation that is seen in the in vivo intestine and are generated directly from human biopsy tissue. This model enables an in vitro study of factors influencing not only transport (Haynes et al., 2022), but also proliferation and differentiation. Consequent of their human origin they maintain a human genome and similar epigenetic markers to their human donor (Lewis et al., 2020). Because of this we were able to observe how activation of AhR, interacting with a human genome, can influence the differentiation of EECs.

### **Conclusion**

In these human intestinal organoid models, we observed a positive influence on the differentiation of EECs via transcriptional and translational upregulation of the EEC marker

protein chromogranin A. Additionally, we supported the ligand gated transcription factor AhR as a critical mediator of this process. This was accomplished through measuring its activity via mRNA expression of one of its primary target genes, Cyp1A1. Expression of Cyp1A1 was significantly increased in response to indole. Further these effects of indole were halted under the presence of the AhR antagonist CH223191. To provide more physiologically relevant evidence that bacterial tryptophan metabolites can improve EEC differentiation, we showed that media conditioned by the probiotic bacteria *L. acidophilus* (María Remes Troche et al., 2020) under the presence of Tryptophan, can also increase the differentiation of EECs in human intestinal organoids. Combined, these results support that hypothesis that the gut microbial metabolite of tryptophan, indole, stimulates EEC differentiation dependent upon activation of AhR.

### **Applications**

The clinical goal of this study is identifying means by which EEC differentiation and secretion of its hormones related to energy balance (i.e., GLP-1, GIP) can be increased. Achieving this would contribute to moving the bodies hunger/satiety feedback mechanisms from obesogenic to healthy, as GLP-1 increases satiety. Recently, medications used in the treatment of obesity have been developed which target GLP-1 receptors (GLP-1R) directly, to induce satiety momentarily, and improve glycemic control. These drugs provide successful treatment and prevention of obesity in both animal and clinical studies (J. Y. Wang et al., 2023), further supporting the clinical goal of this study.

However, the use of GLP-1R agonist drugs requires a patient to regularly take the drug in order to achieve a healthier metabolic set point for hunger and satiety, as the drug is artificially producing this change in metabolic set point. Additionally, recipients of these drugs may experience the adverse effects of nausea, vomiting, or diarrhea (Filippatos et al., 2014). For these

reasons it is important to solve the systemic endogenous problems at the base level. In the case of this study, we examined the human gut microbiome, noticing that indole released from commensal bacteria can aid in achieving greater secretion of incretins like GLP-1 from EECs.

When considering the ability of indole in accomplishing this goal endogenously, it's important to understand that the entirety of the substrate for the bacterial enzyme converting tryptophan to indole (tryptophanase), tryptophan, comes from the diet. Hence, the limiting factors of indole production by gut microbiota are limited by the composition of the diet and the number of bacteria capable of performing the reaction. Thus, to optimally improve the incretin secretion from EECs one must improve the number of probiotics such as *L. Acidophilus* in their intestine and increase their dietary consumption of tryptophan. This can be accomplished by regularly consuming yogurts and fermented foods to increase probiotic bacteria numbers in the gut (Cleveland Clinic, 2022), and by consuming food more rich in proteins and elevated tryptophan such as eggs, white beans, or chicken (Begum, 2022).

### **Future Directions**

Indole has been shown here in an in vitro model of the human intestine to increase the differentiation of EECs. This effect has, however, not been shown in vivo experimental models, where numerous microbes and cell types coexist and interact. In the normal human gut tryptophan metabolism by IECs is in competition with bacteria. Additionally, bacterial tryptophan metabolism will be under the influence of many complex variables.

To further establish the clinical usefulness of the above recommendations to diet, they must be explored in both the in vivo human gut and animal models. A logical approach may first involve correlating incretin secretion from EECs and EEC number, with the levels of *L. acidophilus* and other indole producing probiotics. Tryptophan ingestion and subsequent

bacterial indole production should also be correlated with the incretin secretion from EECs and EEC number. With this information one could experimentally modify the variables of tryptophan and indole producing probiotic ingestion, measuring the consequential changes in EEC differentiation and incretin secretion in response to food.

Accomplishing this task would provide quite strong clinical evidence supporting the recommendation of certain probiotic supplementation and a tryptophan rich diet for those suffering from obesity.

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# Appendix A: IRB Approval Letter



**Office of Research Integrity**  
Institutional Review Board  
One John Marshall Drive  
Huntington, WV 25755

FWA 00002704

IRB1 #00002205  
IRB2 #00003206

November 12, 2021

Uma Sundaram, Ph.D  
Department of Clinical and Translational Sciences, MUSOM

RE: IRBNet ID# 964144-18  
At: Marshall University Institutional Review Board #1 (Medical)

Dear Dr. Sundaram:

<b>Protocol Title:</b>	[964144-18] Regulation of intestinal nutrient and electrolyte transport in chronic diseases.	
<b>Next Annual Report Due:</b>	November 14, 2022	
<b>Site Location:</b>	MU, SMMC, CHH	
<b>Submission Type:</b>	Annual Update	APPROVED
<b>Review Type:</b>	Administrative Review	

The annual update report for the above listed study was approved today by the IRB Coordinator. The next annual report will be due on November 14, 2022. The annual report must be submitted prior to the due date in order to continue with the research. A closure package must be submitted upon completion of the study.

If you have any questions, please contact the Marshall University Institutional Review Board #1 (Medical) Coordinator Margaret Hardy at (304) 696-6322 or hardyma@marshall.edu. Please include your study title and reference number in all correspondence with this office.

Sincerely,

A handwritten signature in blue ink that reads 'Bruce F. Day'.

Bruce F. Day, ThD, CIP  
Director, Office of Research Integrity



Office of Research Integrity

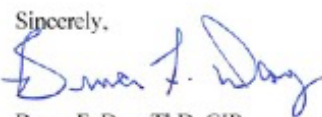
June 23, 2023

Jimmy Hart, B.S.  
37 Township Road 1435  
South Point, OH 45680

Dear Jimmy,

This letter is in response to the submitted thesis abstract entitled "*Gut Microbial Metabolite Indole: A Stimulator of Enteroendocrine Cell Differentiation via Activation of Aryl Hydrocarbon Receptor.*" After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight by the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided, then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,  
  
Bruce F. Day, ThD, CIP  
Director

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## Appendix B: Figure Permissions

Hello James Hart

As the corresponding author, I hereby give permission to reproduce a figure (Figure 2) from the following review article in your Master's thesis:

[Altered intestinal epithelial nutrient transport: an underappreciated factor in obesity modulated by diet and microbiota.](#)

Sundaram S, **Borthakur A.** Biochem J. 2021 Mar 12;478(5):975-995. doi: 10.1042/BCJ20200902.

PMID: 33661278      [Review.](#)

Sincerely

Alip Borthakur



**Alip Borthakur, Ph.D.**

Assistant Professor

Department of Clinical & Translational Sciences

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## Appendix C: Abbreviations

AhR- Aryl hydrocarbon receptor

ANOVA- analysis of variance

B2M-  $\beta$ 2-Microglobulin

BCAAs- Branch-chain amino acids

BMI- Body mass index

CCK- Cholecystokinin

CFUs- Colony forming units

CS- Culture supernatant

CYP1A1- Cytochrome P450 family 1 subfamily A member 1

DNA- Deoxyribonucleic acid

ECL- Enhanced chemiluminescence

EECs- Enteroendocrine cells

Fe- Iron

FTO- an obesity-associated gene

GI- Gastrointestinal

GIP- Glucose-dependent insulintropic peptide

GLP-1R- GLP-1 Receptor

GLP1- Glucagon-like peptide 1

IAA- Indole-3-acetic acid

IAld- Indole-3-aldehyde

IBS- Irritable bowel syndrome

IECs- Intestinal epithelial cells

ISCs- Intestinal stem cells

KO- Knockout

LA- Lactobacillus acidophilus

LEP- Leptin

LEPR- Leptin receptor

LPS- Lipopolysaccharide

MC4R- melanocortin-4 receptor

mRNA- Messenger ribonucleic acid

MRS- Mann-Rogosha-Sharpe

Muc-2- Mucin-2

NAD- Nicotinamide adenine dinucleotide

OXM- Oxyntomodulin

PYY- Peptide YY

qRT-PCR- Quantitative reverse transcription polymerase chain reaction

RNA- Ribonucleic acid

SCFAs- Short-chain fatty acids

SEM- Standard error of the mean

SOCS- Suppressors of cytokine signaling

Src- Sarcoma

TBS- 1X-Tris Buffered Saline

Tcf-4- T-cell factor

TDO- Tryptophan-2,3-dioxygenase

TMA- Trimethylamine

TMAO- Trimethylamine N-oxide

Tph- Tryptophan hydroxylase

TRP- Tryptophan

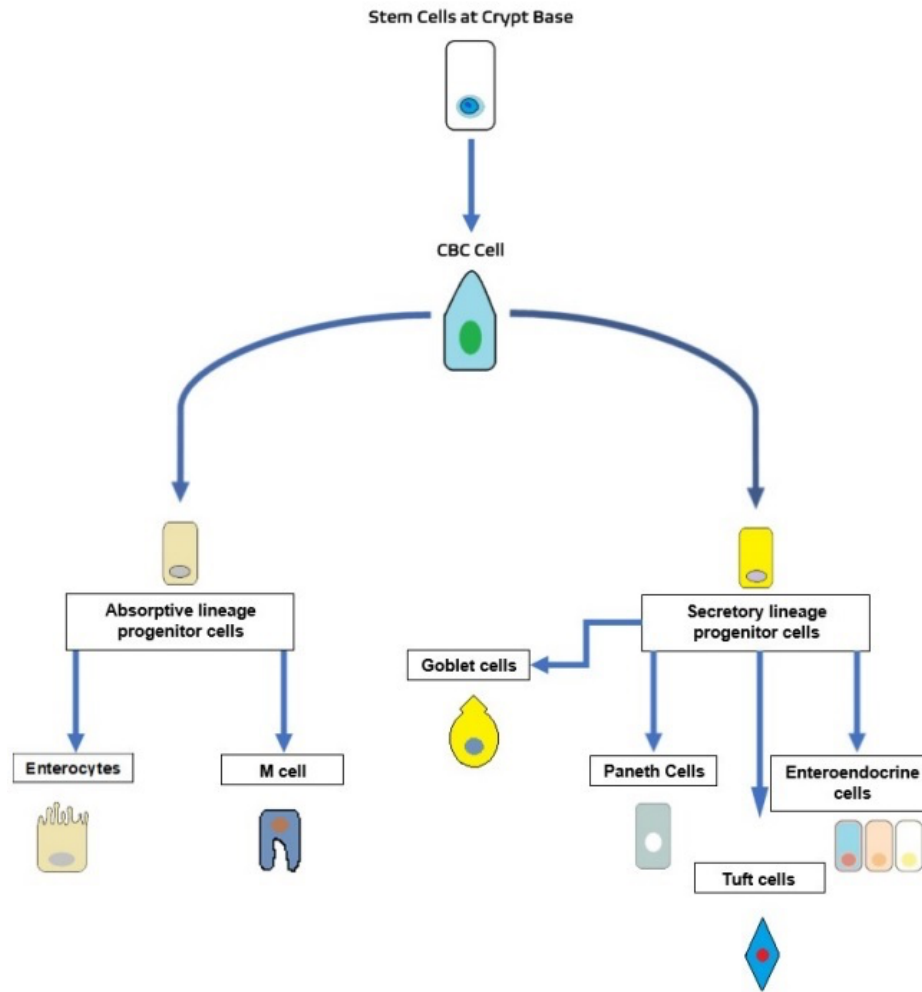
Wnt- Wingless



## Appendix D: Supplemental Figures

**Figure D1**

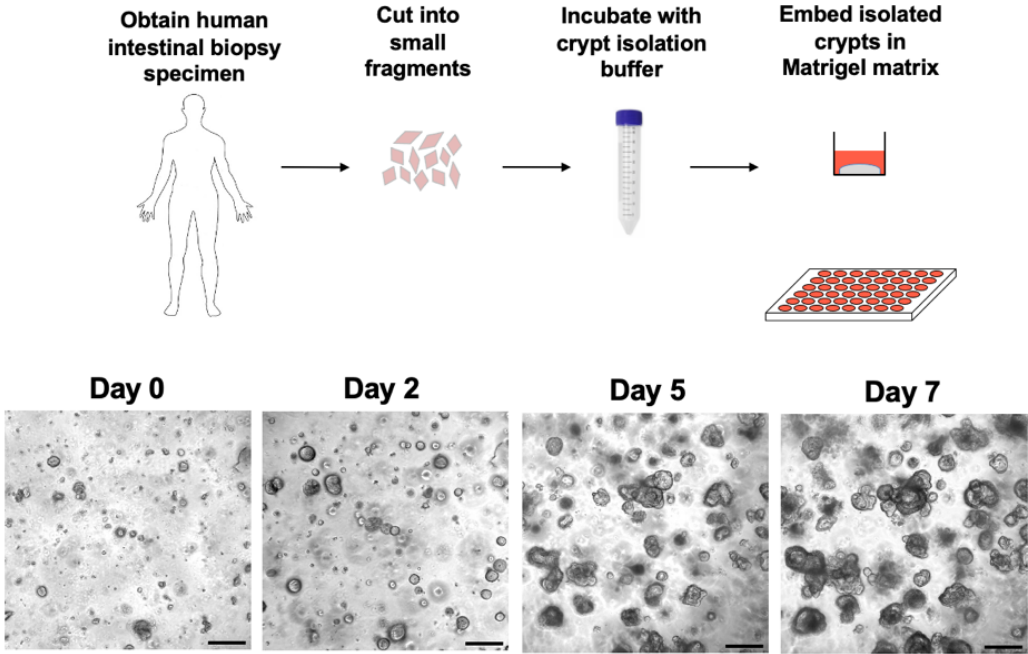
*Flowchart of Intestinal Epithelial Stem Cell Lineages and Fates*



*Note.* The above figure details the various paths of differentiation intestinal epithelial stem cells can take and the various cell type fates corresponding to the lineages they follow. Reprinted with permission from "Altered intestinal epithelial nutrient transport: an underappreciated factor in obesity modulated by diet and microbiota" by S. Sundaram & A. Borthakur, 2021, *The Biochemical Journal*, 478(5), 975–995 (<https://doi.org/10.1042/BCJ20200902>).

**Figure D2**

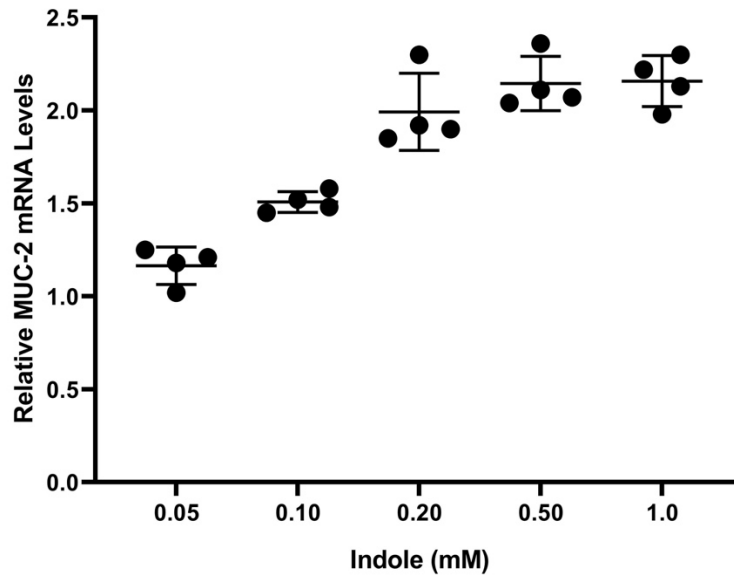
*Diagram of Human Intestinal Tissue to Intestinal Organoid Workflow*



*Note.* The above figure shows an overview of the workflow from human tissue to intestinal organoids as well as example light microscopy images of organoid development from initiation to day 7.

### Figure D3

*Indole Reliably Increases Muc-2 mRNA Expression at minimum 0.2mM*



*Note.* Indole treatment increases expression of Muc-2 mRNA in human intestinal organoids in a dose dependent manner. 0.2 mM is the concentration used as the minimum reliable response dosage.