ASSOCIATION OF CYCLOOXYGENASE 2 GENE POLYMORPHISM IN IRRITABLE BOWEL SYNDROME

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APRIL 2012
BONAFIDE CERTIFICATE

This is to certify that this dissertation work Entitled “Association Of Cyclooxygenase 2 Gene Polymorphism In Irritable Bowel Syndrome” is the original bonafide work done by Dr.S.Siva, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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ABBREVIATION

AGA - Antigliadin antibodies
Anti-tTG - Antibodies to tissue transglutaminase
BHR - Bronchial hyper-responsiveness
CBC - Complete Blood Count
CGRP - Calcitonin Gene Related Peptide
cds - Coding sequences
EMA - Endomysial antibodies
FOBT - Fecal occult blood test
HPA - Hypothalamic-pituitary-adrenal axis
IBS - Irritable bowel syndrome
IBS-D - Diarrhea-predominant IBS
IBS-M - Mixed IBS
IBS-C - Constipation-predominant IBS
IBD - Inflammatory bowel disease
PG - Prostaglandin
RFLP - Restriction Fragment Length Polymorphism
SIBO - Small Intestinal Bacterial Overgrowth
HRQoL - Health-Related Quality of Life.
TSH - Thyroid Stimulating Hormone
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ETHICAL COMMITTEE CLEARANCE

PATIENT CONSENT FORM

PROFORMA
Introduction
Irritable bowel syndrome (IBS) is a functional disorder that affects 10% to 20% of the population worldwide. IBS is characterized by altered bowel habits and abdominal discomfort in the absence of organic disease. No clear diagnostic markers exist for IBS and thus diagnoses are based on the clinical presentation. A relationship between irritable bowel syndrome (IBS) and broncho-pulmonary disease was initially suspected in 1991, when White and co-workers reported bronchial hyper-responsiveness to be more frequent in patients with IBS. Further, Kennedi and colleagues showed an association between symptoms of IBS and those of bronchial hyper-responsiveness. Amra et al reported that patients with IBS have increased airway resistance as compared to healthy subjects, as measured by impulse oscillometry. Two independent results showed bronchial asthma may be more prevalent in IBS patients than in otherwise healthy subjects. Chan et al reported PTGS2.8473 polymorphism is associated with asthma, atopy and lung function. Sanak et al reported increased production of prostaglandins in bronchial asthma in association with T→C transition within the 3′-untranslated region (COX2.8473).

Serotonin (5-HT) is secreted in copious amounts from gut enteroendocrine cells and serves as a critical messenger for gastrointestinal fluid secretion and gut motility. There are seven subclasses of serotonergic receptors, differentiated on the basis of structure, molecular mechanism, and function. The 5-HT1A receptor is a G protein-coupled receptor that is coupled to G\textsubscript{i}/G\textsubscript{o} and mediates inhibitory neurotransmission. Activation of serotonin 5-HT1A receptor in the enteric nervous system suppresses gut motility. Clarke et al reported marked elevations in proinflammatory polyunsaturated fatty acid metabolites like PGE2 in females with Irritable Bowel Syndrome. Kenda et al showed the responsiveness of the 5-HT1A receptor system was reduced by a cyclooxygenase-dependent metabolite of AA.

In this study, the association of COX2.8473 T→C single nucleotide polymorphism (SNP) with IBS was investigated.
Review of Literature
Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder characterized by abdominal pain or discomfort and altered bowel habits\(^\text{18,19}\). As such, IBS occurs in the absence of identifiable physical, radiologic, or laboratory indications of organic disease\(^\text{19,20}\). Characterized as a “brain–gut disorder,”\(^\text{21}\) IBS is associated with such altered physiologic processes as changes in gut motility\(^\text{22,23}\), visceral hypersensitivity\(^\text{24}\) and altered immune activation of the gut mucosa and intestinal microflora\(^\text{25}\). IBS has a reported prevalence of 5%-10% in most of Asia\(^\text{26}\). Although IBS is characterized with abdominal pain and altered bowel activity, it lacks any pathological organic changes. The IBS group was subdivided (Table 1) into diarrhea predominant (IBS-D), constipation predominant (IBS-C), mixed with diarrhea and constipation (IBS-M), or undetermined categories (IBS-U) according to the bowel movement frequency and stool consistency.

Generally, race\(^\text{27}\), gender\(^\text{28-31}\), age\(^\text{2,32,33}\), marital status\(^\text{34,35}\), stress\(^\text{28-30,34-37}\), food\(^\text{1,32,35,38}\), or alcohol and tobacco\(^\text{32}\) use have been considered as risk factors to IBS. IBS accounts for significant health care resource utilization and economic burden. Direct costs are often high in the initial diagnostic phase as historically IBS has been considered a diagnosis of exclusion, prompting sequential testing and invasive procedures in an attempt to identify organic GI disease\(^\text{19,20,39}\). Dean et al\(^\text{40}\) found that IBS-related symptoms reduced work productivity by an estimated 21% per week thus increasing costs to employers. Because IBS is not a mortal illness, the impact on patients is often underestimated. In reality, however, IBS patients have substantially poorer health-related quality of life (HRQoL) than the general population and HRQoL that is on par with that seen in diabetes, depression, and gastroesophageal reflux disease\(^\text{41,42}\).
Table 1

<table>
<thead>
<tr>
<th>IBS Subtype</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. IBS with constipation (IBS-C)</td>
<td>hard or lumpy stools ≥25% and loose (mushy) or watery stools &lt;25% of bowel movements</td>
</tr>
<tr>
<td>2. IBS with diarrhea (IBS-D)</td>
<td>loose (mushy) or watery stools ≥25% and hard or lumpy stools &lt;25% of bowel movements</td>
</tr>
<tr>
<td>3. Mixed IBS (IBS-M)</td>
<td>hard or lumpy stools ≥25% and loose (mushy) or watery stools ≥25% of bowel movements</td>
</tr>
<tr>
<td>4. Unsubtyped IBS</td>
<td>insufficient abnormality of stool consistency to meet criteria for IBS-C, D, or M.</td>
</tr>
</tbody>
</table>

Data on the prevalence of IBS:

Table 2

<table>
<thead>
<tr>
<th>Location</th>
<th>Prevalence (%)</th>
<th>% IBS subtype (C:D:A)</th>
<th>Gender (F:M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North America</td>
<td>17-20*</td>
<td>27.1-29.4:27.1-33.9:26.3135-137</td>
<td>1:1–2:1</td>
</tr>
<tr>
<td>European Union</td>
<td>4†</td>
<td>16:21:63</td>
<td>2:1</td>
</tr>
<tr>
<td>Asia</td>
<td>2.9-15.6</td>
<td>No systematic evaluation has been reported</td>
<td>1:1–1.5:1</td>
</tr>
</tbody>
</table>

* Manning criteria; † Rome II criteria.
Symptom-based criteria

Several sets of criteria have been developed to identify and standardize the diagnosis of IBS (Table 3)\(^{19,20}\). The early Manning criteria\(^{43}\) identified six symptoms that increase the likelihood of an IBS diagnosis but do not stipulate a required number or duration of symptoms. In 1984, Krüs et al\(^{44}\) developed a system of symptoms, physical findings, and laboratory results, and in 1990, an international working group\(^{45}\) developed Rome I criteria in an attempt to reduce unnecessary testing and provide a uniform framework for selecting patients for diagnostic and therapeutic trials in IBS\(^{20,46}\). These criteria were revised in 1999 by the Rome II working group and again in 2006 by the Rome III working group and again in 2006 by the Rome III working group (Table 3)\(^{18,47}\).

Anatomy & Physiology of the Gastrointestinal Tract:

The lower gastrointestinal tract is divided into five parts: the caecum, the ascending colon, the transverse colon, the descending colon, and the rectum. The large intestine (colorectum) begins at the caecum, which is approximately 2–3 inches long and shaped like a pouch. Ileal contents empty into the cecum through the ileocecal valve. The appendix extends from the base of the cecum. The ascending colon rises from the caecum along the right posterior wall of the abdomen, under the ribs to the undersurface of the liver. At this point it turns toward the midline (hepatic flexure), becoming the transverse colon. The transverse portion crosses the abdominal cavity toward the spleen, then goes high up into the chest under the ribs, and turns downward at the splenic flexure. Continuing along the left side of the abdominal wall to the rim of the pelvis, the descending colon turns medially and inferiorly to form the S-shaped sigmoid (sigma-like) colon. The rectum extends from the sigmoid colon to the pelvic floor muscles, where it continues as the anal canal terminating at the anus (Figure 1). The anal canal is approximately 4 cm long.
The large intestine, the site of salt and water absorption, is approximately 5–6 feet long and about 2½ inches in diameter. It is the site of salt and water absorption. Glands secrete large quantities of alkaline mucus into the large intestine, and the mucus lubricates intestinal contents and neutralizes acids formed by bacteria in the intestine. These bacteria aid in decomposition of undigested food residue, unabsorbed carbohydrates, amino acids, cell debris, and dead bacteria through the process of segmentation and putrefaction. Short-chain fatty acids, formed by bacteria from unabsorbed complex carbohydrates, provide an energy source for the cells of the left colon. Maintenance of potassium balance is also assigned to the colon, where the epithelium absorbs and secretes potassium and bicarbonate.

The sympathetic and parasympathetic nervous systems innervate the gastrointestinal tract. Both carry sensory stimuli, though it appears that spinal affrent nerves in the dorsal horn of the spinal cord process pain.

The physiology of sensation in the gut is multifaceted. Enteroendocrine cells transmit mechanical and chemical messages. The communication between gut and brain results in reflex responses mediated at three levels—prevertebral ganglia, spinal cord and brainstem. 5-HT, substance P, CGRP, norepinephrine, kappa opiate and nitric oxide are all involved in the perception and autonomic response to visceral stimulation. Sensation is conveyed from the viscus to the conscious perception via neurons in vagal and parasympathetic fibers (Figure 2). Afferent nerves in the dorsal root ganglion synapse with neurons in the dorsal horn. These signals result in reflexes that control motor and secretory functions as they synapse with efferent paths in the prevertebral ganglia and spinal cord. Pain is processed through spinal afferents in the dorsal horn. Ultimately, stimulation of the brainstem brings sensation to a conscious level. Bidirectional signaling between the brainstem and the dorsal horn mediate sensation. The descending pathways are primarily adrenergic and serotonergic and affect incoming stimuli. End organ sensitivity, stimulus
intensity changes or receptive field size of the dorsal horn neuron and limbic system modulation are the mechanisms involved in visceral hypersensitivity.

**Normal flora and host defences:**

The normal gut is host to several kilograms of bacteria, most of which reside in the colon. Bacterial counts in the stomach number 10^{1-2} colony-forming units (cfu)/ml, while in the jejunum 10^{3-4} cfu/ml, largely of pharyngeal organisms, is normal. As one progresses toward the ileocecal valve, counts rise, and within the ileum 10^{5-6} is normal. However, at the ileocecal valve, there is an abrupt increase in numbers, which rise to 10^{10-13} cfu/ml, with a marked change in the nature of organisms, which become anaerobic/facultative anaerobic, with coliforms and anaerobes predominating. Throughout the gut, there are extensive innate defensive mechanisms, which include both gastric acid, bile, lysozyme, and defensins in the upper gut and tight intraepithelial junctions and mucus layers in the lower gut. There is also an acquired immunity based on immunoglobulin secretion (mainly IgA) and cell-mediated responses. Breaches of these defenses by pathogenic organisms produce acute inflammation with release of cytokines and recruitment of circulating inflammatory cells. In addition to breaches in the epithelium, there is also extensive damage to the lamina propria and submucosa together with the associated nerves. During the process of healing, metaplastic changes occur with increases in enteroendocrine cells, Paneth cells, and chronic inflammatory cells. Furthermore, remodeled nerves often show aberrant expression of receptors and neuropeptides.

**Pathophysiology of IBS:**

The biopsychosocial model of IBS integrates a number of psychosocial, motility, sensory abnormalities and abnormalities in central nervous system processing of visceral pain as the causes of abdominal pain and altered bowel habits (Figure 3).
Figure 3

Psychosocial abnormalities → Motility abnormalities → Sensory abnormalities → CNS processing abnormalities → IBS → Pain

Figure 4

1. Pressure in the rectal lumen
2. Serotonin released from enterochromaffin cells (epithelial cells), stimulates nerve impulse. Resulting in rectal contraction and possibly pain.

Enterochromaffin cell
Afferent nerve fiber
Serotonin impulse
Psychosocial

Approximately 40–60% of patients with IBS who seek medical care also report psychiatric symptoms, such as depression, anxiety, or somatization. Interestingly, however, psychiatric symptoms in patients with IBS in the general population are not as prevalent. It is thought that these psychiatric disturbances influence coping skills and illness-associated behaviors. A history of abuse (physical, sexual, or emotional) has been correlated with symptom severity. More than half of patients who are seen by a physician for Irritable Bowel Syndrome report stressful life events coinciding with or preceding the onset of symptoms.

Neurotransmitters

IBS patients demonstrate significant differences in pain perception, and a variety of perceptual abnormalities related to gastrointestinal stimuli may be more frequent in irritable bowel sufferers. This sensitivity develops as a result of visceral hyperalgesia. Studies evaluating somatic stimuli have demonstrated that the lower tolerance for pain in patients with IBS occurs primarily in the bowel. Recent studies associate neurotransmitters with IBS. Serotonin is located in the central nervous system (5%) and the gastrointestinal tract (95%), and when it is released into the body it results in the stimulation of intestinal secretion and peristaltic reflex (Figure 4) and in symptoms such as abdominal pain, bloating, nausea, and vomiting. These preliminary studies suggest increased serotonin levels in the plasma and in the rectosigmoid colon of patients with IBS.

Stress and Irritable bowel syndrome:

Stress, defined as an acute threat to the homeostasis of an organism, real (physical) or perceived (psychological) and posed by events in the outside world or from within, evokes adaptive responses that serve to defend the stability of the internal environment and to ensure the survival of the organism. Numerous reports in the literature provide evidence for a prominent role of stress in the pathophysiology and clinical
presentation of IBS symptoms. Various stressors induce characteristic changes in gastrointestinal motor function. Moreover, manipulation of attention and changes in arousal level produced by stress, distraction, and relaxation has been reported to alter visceral perception. Different types of stressors play a role in 1) permanent enhancement of stress responsiveness (pathological stress), 2) transient symptom exacerbation, and 3) symptom perpetuation (symptom-generated stress). Early life stress and trauma, in the form of abuse, neglect, or loss of the primary caregiver, play a major role in the vulnerability of individuals to develop functional gastrointestinal (GI) disorders later in life. Acute, life-threatening stress episodes in adult life (rape, posttraumatic stress syndrome) are also important risk factors in the development of functional GI disorders. In the genetically predisposed individual, both early life stress and severe life-threatening stress (referred in this article as “pathological stressors”) can result in permanent, irreversible enhancement of the responsiveness of central stress circuits and therefore vulnerability to development of functional (as well as affective) disorders later in life. Other types of stressors occurring throughout the life of an individual, which may result only in transient changes in stress responsiveness, clearly play a role in symptom exacerbation. For example, psychosocial stressors in the form of sustained, threatening life events have been associated with onset and symptom exacerbation in IBS. “Physical” or interoceptive stressors of the digestive system, such as enteric infections, trauma, and surgery, may play a similar role in symptom exacerbation in the predisposed individual. Finally, in the affected patient, fear conditioning and interoceptive conditioning are likely to play important roles in triggering stress responses to situations and contexts that by themselves are not threatening or stressful. For a large number of IBS patients, the positive-feedback loop of conditioned fear responses to interoceptive stimuli or contextually conditioned stimuli of symptom-generated stressors may play a primary role in symptom chronicity.
The traditional concept of stress has focused on the subjective conscious feelings, thoughts, beliefs, and memories reported by some individuals in association with stressful life events. However, the major breakthroughs in this area have occurred through an understanding of the biological mechanisms that are responsible for the detrimental effects of certain stressful life events on health. The organism’s response to stress is generated by a network comprised of integrative brain structures, in particular, subregions of the hypothalamus (paraventricular nucleus, PVN), amygdala, and periaqueductal gray. These structures receive input from visceral and somatic afferents and from cortical structures, in particular, the ventral subdivision of the anterior cingulate and the medial prefrontal (ventromedial and orbitofrontal) cortex. This integrative network provides outputs to the pituitary and to pontomedullary nuclei, which in turn mediate the neuroendocrine and autonomic output to the body, respectively. This central stress circuitry is under feedback control via ascending monoaminergic projections from these brain stem nuclei, in particular, serotonergic (raphe nuclei) and noradrenergic (NA) (including locus ceruleus) nuclei, and via circulating glucocorticoids, which exert an inhibitory control via central glucocorticoid receptors located in the medial prefrontal cortex and hippocampus. The parallel outputs of this central circuitry (“emotional motor system,” EMS), which is activated in response to various stressors, include responses of the autonomic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis, endogenous pain modulatory systems, and ascending aminergic pathways. One important chemical mediator of the central stress response is corticotropin-releasing factor (CRF) (and probably related currently unknown molecules) located in certain effector neurons of the PVN, the amygdala, and the locus ceruleus complex. CRF secretion by PVN neurons is under positive-feedback regulation by central NA pathways (including those originating from locus ceruleus), thereby forming a bidirectional positive-feedback loop between the CRF and NA systems. Central injection of
CRF can reproduce behavioral and physiological responses similar to those seen in response to acute psychological stress, and inhibition of CRF-mediated responses by antagonists or in knockout animals results in a decrease in the animals’ response to stress.50,51

**Enhanced responsiveness of central stress circuits:**

The responsiveness of the EMS is likely to be under partial genetic control, and it shows considerable plasticity in response to early life events and to certain types of pathological stress. For example, studies in animals and humans clearly demonstrated that certain types of pathological stress can alter the responsiveness of feedback systems by downregulation of preand/ or postsynaptic receptors [adrenergic, serotonergic, glucocorticoid receptors (GC)] and, in the most severe forms, by structural changes in certain brain regions. Thus pathological stress can not only activate but also fundamentally change the responsiveness and output of the central stress circuits. These alterations could affect the individual output pathways of the EMS differentially and in different directions, for example, an increase or decrease in target-specific sympathetic and vagal outputs, up- or downregulation of the HPA axis, and up- or downregulation of pain perception. Some of the best-characterized alterations in this central adaptation to pathological stress are an increase in CRF synthesis and secretion, an increase in the activity and sensitivity of central NA systems, and downregulation of GC suggestive of an enhanced HPA response to stress. In contrast, an upregulation of GC has been found in animals exposed to “early handling” stress and in patients with posttraumatic stress disorder (PTSD), which supports an enhanced negative-feedback control of cortisol and a blunted HPA response to stress. As a consequence of these alterations in the central stress circuitry, secondary changes in receptor systems can occur in spinal or peripheral target cells of the output systems. Thus, in cases of pathological stress resulting in permanent changes in the central stress circuitry, lifelong changes in
Changes in autonomic nervous system responses:

In the most common functional GI disorders, IBS and functional dyspepsia (FD), persistent alterations of autonomic responsiveness are likely to play a role in altered bowel habits and alterations in gastric emptying, respectively. Evidence for such enhanced responsiveness of autonomic responses in IBS includes increased responses of distal colonic motility in response to laboratory stress and possibly food intake and delayed gastric emptying in a subset of patients. A model of IBS, taking into account altered autonomic regulation of gastric and distal colonic function and based on an upregulation of CRF-containing neurons in Barrington’s nucleus (part of the locus ceruleus complex), was recently reported by Valentino and co-workers. Although descending CRF-containing projections from this pontine nucleus to the distal colon may mediate increased stress- and food-induced motor responses of the distal colon, ascending projections to the locus ceruleus and to the forebrain may be responsible for mediating arousal and shifting attention to visceral afferent stimuli. Increased expression of CRF message and release of CRF in IBS patients, or a subset of patients, is also consistent with the reported evidence for certain increased sympathetic responses. Changes in the frequency of high-amplitude propagated contraction (HAPC) in the colon, presumably via alteration in vagal colonic regulation, may play an important role in diarrhea and slow-transit constipation, thereby determining the predominant bowel habit pattern in IBS. There is evidence that decreased cardiovagal tone is present in a subset of patients with IBS, in particular in female patients with constipation-predominant bowel habit and more severe symptoms. The correlation of changes in cardiovagal tone and vagal regulation of the intestine is emphasized by the recent demonstration in patients with functional constipation of parallel changes in cardiovagal tone and autonomic regulation of
whole gut transit and distal colonic mucosal blood flow. Thus, although enhanced sacral parasympathetic modulation of the distal colon, reflecting enhanced responsiveness of neurons within the locus ceruleus complex, may be shared by all IBS patients, alterations in vagal output to the small intestine and proximal colon may be variable, depending on severity and the predominant bowel habit.

**Neuroendocrine changes:**

Preliminary evidence for alterations in HPA axis function was demonstrated in diarrhea-predominant IBS patients who showed decreased 24 h plasma cortisols, blunted cortisol responses, and normal ACTH responses to noxious rectosigmoid distension. In contrast, Heitkemper et al reported that urine cortisol levels obtained immediately on rising were significantly higher in a subgroup of IBS women compared with control women. Even though a thorough characterization of HPA axis responses in patients with functional gastrointestinal disorders has not been reported, these preliminary findings suggest the pattern of sensitized GC feedback also reported in patients with PTSD, fibromyalgia, and chronic fatigue syndrome. However, HPA responses at baseline and in response to provocation in these patients have been conflicting, which may be caused in part by methodological differences and the presence of comorbid depression in some patients. There is significant overlap in the epidemiology of all these conditions with IBS. Other evidence for central alterations in neuroendocrine responses in IBS comes from reports of abnormal neuroendocrine challenge tests. Existing data support neuroendocrine alterations in IBS and other overlapping syndromes, but further well-designed studies are needed to fully characterize these alterations.
Possible relevance of autonomic and neuroendocrine changes for intestinal immune modulation:

Although it is not known currently whether these HPA axis changes are an epiphenomenon or play a role in symptom generation and pathophysiology of these syndromes, one may speculate about their possible role (in conjunction with alterations in autonomic gut regulation) in the observed findings in postinfectious IBS patients. The reported persistence of chronic inflammatory mucosal changes after eradication of the infectious organism and increased intestinal permeability and hyperplasia of enterochromaffin cells are consistent with an inadequate physiological response to acute gut inflammation, in particular an inadequate cortisol (and possibly an altered sympathetic) response. Stress-related alterations in cytokine networks, in particular a suppression of cellular immunity and a shift toward humoral immunity [alteration in T helper (Th)1/Th2 balance], have been reported. Multiple reports in the literature on increased intestinal mast cell numbers in IBS patients are consistent with such a Th2 shift.

Changes in pain modulation:

Evidence suggestive of alterations in stress-induced modulation of viscerosomatic sensitivity comes from human and animal studies. IBS patients show cutaneous normo- or hypoalgesia combined with visceral hypersensitivity. A similar pattern was also seen in a recently described rat IBS model in response to an acute psychological stressor. Preliminary results from the use of psychological laboratory stressors in healthy volunteers suggest a stress-induced increase in colonic or rectosigmoid sensitivity to distension. Even though all published human studies are open to methodological criticism, they are consistent with reported findings in animals of a differential viscerosomatic pain modulation. It is of interest to note that patients with bulimia (who, in contrast to IBS
patients, have a hyperactive HPA axis) show cutaneous hypoalgesia as well, which precedes symptom exacerbations.

**Changes in regional brain activation:**

Functional brain imaging studies of IBS patients have shown decreased activation of ventral subdivisions of the anterior cingulate cortex (ACC) and increased activation of the dorsal subdivision. Because dorsal ACC is inhibited by intense visceral stimuli in healthy control subjects, increased dorsal ACC activation may be related to alterations in attentional processes in IBS in regard to visceral sensory events. Decreased ventral cingulate/medial prefrontal cortex activity was also reported in patients with depression and PTSD, both affective disorders commonly associated with IBS. Southwick et al. reported a decrease in prefrontal and orbitofrontal cortical metabolism in patients with PTSD in response to the α2-antagonist yohimbine. Together with results from preclinical studies showing decreased metabolism in cortical regions with high NA release, these results are consistent with enhanced NA release in these brain regions in PTSD patients. One may speculate that the decreased activation in ventral anterior cingulate, ventromedial frontal cortex, and hippocampus seen in IBS patients may also be related to enhanced NA release from locus ceruleus projections in response to stress, consistent with the Valentino model.

Recent evidence suggests that regional brain activation in response to visceral stimulation may differ between male and female IBS patients. Although most brain regions showed similar activity patterns in male and female patients, differences were seen primarily in the insular and anterior cingulate cortices.

**Corticotropin-releasing hormone (CRH) & IBS**

Corticotropin releasing hormone (CRH) is considered to be a major mediator of stress responses in the brain-gut axis. In particular, stress related activation of CRH receptors has been reported to produce alterations in gastrointestinal function. In addition, physical or
psychological stress is known to delay gastric emptying, accelerate colonic transit and evoke colonic motility in rats. Stress is processed in the brain and the signal is conducted to the paraventricular nucleus (PVN) of the hypothalamus. CRH is released in the PVN and stimulates adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland. ACTH stimulates the adrenocortex and releases cortisol. At the same time, CRH activates sympathetic nervous system and stimulates cardiovascular system. CRH also activates sacral parasympathetic outflow and stimulates colonic motility. Recently, distention of the colon is known to activate the CRH system in the brain. Therefore, visceral stimulation is interpreted as interoceptive stress. Effect of CRH is mediated via CRH receptors in the cell membrane of effector organs. CRH receptor is a seven transmembrane G-protein coupled receptor. Activated G-protein stimulates adenylate cyclase and increases intracellular cyclic AMP. CRH receptors are expressed in the various brain regions. They are the cerebral cortex, cerebellum, hypothalamus, anterior pituitary, amygdala, hippocampus, locus ceruleus, lateralseptum, and others. CRH receptors are also expressed in the peripheral organs. They are heart, arterial smooth muscle, lung, spleen, stomach, intestine, adrenal glands, kidney, skin, skeletal muscle, testis, ovary, and uterus, and others. In IBS patients, exogenous administration of CRH induced robust colonic motility. Besides, exogenous administration of CRH induced exaggeration of ACTH secretion in IBS patients. This finding was replicated by Dinan et al.

Inflammation of the gut and CRH

Chronic low-grade inflammation or discrete inflammation of the gut mucosa combined with psychosocial stress may trigger the sensitization of the lower gastrointestinal tract in IBS. Several reports indicated that there is low-grade inflammation of the colonic mucosa in IBS patients. Increase in intraepithelial lymphocytes, CD3 positive cells, and CD25 positive cells was found in the colonic mucosa of IBS patients. Activated immune system may result
in elevated plasma cytokines in the peripheral blood in IBS patients. Dinan et al.\textsuperscript{69} reported increase in plasma interleukin-6 level in IBS patients. There was no abnormality in the plasma level of TNF-\(\alpha\). Interestingly, the plasma level of interleukin-6 positively correlated with the CRH stimulated ACTH level. Some links between macrophage and colonic function may be present. Studies by Muramatsu et al.\textsuperscript{74} proved mRNA of the CRH family peptide urocortin and CRH receptors in the human colonic mucosa. The major source of urocortin in the human colonic mucosa is macrophages. Furthermore, Wood’s group recently proved the existence of CRH immunoreactivity and CRH receptors in the myenteric plexus of the guinea pig.\textsuperscript{75} There are abundant CRH-R1 positive cells in the myenteric neurons. Most of them are excitatory neurons which enhance colonic and intestinal motility. Besides, there is some evidence that peripheral CRH induces inflammation via an increase in intestinal permeability.\textsuperscript{76} Degranulated mast cells may play a role in the proinflammatory action of CRH. On the other hand, CRH-R2 has been proven to have an anti-inflammatory action\textsuperscript{77} CRH-R2-deficient mice showed increased paw edema after the exposure to the heat stimuli. Besides, CRH-R2 has anti-nociceptive action.\textsuperscript{78} Administration of CRH-R2 agonist human urocortin2 inhibited spinal expression of immunoreactivity of the extracellular signal-regulated kinase 1/2 evoked by the colorectal distention in rats. There are functional differences between CRH-R1 and CRH-R2\textsuperscript{79} In the brain, R1 stimulation causes anxiety whereas R2 stimulation induces anxiolysis. In the gut motility, R1 stimulation evokes colonic motility whereas R2 stimulation inhibits gastric emptying. R1 mediates visceral nociception whereas R2 may reduce visceral perception. Finally, activation of CRH-R1 causes proinflammatory response, whereas stimulation of CRH-R2 provokes anti-inflammatory changes.
Infections:

Other theories concerning IBS associate the inflammation of enteric mucosa or neural plexuses with symptoms. It is hypothesized that inflammatory cytokines may activate peripheral sensitization or hypermotility. One group of researchers was able to predict the development of IBS in patients with infectious enteritis in the presence of stressful life events and hypochondriasis. Researchers in Ontario recently demonstrated that post infection inflammation (Trichomonas spiralis) alters visceral sensitivity. In this particular study, NIH Swiss mice were infected with T spiralis. Six days after infection the mice experienced jejunal enteritis, which returned to normal after 28 days. Using a latex balloon placed in the distal colon, investigators found hyperalgesic sensory response following distension that persisted despite the lack of acute inflammation. Rapid small bowel and colonic transit times have been reported in patients with diarrhea-predominant IBS. Patients with constipation-predominant IBS may have a component of disordered defecation, resulting, at least in part, from abnormal function of the pelvic floor and anal sphincter muscles. Another factor in motor dysfunction is the abnormal passage and handling of gas.

Colonic and rectal hypersensitivity (also called “visceral hyperalgesia”) are also important factors in the causation of symptoms. Enteric propulsion and sensation are, in part, mediated by acetylcholine and serotonin (5HT). A number of studies have reported accelerated small bowel and colon transit as well as exaggerated bowel motility patterns in those with diarrhea predominant IBS (IBS-D). Likewise, several studies have reported delayed bowel transit in those with constipation predominant IBS (IBSC). Visceral hypersensitivity to mechanical distension can often be identified in IBS patients. Unfortunately, visceral hypersensitivity does not occur commonly enough to be considered a biomarker of IBS. There is recent evidence to suggest central dysregulation of emotional arousal and pain modulation in IBS.
Integrative model of Irritable Bowel Syndrome (IBS) pathophysiology:

IBS can develop from centrally dominant factors such as stress or luminal factors like dysbiosis triggering an altered immune response. Stress alters gastrointestinal motility mediated through the hypothalamic-pituitary-adrenal (HPA) axis, and these motility abnormalities can modify the microbiota with the subsequent immune activation in the mucosa and stimulation of nerve terminals, generating symptoms of IBS. On the other hand, dysbiosis related to gastrointestinal infections, small intestine bacterial overgrowth or antibiotics may increase the epithelial permeability leading to contact between pathogens-associated molecular patterns (PAMPs) and toll-like receptors (TLRs) in the deeper layers of the gut with the subsequent host immunity response and IBS generation or symptom exacerbation.

Clinical presentation

The hallmark clinical feature of IBS is abdominal pain associated with changes in bowel habits. Patients with symptoms consistent with constipation-predominant IBS may describe bloating, feelings that their bowel is being incompletely evacuated, and straining, whereas those with the diarrhea-predominant form typically report abdominal pain, gas, urgency, and loose stools, with more than 30% experiencing loss of bowel control. The typical patient is a young woman with abdominal discomfort that is relieved by passage of multiple loose liquid stools. Her symptoms will have been present for more than 3 months and may be exacerbated by factors such as fatty foods or stress. Typically, no alarm features of organic disease, such as unintentional weight loss or rectal bleeding, are present. The clinical course of IBS is chronic although symptoms are extremely variable and fluctuate over time. Nearly half of IBS patients report experiencing daily episodes, whereas about 75% experience at least two episodes per week. Symptoms of IBS may overlap with symptoms found in other disorders, such as chronic constipation, functional dyspepsia,
gastroesophageal reflux disease, inflammatory bowel disease (IBD), celiac disease, and lactose intolerance. IBS can coexist with other functional disorders, most notably fibromyalgia, chronic fatigue syndrome, temporomandibular joint disorder, and chronic pelvic pain, and psychological conditions, such as anxiety, symptom-related fears, and somatization. The differential diagnosis of IBS can be broad and may include IBD, colorectal cancer, enteric infections, systemic hormonal disturbances, and diseases associated with malabsorption. Alarm features that have traditionally increased suspicion for organic disease include rectal bleeding, weight loss, iron deficiency anemia, nocturnal symptoms, and a family history of such organic diseases as colorectal cancer, IBD, and celiac disease. However, with the exception of anemia and weight loss, which have good specificity for organic disease, most alarm features have poor overall accuracy for organic pathology, including colorectal cancer. Evidence indicates that sequential testing is unlikely to uncover the underlying GI organic disease in patients without “alarm features,” and both the American Gastroenterological Association (AGA) and American College of Gastroenterology (ACG) recommend using accepted symptom-based diagnostic criteria to make a positive diagnosis of IBS rather than an exhaustive diagnostic investigation.

Clinical observations indicate that a subset (about 10–25%) of the subjects exposed to enteric infections in an individual or a community setting go on to develop predominantly the symptoms of diarrhea-predominant IBS (IBS-D). PI-IBS is characterized by the acute or new onset of symptoms in the presence of previously normal bowel function. In individuals with PI-IBS, abnormal bowel habits typically persist continuously after the acute infectious episode, although they may wax and wane. Although the severity of symptoms diminishes, compared with that during the acute infectious episode, bowel function before the episode is not regained. Weight loss and bleeding with defecation, which may occur during the acute diarrheal episode that precipitates PI-IBS, do not characterize PI-IBS. Persistent
weight loss and/or rectal bleeding should stimulate investigation of alternative diagnoses. Experimental evidence suggests that chronic inflammation following acute bacterial infection has a pathophysiological role in the development of PI-IBS. Subjects with PI-IBS appear to be unable to down-regulate intestinal inflammation. In a recent study of unselected patients with IBS, there was a decreased prevalence of the anti-inflammatory cytokines IL-10 and transforming growth factor–β among these patients, which implies that they were more susceptible to prolonged and severe inflammation. Markers of mucosal inflammation are consistently elevated in patients with PI-IBS. Both during and 3 months after an episode of acute gastroenteritis, the inflammatory cytokine IL-1β was present in higher levels in the rectal mucosa of 8 patients who developed PI-IBS, compared with 7 patients whose bowel habits returned to normal. Moreover, at the 3-month assessment, IL-1β levels were elevated in the patients with PI-IBS, but not in the patients whose bowel habits normalized after acute gastroenteritis, compared with 18 control subjects who had not had gastroenteritis for at least 2 years before the study. On the basis of these findings, the authors suggest that inflammation plays a role in causing PI-IBS and that patients who develop PI-IBS may be more susceptible to inflammatory stimuli than those who do not. The results of macroscopic and conventional histological assessments of the intestinal mucosal of patients with PI-IBS generally appear to be normal within 2 weeks of the acute infectious illness, but chronic inflammation as revealed by quantitative histological analysis persists. In a study of 21 patients who had experienced acute Campylobacter enteritis and 12 control subjects with no bowel symptoms after Campylobacter enteritis, numbers of intraepithelial T lymphocytes and enterochromaffin cells in rectal biopsy specimens were elevated, compared with cell counts in control subjects, at 2, 6, and 12 weeks after the episode of enteritis. In a subset of 7 patients who had symptoms for 1 year after the episode of enteritis, counts of both cell types remained significantly higher than those noted for control subjects. Intraepithelial T
lymphocytes and enterochromaffin cells were also increased in a separate group of 10 patients with PI-IBS. Cell counts in the latter group were comparable to those in the 7 patients who had symptoms 1 year after the episode of *Campylobacter* enteritis. These signs of persistent local inflammation were accompanied by increased small-bowel permeability, as reflected by an elevated ratio of urinary excretion of lactulose to mannitol. Together, the increases in T lymphocytes and enterochromaffin cells and in small-bowel permeability reflect persistent mucosal inflammation. These findings are corroborated by results of a study of 28 patients with newly diagnosed PI-IBS after *Campylobacter* infection, 28 age- and sex-matched control subjects who were asymptomatic after *Campylobacter* infection, and 34 healthy volunteers. Enterochromaffin cell counts and T lymphocyte counts in rectal biopsy specimens were higher in patients with PI-IBS than in either recovered control subjects or healthy volunteers. Enterochromaffin cell hyperplasia, which is thought to be a relatively nonspecific response to mucosal injury and inflammation, possibly contributes to the symptoms of PI-IBS through serotonin-mediated effects. Enterochromaffin cells are the source of nearly all intestinal mucosal serotonin, which stimulates enteric secretions, activates visceral sensory afferents, and mediates peristalsis.

Why only less than a quarter of the subjects exposed to enteric infections develop the chronic symptoms of IBS has remained an enigma. However, two major risk factors that predispose the individuals to develop postinfectious IBS (PI-IBS) symptoms following an enteric infection have emerged. 1) The duration of enteritis lasting greater than 3 wk significantly increases the risk for developing PI-IBS over a duration lasting less than 1 wk. 2) The presence of comorbid psychiatric disorders or a lifetime history of anxiety and depression at the time of infection increases the risk of developing PI-IBS. The longer duration of enteritis reflects its severity of inflammation. The psychosomatic disorders represent dysregulation/impairment of the central nervous system. Stress resulting from...
psychological trauma or physical insult in early stages of life is known to result in persistent neural disorders in adulthood. Therefore, early life stress has been used extensively to investigate major depressive disorders and posttraumatic stress disorders. More recently, neonatal maternal separation and colonic irritation have been used to model visceral hypersensitivity in response to colorectal distension. The immaturity of the stress response system in neonates is, therefore, a risk factor for the development of persistent adverse effects later in life. However, the molecular mechanisms of persistent cellular dysfunction long after the neonatal insult remain unknown.

**Traditional diagnostic tests for IBS**

The traditional diagnostic work-up of IBS includes common tests that may be helpful in differentiating IBS with alarm features of organic disease although they are not recommended in patients with typical IBS symptoms who do not have alarm features. The choice of test is usually guided by the nature and severity of symptoms and the patient’s expectations and concerns. Advantages and disadvantages of these traditional tests are summarized in Table 4. Newer diagnostic tools, such as the examination of stool forms, fecal inflammatory markers, and the use of serum biomarker, may be useful adjuncts to traditional evaluations of patients with suspected IBS. These simple tests may allow clinicians to distinguish IBS from non-IBS disorders in a cost-effective manner, possibly facilitating a paradigm shift from approaching IBS as a diagnosis of exclusion to one of inclusion. Already used routinely in some European countries, fecal calprotectin and lactoferrin are highly sensitive and specific for intestinal inflammation and can differentiate IBD from IBS. Recently, an IBS diagnostic panel composed of 10 serum biomarkers that are linked to multiple regulatory pathways and proteins associated with IBS has been developed.
Table 4

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<tr>
<th>Tests</th>
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<th>Disadvantages</th>
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<tr>
<td>Stool for ova and parasites</td>
<td>Noninvasive, inexpensive</td>
<td>Limited value in identifying organic disease in patients without alarm features</td>
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<td>May be useful in patients with clinical features, symptom pattern, and geographic area suggestive of infection</td>
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<tr>
<td>Hydrogen breath tests</td>
<td>May be useful when lactose maldigestion is suspected</td>
<td>Need for specialized equipment, dedicated space, and technical support</td>
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<td>Noninvasive</td>
<td>False-positive results possible in smokers and those with poor oral hygiene</td>
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<td>Limited value in identifying organic disease in patients without alarm features</td>
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<tr>
<td>Celiac serologies (anti-tTG, EMA, AGA)</td>
<td>Routine use in IBS-D and IBS-M patients helpful in identifying celiac disease</td>
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<td>Abdominal imaging</td>
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<td>Low diagnostic yield in patients without alarm features</td>
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<tr>
<td>Endoscopy/colonoscopy</td>
<td>Useful for identifying organic diseases (particularly IBD, colorectal cancer, and microscopic colitis) in patients with IBS-D and alarm features Can exclude mechanical obstruction in patients with IBS-C and alarm features Upper endoscopy with biopsies may be useful in detecting celiac disease or SIBO</td>
<td>Expensive</td>
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<tr>
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<td>Low diagnostic yield in patients without alarm features</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal findings not associated with improved HRQoL</td>
</tr>
</tbody>
</table>

Burbige, E.J., *Irritable bowel syndrome: diagnostic approaches in clinical practice*
**Routine blood and stool hemoccult tests**

The AGA recommends a screening of complete blood count and stool hemoccult for patients presenting with IBS symptoms of short duration, family history of colorectal cancer or IBD, older age at symptom onset, or lack of concurrent psychosocial difficulties. However, these tests offer little value in identifying organic disease in patients with typical IBS symptoms but no alarm features. Further, a causal relationship between the thyroid dysfunction and IBS symptoms was not established. Stool examinations for ova and parasite stool examinations may be useful if the patients’ symptom pattern, geographic area, and clinical features (eg, diarrhea in an area of known endemic infection) suggest an infectious etiology. However, such tests are not recommended for routine use in patients without alarm features or infection, as findings are significant in fewer than 2% of patients with IBS.

**Carbohydrate breath test**

The carbohydrate breath test has been used by gastroenterologists for many years to detect lactose malabsorption. The test measures exhaled levels of hydrogen and/or methane produced during the metabolism of carbohydrate substrates by intestinal bacteria. Breath testing for lactose intolerance may be useful in patients with typical IBS symptoms and suspected lactose maldigestion. It is important, however, to establish that dietary intake of lactose is 240 mL of milk (or equivalent) per day before testing for lactose intolerance, as Suarez et al have shown that patients with documented lactose malabsorption are able to tolerate 240 mL of milk per day with minimal or no symptoms. Despite the frequent occurrence of lactose intolerance in patients with IBS, no causal relationship has been established between lactose intolerance and IBS symptoms. However, given the possibility that IBS patients are more sensitive to the clinical consequences of lactose maldigestion,
the ACG IBS Task Force has recommended considering a lactose hydrogen breath test in patients whose history and food diary review suggest potential lactose maldigestion\textsuperscript{19}. Recently, carbohydrate breath test has been used in an attempt to identify patients with small intestinal bacterial overgrowth (SIBO)\textsuperscript{124}. Emerging evidence suggests that SIBO plays a pathogenic role in IBS,\textsuperscript{127,128} although this remains contentious. The use of carbohydrate breath test to diagnose SIBO in IBS patients has yielded conflicting results. The ACG IBS Task Force does not recommend breath testing for SIBO in patients with IBS.\textsuperscript{19}

**Imaging**

Neither abdominal nor colonic imaging tests are likely to reveal the structural abnormalities that explain symptoms of IBS in patients with no alarm features.\textsuperscript{19,101}

**Endoscopy or colonoscopy**

Studies evaluating endoscopic investigation for suspected IBS do not support their routine use in patients without alarm features. Patients younger than 50 years with IBS symptoms but no alarm features do not require routine colonic imaging. However, patients aged > 50 years should follow expert recommendations for colonic imaging for colorectal cancer screening\textsuperscript{19,129,130} and those aged > 50 years with alarm features should undergo colonoscopy to rule out organic disease.\textsuperscript{19} Patients with IBS-D and alarm features should be examined specifically for colorectal cancer and IBD and potentially undergo random mucosal biopsies to rule out microscopic colitis, which has a prevalence of 2.3% among patients with IBS-D.\textsuperscript{19,131} The prevalence of microscopic colitis is highly age-dependent as shown in a Swedish epidemiology study of 1,018 patients presenting with nonbloody diarrhea who underwent colonoscopy; 10% overall, but 20% of those >70 years, received a diagnosis of microscopic colitis.\textsuperscript{132} Patients with IBS-C should be evaluated for mechanical obstruction, which can be done by colonoscopy, virtual colonography, or barium enema. Upper
endoscopy with small bowel biopsies can be considered to test for celiac disease or SIBO in patients with laboratory or stool findings suggestive of malabsorption.\textsuperscript{19}

**Examination of stool forms**

Pimentel et al\textsuperscript{118} evaluated the variations in frequency and consistency of bowel habits over time to distinguish diarrhea secondary to IBS from diarrhea associated with active celiac disease, ulcerative colitis (UC), and Crohn’s disease. The basis for this testing is that patients with IBS-D, whose bowel function varies with changes in neuromuscular function, will more likely experience irregular bowel function and stool form than those with non-IBS causes of diarrhea. Sixty-two IBS-D patients and 37 non-IBS patients (UC, Crohn’s disease, celiac disease) completed a questionnaire on their bowel habits and stool forms during the preceding week. More IBS than non-IBS patients reported daily variations in stool form and frequency of bowel habits.\textsuperscript{118} Further, 81\% of IBS patients and 41\% of those without IBS reported having at least three stool forms per week; the difference of three stool forms per week has a sensitivity of 68\% and specificity of 84\% in differentiating IBS.\textsuperscript{118} The success of this simple tool in distinguishing between IBS and non-IBS diarrheal disease may help avoid unnecessary diagnostic testing.\textsuperscript{118}

**Fecal markers**

Increasing evidence supports the utility of fecal calprotectin and lactoferrin in differentiating between IBD and IBS because these markers can identify active intestinal inflammation in IBD\textsuperscript{96,121,133-135}. Easily measured in feces, calprotectin and lactoferrin are stable, neutrophil-derived proteins that increase in concentration in response to leukocyte migration into the gut\textsuperscript{121,134,136}. Although the elevated levels of fecal calprotectin and lactoferrin are potentially valuable when screening for intestinal inflammation and discriminating between organic and functional diseases\textsuperscript{119,121} either the cause of the inflammatory process leading to the elevations can be discerned with these markers nor are
the elevations able to “rule in” a diagnosis of IBS, given that differences between IBS and healthy controls are not significant.

**Serologic markers**

A number of altered physiological pathways have been identified in patients with IBS. The major pathogenic processes include altered gut motility and visceral hypersensitivity, which are believed to be due to the dysregulation of brain–gut axis pathways,\textsuperscript{137} immune dysregulation in the GI tract,\textsuperscript{138} altered gut flora\textsuperscript{19,127,139,140} and complex interactions between neuronal and hormonal factors\textsuperscript{141}. These alterations have prompted investigations of IBS biomarkers that may help elucidate the pathophysiology of the disorder\textsuperscript{138} and aid in the diagnosis of the disease\textsuperscript{141}. Lembo et al\textsuperscript{141} described a panel of 10 serum biomarkers that may be useful to help differentiate IBS from non-IBS disease and healthy controls. The panel includes interleukin-1\(\beta\), growth-related oncogene-\(\alpha\), brain-derived neurotrophic factor, antihuman anti-tTG, tumor necrosis factor-like weak inducer of apoptosis, tissue inhibitor of metalloproteinase-1, neutrophil gelatinase-associated lipocalin, antibody to bacterial flagellin (anti-CBir1), anti-\textit{Saccharomyces cerevisiae} antibody (ASCA-IgA), and antineutrophil cytoplasmic antibody (ANCA).\textsuperscript{33} Six of these biomarkers have been associated with metabolic dysregulation in IBS, whereas others (ASCA, ANCA, and anti-CBir1) have typically been associated with IBD. Notably, one study demonstrated elevated levels of anti-CBir1 in a subset of patients with postinfectious IBS, presumably the result of bacterial infection and immune system activation\textsuperscript{142}. The role of inflammatory processes in IBS and concomitant changes in biomarker expression need to be more fully evaluated, especially as they relate to potential changes over the course of the disease. Likewise, anti-tTG is a serum antibody known to be highly specific for only celiac disease. After the biomarkers were identified, a predictive modelling tool was used to discern patterns of serum concentrations that best differentiated IBS patients from those with non-IBS GI disease (IBD,
non-IBS functional GI disorders, and celiac disease) and healthy controls, leading investigators to conclude that a positive test result can help confirm a suspicion of IBS although the sensitivity is insufficient for a negative test to reliably exclude the diagnosis 141.

**Prostaglandins:**

Prostaglandins (PG) are synthesized by the sequential action of phospholipases, cyclooxygenases (COX)-1 and COX-2, and specific terminal synthases, and exert their diverse biological effects through several membrane receptors (Figure 5).

In particular, PGE2 is involved in many normal and pathological pathways that are mediated by four different E prostanoid receptors (EP1–4). Prostanoids mediate a variety of cellular interactions in physiological and pathological processes, including hemostasis and thrombosis; glomerular filtration and water balance; ovulation, embryo implantation, and development; initiation of labor or abortion; and inflammation and modulation of immune responses. Prostanoids are biologically active metabolites of arachidonic acid (AA). In response to different stimuli (i.e., physical, chemical, hormonal, cytokines, etc.), AA is mobilized from membrane phospholipids through the action of phospholipases (PL) and is then converted to prostaglandin (PG)H2 by cyclooxygenase (COX)-1 or COX-2. COX isozymes catalyze a two-step reaction, first cyclizing AA to form PGG2 and then reducing the 15-hydroperoxy group to form PGH2. Cell-specific isomerases or reductases catalyze the conversion of PGH2 to biologically active end-products including PGE2, PGF2α, PGD2, PGI2 and thromboxane (TX) A2, known collectively as prostanoids. Prostanoids are produced as needed in the cell of origin, act primarily as autacoids on the parent cell and/or neighboring cells, and have very short half-lives. COX-1 and COX-2 isozymes catalyze the same reactions, show approximately 60% identity in their amino-acid sequence within a given species, but are encoded by two different genes, located in different chromosomes. They seem to have different functions even within the same cell type. 143, 144 COX-1-dependent
Figure 5

Cell membrane phospholipids

\[ \text{Phospholipase A2} \]

\[ \text{Arachidonic acid} \]

\[ \text{Cyclooxygenase-1} \]
\[ \text{Cyclooxygenase-2} \]

\[ \text{PGG2} \]

\[ \text{PGH2} \]

\[ \text{TXS} \]
\[ \text{ePGES} \]
\[ \text{mPGES-2} \]
\[ \text{PGIS} \]
\[ \text{PGDS} \]

\[ \text{TXA2} \]
\[ \text{PGF2\textsubscript{\alpha}} \]
\[ \text{PGE2} \]
\[ \text{PGD2} \]
\[ \text{PGI2} \]

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Arthritis Research & Therapy
prostanoids serve a number of physiologic “housekeeping” functions, such as modulation of platelet aggregation and cytoprotection in the gastrointestinal mucosa.\(^\text{143}\) In addition, the expression of COX-1 is developmentally regulated in many different tissues including thymus\(^\text{143}, \text{145}\), and small changes in expression (e.g., 2- to 4-fold increase) can occur after stimulation with hormones or growth factors\(^\text{143}\). In contrast, COX-2 is induced in macrophages, fibroblasts, vascular endothelial cells, and smooth muscle cells by various cytokines, endotoxins, growth factors, or tumor promoters.\(^\text{144}\) Therefore, COX-2-dependent PGs play a major role in inflammation and cell proliferation. The protein expression of specific down-stream PG synthases, such as the PGE\(_2\) synthases (PGES), could be induced, leading to an overall increase in enzymatic activity PGE\(_2\) production.\(^\text{145,146}\) Two isoforms of PGES have been described—one membrane-associated (mPGES) and one cytosolic (cPGES).\(^\text{145,146}\) and COX-1 and COX-2 exhibit differential “functional” coupling to each isoform of the PGES in different cell types, including platelets.\(^\text{145-148}\) There has been no demonstrated physical binding between COX and PGES; rather, it is more likely that intracellular colocalization of these enzymes creates a local zone where AA is processed by physically close (but not coupled) enzymes. In addition, intracellular distribution of both COXs and PGES appears compartmentalized, in that COX-2 and mPGES are mainly membrane-associated and localized in the perinuclear envelope.\(^\text{147}\) Intracellular compartmentalization and functional coupling create preferential and selective “routes” for AA in response to different biological demands. Indeed, PGE\(_2\) and PGI\(_2\) appear to be the main prostanoids produced by COX-2, whereas COX-1 can generate all of the PGs\(^\text{147,148}\), depending on the availability of other enzymes present. Finally, the complex regulation and tissue specificity of prostanoid actions is enriched by a variety of cell receptors which trigger different intracellular signalling.\(^\text{149}\) PG receptors were first characterized pharmacologically and classified based on their sensitivity to five primary prostanoids (i.e., PGE\(_2\), PGI\(_2\), TXA\(_2\),
PGD$_2$ and PGF$_{2\alpha}$, and termed EP (for, E type prostanoid receptor), IP, TP, DP, and FP, respectively.$^{149}$ Among prostanoids, PGE$_2$ has the most receptors: four subtypes of EPs have been characterized so far: EP1, EP2, EP3, and EP4, defined on the basis of their pharmacological profiles.$^{149}$ EPs are encoded by distinct genes and have divergent amino-acid sequences, but all bind PGE$_2$ with higher affinity than other prostanoids. Thus, based on multiple receptor subtypes, PGE$_2$ can trigger several different intracellular signal transduction paths and has diverse final effects, which sometimes seem to be even functionally opposing within the same cell or organ. Activation of the EP1 receptor most likely increases intracellular Ca$^{2+}$, through G$_q$, phospholipase C (PLC)/inositol triphosphate signaling, and protein kinase C (PKC) activity. EP1-mediated Ca$^{2+}$ increases, however, might not be solely dependent on G$_q$ activity.$^{149}$ EP2 and EP4 stimulate adenylate cyclase via G$_s$, leading to the production of adenosine 3',5'-monophosphate (cyclic AMP, cAMP), which then activates the cAMP-dependent protein kinase (PKA).$^{150}$ Stimulation of EP4 is also known to activate phosphoinositide 3'-kinase (PI3K).$^{150}$ At variance with other EPs, the EP3 has multiple splice variants, each having a unique C-terminal cytoplasmic tail $^{149}$, which adds more complexity to EP3-mediated signaling. EP3 generally inhibits adenylate cyclase through the activation of Gi (a pertussis toxin-sensitive G protein); however, EP3 is likely to signal through G-protein–Rho interactions as well.$^{149}$ The complexity of the final response to PGE$_2$ is further complicated by evidence that multiple EPs are often coexpressed or induced in the same cell or organ. The physiological effects of prostaglandins are mediated in part by G-protein-coupled prostanoid receptors, a family of rhodopsin-like seven transmembrane spanning receptors (GPCRs). Activation of a given prostaglandin receptor by its cognate ligand may elicit varying responses in different cell types and tissues. Moreover the existence of multiple receptors coupling to different signal transduction pathways for a given prostaglandin (e.g., EP1–4 for PGE2 and DP/CRTTH2 for PGD2) allows for potential synergism or antagonism.
between prostanoid receptor. Given their structural similarities, that prostaglandins may activate more than one subtype of prostaglandin receptor. Complexity is also observed in modulation of the immune response by PGE2, whereby activation of specific EP receptors has been shown to regulate the function of many cell types including macrophages, dendritic cells, T and B lymphocytes leading to both pro- and anti-inflammatory effects.

**PTGS2 (COX-2) gene**

PTGS2 gene is mapped to chromosome 1q25.2-q25(Figure 6).

**Homo sapiens PTGS2 gene for prostaglandin endoperoxide synthase-2, complete cds**

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COX-2 enzyme

**Catalytic activity:**  
Arachidonate + AH(2) + 2 O(2) = prostaglandin H(2) + A + H(2)O

The first rate-limiting step in the conversion of arachidonic acid to prostaglandins is catalyzed by PTGS. The cyclooxygenases oxygenate arachidonic acid (AA) in the committed step of prostaglandin biogenesis. Substitutions of I434V, H513R, and I523V constitute the only differences in residues lining the cyclooxygenase channel between COX-1 and COX-2. These changes create a hydrophobic pocket in COX-2, with Arg-513 located at the base of the pocket, which has been exploited in the design of COX-2-selective inhibitors. Tazawa et al. (1994) isolated the entire PGHS2 gene and its 5-prime flanking region and showed that it contains 10 exons and spans 7.5 kb. PTGS2 is a protein that is expressed as part of the acute response to inflammation$^{151}$ and is the main enzyme responsible for transforming arachidonic acid into prostaglandins. Expression of PTGS2 is tightly controlled, with basal levels low to nonexistent in most tissue types. In addition, PTGS2 mRNA has a very short half-life regulated by sequence-specific elements in the 3’ untranslated region (UTR) of the gene.$^{152,153}$ PTGS2 is a very small gene with only five
This ribbon diagram shows the three-dimensional structure of the cyclooxygenase or COX-2 enzyme.
known common polymorphisms; The SNPs are located at base pairs 926; 3,050; 5,209; 8,473; and 9,850.

COX-2 exists as a homodimer, each monomer with a molecular mass of about 70 kDa. The tertiary and quaternary structures of COX-1 and COX-2 enzymes are almost identical. Each subunit has three different structural domains: a short N-terminal epidermal growth factor (EGF) domain; an α-helical membrane-binding moiety; and a C-terminal catalytic domain. COX enzymes are monotopic membrane proteins; the membrane-binding domain consists of a series of amphipathic α helices with several hydrophobic amino acids exposed to a membrane monolayer. COX-1 and COX-2 are bifunctional enzymes that carry out two consecutive chemical reactions in spatially distinct but mechanistically coupled active sites. Both the cyclooxygenase and the peroxidase active sites are located in the catalytic domain, which accounts for approximately 80% of the protein. The catalytic domain is homologous to mammalian peroxidases such as myeloperoxidase. Different ligands bind either the allosteric or the catalytic subunit (Figure 8). Allosteric subunit binds a non-substrate, activating FA (e.g., palmitic acid). The allosteric subunit with bound fatty acid activates the catalytic subunit by decreasing the Km for AA. COX-2 exists as a homodimer, each monomer with a molecular mass of about 70 kDa. It has been found that human PGHS-2 functions as a conformational heterodimer having a catalytic monomer (E-cat) and an allosteric monomer (E-allo). Heme binds only to the peroxidase site of E-cat while substrates, as well as certain inhibitors, bind the COX site of E-cat. E-cat is regulated by E-allo in a way dependent on what ligand is bound to E-allo. Substrate and non-substrate fatty acid (FAs) and some COX inhibitors preferentially bind to the COX site of E-allo. AA can bind to E-cat and E-allo, but the affinity of AA for E-allo is 25 times that for E-cat. Palmitic acid, an efficacious stimulator of huPGHS-2, binds only E-allo in palmitic acid/murine PGHS-2 co-crystals. Non-substrate FAs can potentiate or attenuate COX
Figure 8
inhibitors depending on the fatty acid and whether the inhibitor binds E-cat or E-allo. Studies suggest that the concentration and composition of the free fatty acid pool in the environment in which PGHS-2 functions in cells, also referred to as the FA tone, is a key factor regulating the activity of PGHS-2 and its response to COX inhibitors. COX enzymes produce PGH2 from AA in two consecutive chemical reactions. PGH2 is converted to other prostanoids by tissue-specific enzymes called isomerases. In the first step of the reaction, two moles of oxygen are added to arachidonic acid to yield PGG2. This is referred to as the COX reaction. The latter is followed by the peroxidase reaction, which reduces PGG2 to give PGH2 (Figure 9). The COX reaction and the peroxidase reaction occur at two different locations in COX enzymes. Both the peroxidase and the cyclooxygenase activities are inactivated during catalysis by mechanism-based, first-order processes, which means that PGHS-2 peroxidase or cyclooxygenase activities fall to zero within 1–2 minutes, even in the presence of sufficient substrates.

Mechanism

The conversion of arachidonic acid to PGG2 can be shown as a series of radical reactions analogous to polyunsaturated fatty acid autoxidation (Figure 10). A hydroperoxide oxidizes the heme to a ferryl-oxo derivative that either is reduced in the first step of the peroxidase cycle or oxidizes Tyrosine 385 to a tyrosyl radical. The tyrosyl radical can then oxidize the 13-pro(S) hydrogen of arachidonic acid to initiate the COX cycle. The 13-pro(S) hydrogen is abstracted and dioxygen traps the pentadienyl radical at carbon 11. The 11-peroxyl radical cyclizes at carbon 9 and the carbon-centered radical generated at C-8 cyclizes at carbon 12, generating the endoperoxide. The allylic radical generated is trapped by dioxygen at carbon 15 to form the 15-(S) -peroxyl radical; this radical is then reduced to PGG2 . This is supported by the following evidence: 1) a significant kinetic isotope effect is observed for the abstraction of the 13-pro (S )-hydrogen; 2) carbon-centered radicals are
trapped during catalysis\textsuperscript{162} \(3\) small amounts of oxidation products are formed due to the oxygen trapping of an allylic radical intermediate at positions 13 and 15.\textsuperscript{163,164} Another mechanism is the 13-pro (S)-hydrogen is deprotonated and the carbanion is oxidized to a radical is theoretically possible. However, oxygenation of 10,10-difluoroarachidonic acid to 11-(S)-hydroxyeicosa-5,8,12,14-tetraenoic acid is not consistent with the generation of a carbanion intermediate because it would eliminate fluoride to form a conjugated diene.\textsuperscript{165}\[15\]

The absence of endoperoxide-containing products derived from 10,10-difluoroarachidonic acid has been thought to indicate the importance of a C-10 carbocation in PGG2 synthesis\textsuperscript{166} However, the cationic mechanism requires that endoperoxide formation comes before the removal of the 13-pro (S)-hydrogen. This is not consistent with the results of the isotope experiments of arachidonic acid oxygenation.\textsuperscript{167}

In genotype association studies, the relationship between genotype and clinical phenotype includes “black box” intermediaries such as the effect of the environment and other unknown biological factors (Figure 11). Lower panel: In genotype-endophenotype association studies, the relationship between candidate genes that affect the biomarkers (endophenotype) of interest is explored with confidence that the biomarkers are associated with a known (often robust) relationship with the main manifestations of the clinical phenotype. Since the biomarker functions are measurable and have a defined coefficient of variation, the size of the function that is detectable can be used to inform the much smaller sample size for the genetic association study.

By studying the genetic associations between candidate genes and in depth physiological functions, it is possible to identify genetic susceptibility or, at least, to generate hypotheses that can be tested to evaluate the role of the candidate mechanism.

The single genetic variation most intensely studied in search of an association with IBS is 5-HTTLPR. This polymorphism is located in the promoter region of
the gene \textit{SLC6A4} responsible for the synthesis of 5-HT transporter, SERT. The latter is central to fine-tuning brain 5HT neurotransmission, and is abundant in cortical and limbic areas, and affects emotional aspects of behavior.\textsuperscript{168} This polymorphism (s) results in short (s) and long (l) allele, based on the presence or absence of a 44 bp insertion.\textsuperscript{168,169} The \textit{SLC6A4} gene is located on chromosome 17q11.1–17q12 and organized into 14 exons spanning ~ 38 kb.\textsuperscript{169} A study from Mayo Clinic did not demonstrate a significant association of any 5-HTTLPR allele and IBS; this contrasted with the results of the small study by Pata et al. However studies from Western countries and Asia provided contradictory results.\textsuperscript{170} A meta-analysis of most of the studies currently in the literature suggests that among Caucasians and Asians, odds ratio for IBS in subjects for SS or LS vs LL (odds ratio [OR] 1.0, 95% CI 0.8–1.2) and for SS vs LL or LS (OR 1.0, 95% CI 0.7–1.4) are not significant.

Another genetic variation related to 5-HT control is STin2 VNTR, which is located in intron 2. It consists of variable number \textsuperscript{168,171,172} identical 17-bp segments\textsuperscript{173}; the 10/12 genotype has been associated with IBS in one study.\textsuperscript{174} However, other studies found no association between STin2 VNTR and IBS.\textsuperscript{175–177}

A SNP, rs25531, located immediately upstream of 5-HTTLPR is strongly linked, yet has opposing effects on SERT expression. The rs25531 G allele lowers SERT transcription compared with the A-allele and occurs most frequently with 5-HTTLPR – L. Carriers of the G allele (minor allele frequency 10%) of rs25531 had increased odds [OR 3.3 (95% CI 1.1–9.6)] of having IBS compared with healthy controls.\textsuperscript{173} The A:G allele frequency (percent) in controls in this study was 96:4, in contrast to the IBS patients (89:11). Given the relatively small numbers of patients (186 with IBS) in the study, this result requires confirmation, particularly because G allele frequencies of 10% and 18% are reported in two studies (that included 20 and 190 patients respectively) conducted in the United States and
reported in NCBI\textsuperscript{178} and these G allele frequencies are closer to the 11% observed in the IBS cohort studied in the Northwest region of the United States.

In a study of 54 patients with Rome I positive IBS and 107 healthy individuals, Pata et al reported that increased risk of IBS in patients with homozygous C allele of the 102 T/C polymorphisms or homozygous A allele of the -1438 G/A polymorphism of the 5-HT2A receptor gene. In addition, the T/T genotype of 102 T/C polymorphism may be associated with more severe pain in patients with IBS.\textsuperscript{179}

In a collaborative study between centers in England and Germany, investigating the potential role of genetic variation in 5-HT3 receptors, an initial study was conducted in 200 IBS patients and 100 healthy controls from the UK. The novel \textit{HTR3E} 3'-UTR variant c.*76G>A (rs62625044) was associated with female IBS-D. This association was confirmed in a replication study, including 119 IBS-D patients and 195 controls from Germany. Using a reporter assay, Kapeller et al showed that c.*76G>A affected the binding of microRNA-510 (miR-510) to the \textit{HTR3E} 3'-UTR and caused elevated protein expression in 2 different cell lines. In addition, HTR3E and miR-510 co-localize in enterocytes as shown by in situ hybridization and RT-PCR.\textsuperscript{180}

**α2-Adrenergic Receptors**

Adrenergic receptor and 5-HTTLPR genotypes were evaluated in an original cohort\textsuperscript{171} consisting of 274 IBS and 120 controls. There was significant association between IBS-C and the α2C Del 322–325 deletion, which alters the coding region of the gene. This variation results in a receptor that has markedly decreased agonist-mediated responses \textit{in vitro}.\textsuperscript{181} There is also evidence that the same variation results in altered cold pain perception without affecting cognition.\textsuperscript{182}

There was a non-significant association of IBS-C with the α2A -1291 C>G (rs1800544) genotype. These provocative associations demonstrated between genetic
variations in α2A and α2C adrenergic receptors and IBS-C have not been assessed by other groups.

**Cathechol-o-methyl transferase**

Catechol-O-methyltransferase (COMT), which catalyzes the transfer of a methyl group from S-adenosyl methionine to catecholamines (such as dopamine, norepinephrine and epinephrine) and their inactivation, is a key regulator of pain perception, cognitive function, and affective mood. Three common haplotypes of the human COMT gene, consisting of two synonymous and one non-synonymous SNPs, code for differences in COMT enzymatic activity due to a reduced amount of translated protein and are associated with pain sensitivity. A Valine158Methionine (Val158Met) allele has been identified in the COMT gene associated with a three-to-four fold decline of the COMT activity compared with that of the non-Val158Met allele. In a recent study, interaction of gender, age, COMT Val158Met polymorphism was found in dyspepsia.

**G-proteins**

G protein-coupled receptors are present on every excitable cell and every cell that is susceptible to regulation in the body. It is estimated that 80% of ligand-receptor interactions are mediated through G-protein coupled receptors; activation of the receptor leads to production of the βγ heterodimer from the heterotrimeric G protein. This is catalyzed by GNβ3. Initial studies suggested an association between GNβ3 C825T and dyspepsia however, we did not identify an association of IBS with GNβ3 C825T. The latter finding has been replicated in a smaller study. G protein coupled receptors are also important
potential sites for drug action. A preliminary report suggests that that the CC genotype, which results in decreased intracellular signal transduction, may predict response to therapy in patients with functional dyspepsia treated, based on their predominant symptom, with proton pump inhibitors, prokinetics, spasmodytics and tricyclic antidepressants. At 12 months’ follow up, logistic regression analysis showed that the CC genotype was associated with response to therapy. 189

**Mitochondrial DNA**

Mitochondrial (mt) genome is involved in generation of energy in tissues including the brain, nerves and muscles. There are >100 pathogenic point mutations and many rearrangements associated with multi-system or tissue-specific diseases, including conditions rarely seen by gastroenterologists such as mitochondrial neurogastrointestinal encephalopathy (which presents with pseudo-obstruction and small bowel diverticulosis) and Kearns-Sayre syndrome (which presents with high dysphagia).

Since mitochondria are almost exclusively maternally inherited and IBS is more commonly encountered in females, it was hypothesized that mtDNA SNPs could confer risk to IBS. Most mtDNA SNPs are found in the hyper-variable region of non-coding control region “D loop” (including 16519 C>T SNP). The 3010 SNP is located in the 16S ribosomal RNA gene. These two SNPs, 16519C>T and 3010G>A, have been associated with migraine and cyclic vomiting syndrome, which are commonly encountered in patients with IBS. In addition, 16519T alone is associated with diabetes and with a poorer prognosis in individuals with pancreatic cancer.

Given this background, we explored the association of any FGID (vs health) with H haplogroup, defined by the presence of 7028C polymorphism. A non-significant lower odds for any FGID in haplogroup H (relative to all other haplogroups) was observed (OR [95%CI]) = 0.8 [0.6, 1.1]). Constipation-predominant IBS and alternating constipation and
diarrhea IBS are less prevalent in individuals with the 7028C mtDNA polymorphism than in individuals with 7028T. Among those with 7028C, non-specific abdominal pain (chronic abdominal pain or dyspepsia) was significantly associated with 3010A compared with 3010G. (No significant associations of mtDNA genotypes tested were detected with small bowel or colonic transit, rectal compliance, and motor or sensory functions. The relationship of mtDNA and manifestations of IBS require further study.

**SCN5A**

The *SCN5A*-encoded Na$_v$1.5 Na$^+$ channel is expressed in interstitial cells of Cajal and smooth muscle in the circular layer of the human intestine. Mutational analysis was performed on genomic DNA in 49 subjects with IBS associated with at least moderately severe abdominal pain. One patient had a loss-of-function missense mutation, G298S, that was not observed in 1,500 healthy control subjects. Na$^+$ currents were recorded from the four common human *SCN5A* transcripts in transfected HEK-293 cells. The G298S-*SCN5A* missense mutation caused a marked reduction of whole cell Na$^+$ current and loss of function of Na$_v$1.5, and the authors suggest *SCN5A* as a candidate gene in the pathophysiology of IBS.$^{193}$ However, the mutation appears to be rare even among a subset with at least moderately severe pain in IBS.

**Cannabinoid Metabolism**

The endocannabinoid anandamide is produced, metabolized and released from the postsynaptic membrane. The released anandamide stimulates the cannabinoid (CB) receptor on presynaptic membrane to modulate its function, such as the production of transmitters like acetylcholine. The rate limiting enzyme for metabolism of anandamide is fatty acid amyl transferase (FAAH). If this enzyme does not work well, there is more anandamide that reaches the presynaptic membrane, and greater effect on the transmitter released from the
presynaptic neuron. A common SNP in FAAH is FAAH C385A. This SNP is significantly associated with D-IBS and with rapid colon transit.\textsuperscript{194}

**SLC6A4**

5-HTTLPR genotype (s allele) is associated with higher pain sensory ratings during rectal distension studies in health and IBS.\textsuperscript{195} The higher pain sensation is not the result of a decrease in rectal compliance since the same study identified that rectal compliance is increased in patients carrying the s allele of 5-HTTLPR.\textsuperscript{195} The 5-HTTLPR SS genotype is also associated with greater regional cerebral blood flow in response to colorectal distension in patients with IBS, with regional increases most pronounced in the left anterior cingulate cortex, right parahippocampal gyrus and left orbitofrontal cortex, which may represent increased activity in the emotional motor system of the brain.\textsuperscript{196}

**Neuropeptide S receptor 1**

Neuropeptide S receptor 1 (NPSRI) gene maps to a region of chromosome 7 and is associated with asthma and inflammatory bowel disease.\textsuperscript{197} NPSRI is expressed on the intestinal epithelium, and is up-regulated in inflammation. It may conceivably be associated with IBS given the increasing evidence of association with minor inflammation or prior infection with IBS. In a study of 18 NPSRI polymorphisms\textsuperscript{198} that span the gene in 699 participants (~2/3 patients, 1/3 healthy controls), NPSRI\_RS1419793 was significantly associated with colonic transit. While the mechanisms are unclear, whereby NPSRI SNPs might result in altered motor functions, the data suggest that specific NPSRI alleles might act as genetic risk factors for colonic diseases that are associated with changes in epithelial barrier function, including IBD and IBS.

The COX2.8473 SNP is located downstream of the stop codon, in the 3’-UTR region. Binding of proteins to the 3’-UTR can control mRNA stability and degradation, and this may
be affected by polymorphisms.\textsuperscript{199,200} This region is characterized by multiple repeats of AU-rich elements, which are also found in several other genes encoding inflammatory mediators (cytokines and protooncogenes), whose mRNA is very unstable. It may be possible that the T→C substitution at COX2.8473 stabilizes the mRNA of COX2, thus resulting in a larger amount of protein produced and therefore an increased pro-inflammatory stimulus.
Aim of the Study
Mild inflammation is a component in the pathogenesis of Irritable Bowel Syndrome. COX2 gene polymorphism is associated with increased production of prostaglandins. Activation of serotonin 5-HT$_{1A}$ receptor in the enteric nervous system suppresses gut motility. 5-HT$_{1A}$ receptor responsiveness is reduced by arachidonic acid metabolite.

As there is increased incidence of bronchial asthma in patients with Irritable Bowel Syndrome, the polymorphism associated with increased production of prostaglandin $PTGS2.8473T\rightarrow C$ SNP is investigated in this study to know whether the same polymorphism is associated with patients of Irritable Bowel Syndrome.

$PTGS2.8473 \ T\rightarrow C \ SNP \ \rightarrow \uparrow PGE2 \ \rightarrow \downarrow 5$-HT$_{1A}$ responsiveness $\ \rightarrow \uparrow$ Gut motility
Materials and Methods
MATERIALS AND METHODS

STUDY POPULATION

CASES

The study sample comprised 50 unrelated Irritable Bowel Syndrome patients (30 male, 20 female) of Mean age of 40.56 ± 11.36 years. Inclusion criteria was individuals aged between 18 and 65 years who satisfied Rome II criteria for IBS. Organic gastrointestinal diseases and clinically significant systemic diseases, individuals with known lactose intolerance or immunodeficiency or who had any recent transient illness (i.e. within 2 weeks or participation in the study) such as viral illnesses or chest infections were excluded.

CONTROL SUBJECTS

Controls were recruited from outpatient department during their visit for non gastric illness. Age, Sex were matched.

METHODS

2 mL of blood was obtained by Venipuncture & collected in EDTA tub & was centrifuged at 2000 rpm for twenty minutes to get the buffy coat for DNA extraction

BUFFY COAT SEPARATION

Buffy coat was separated by centrifugation of EDTA tubes at 2000 revolutions for 20 minutes. Buffy coat was transferred to 2mL eppendorf and was used for DNA extraction.

DNA EXTRACTION BY MODIFIED HIGH SALT METHOD

RBC Lysis:

400µL of buffy coat in a 2mL eppendorf is mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion until red cells are lysed for about 10 minutes

The cells are centrifuged at 4000rpm for 10minutes.

The white cell pellet is washed with 800µL of 0.17M ammonium chloride solution. The procedure is repeated till a clear white cell pellet is obtained.
**WBC Lysis**

To the pellet 500 µL of TKM I solution is added. It is centrifuged at 10,000rpm for 10 minutes.

**Nuclear Lysis**

Discard the supernatant. To the pellet add 500 µL of TKM II solution. To that add 300 µL of 6M Nacl and 50 µL of 10% SDS.

Mix well (vortex), Centrifuge at 10,000 rpm for 10 minutes.

Save the supernatant. Transfer it to 1.5mL eppendorf.

**DNA Precipitation**

To the supernatant double the volume of 100% ethanol is added.

The sample is stored at -20°C for 1 hour.

Then it is centrifuged at 10,000 rpm for 20 minutes at 4°C in a refrigerated centrifuge.

The supernatant is discarded. To this 500 µL of 70% ethanol is added. The pellet is mixed and centrifuged at 10,000 rpm for 10 minutes at 4°C.

Supernatant is discarded and the pellet is air dried.

**Storage**

To the pellet 30 µL of LTE buffer is added and the extracted DNA is stored at -20°C for future use.

**Identification**

Extracted DNA was identified by 0.8% agarose gel electrophoresis with a constant voltage of 7V/cm and comparison with a known molecular weight 1kb DNA ladder.

**Concentration of extracted DNA**

Concentration of extracted DNA was estimated using UV spectroscopy at 260nm. The absorbance at 260nm was 0.0203. Concentration was calculated using the formula: 1 OD is equivalent to 50µg/mL.
Conc. of DNA = absorbance × 50µg/mL × dilution factor

= 0.0203 × 50 × 100

= 101.5 ng / µL

Purity of extracted DNA was assessed by 260/280 ratio and it was found to be > 1.7

POLYMERASE CHAIN REACTION

177 bp fragment of COX2 gene was amplified using 10,

Forward primer – 5'-GAAATTTAAAGTACTTTTGAT

Reverse primer -5'-CTTTACAGGTGATTCTACCC

Primers are supplied in lyophilized form. Autoclaved distilled water is used to prepare 100 × concentrations i.e. 10times the molecular weight of primer is the volume of water required to prepare 100 × concentrations which is 100µmolar solution.

From this stock solution 10 × concentration is prepared as the working solution for PCR.

MASTER MIX:

Genei Red Dye master mix in the following composition was used.

Master Mix consists of a unique inert red dye in addition to basic components necessary for PCR.

Reaction buffer consisted of Tris Hcl - 10mM at pH 8.3

KCl - 50mM

MgCl2 - 1.5mM acts as catalyst.

dNTP’s were used in a concentration of 2.5mM each.

Taq polymerase in a concentration of 1.5 U.

Primers were used in a concentration of 5 pmol and DNA was used in a concentration of 200ng.

PCR was carried out in a reaction volume of 50 µL with the following components;
Components | Quantity
--- | ---
*In PCR vial* |  
Master mix | 25µl
SNP specific Primer - forward (10pmoles/µl) | 1µl
SNP specific Primer - reverse (10pmoles/µl) | 1µl
Genomic DNA | 1µl
Water, nuclease free | 22µl
Total volume | 50µl

Amplification was carried out in an Bioneer thermal cycler with the following cycling conditions.

**Initial Denaturation:** 94ºC for 3 min

**Denaturation:** 94ºC for 1 min

**Annealing:** 50ºC for 1 min

**Extension:** 72ºC for 1 min

**Final extension:** 72ºC for 5 min

Amplified product – amplicons of 177 bp was identified by 2.5% agarose gel electrophoresis by comparison with a known 100bp DNA ladder. Figure13.

**AGAROSE GEL ELECTROPHORESIS**

PCR product is run on 2.5% agarose gel in a 30 mL agarose cast as follows: 0.75g of agarose is weighed and dissolved in 30mL of TAE buffer with a pH of 8.0.

It is microwaved for 60 secs, cooled and 1.5 µL of ethidium bromide (10mg/mL) is added. It is poured into a cast and allowed to solidify for 15 min before it is kept in the electrophoresis tank.

8 µL of PCR product is loaded onto wells and 4 µL of 250 bp DNA ladder is loaded onto single well as a marker. It is electrophoresed at 8V/cm for 45min and visualized under UV illumination.
RESTRICTION DIGESTION OF PCR PRODUCTS (Figures 14 to 16)

COX2 gene polymorphism was detected by digestion of the PCR amplified product with the Bcl1 restriction enzyme (1 unit for 1 hour)

Principle of Bcl1 enzyme digestion

T allele does not have the restriction site hence will yield a 177bp fragment

C allele has the restriction site, hence gets cleaved to give 156 bp and 21 bp fragment.

Heterozygous individuals (TC) have 177 bp, 156 bp, 21 bp fragments

Analysis was done using a 25 bp DNA ladder.

Procedure

10 µL of PCR product is aliquoted in an eppendorf and 1U of Bcl1 enzyme is added. The entire procedure is carried out in ice. The contents are mixed thoroughly.

The eppendorf is then placed in a 37°C waterbath for 1 hour and reaction is stopped by adding 5 µL of gel loading dye and mixed thoroughly. After digestion with restriction enzyme, products were separated in 3% agarose gel, stained with Ethidium bromide & the gel is visualized in UV illumination and the genotypes are identified by comparison with known molecular weight DNA ladder (25 bp) and identifying the various fragments.
Statistical Analysis
STATISTICAL ANALYSIS

Allele frequencies were calculated by allele counting.

Age, Sex, Comorbidity with mental disorders, H/O Asthma, H/O Fibromyalgic symptoms, H/O Sexual and physical abuse, H/O Major life stress were compared between control subjects and patients by students t test.

Genotype frequency distribution between cases and controls were compared with a $\chi^2$ test for 2*2 contingency table.
Results
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<td>Y=1;N=49</td>
<td>TT=30;TC=14;CC=6</td>
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### TABLE 5

**CHARACTERISTICS OF PATIENTS WITH IBS AND OF CONTROL SUBJECTS (students t test)**

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<th>Variables</th>
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<th>P value</th>
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<td>Age</td>
<td>39.84 ± 11.99</td>
<td>40.56 ± 11.37</td>
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<tr>
<td>Sex Male</td>
<td>30(60%)</td>
<td>30(60%)</td>
<td>1.00</td>
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<tr>
<td>Female</td>
<td>20(40%)</td>
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<tr>
<td>Comorbidity with mental disorders</td>
<td>28(56%)</td>
<td>26(52%)</td>
<td>0.68</td>
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<tr>
<td>Fibromyalgia</td>
<td>26(52%)</td>
<td>28(56%)</td>
<td>0.68</td>
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<tr>
<td>Asthma</td>
<td>3(6%)</td>
<td>2(4%)</td>
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<td>Sexual and physical abuse</td>
<td>10(20%)</td>
<td>6(12%)</td>
<td>0.28</td>
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<td>Major life stress</td>
<td>2(4%)</td>
<td>1(2%)</td>
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TABLE 6: GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES OF COX GENE

<table>
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<td>Chi sq=0.09</td>
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<td>T+*</td>
<td>43</td>
<td>44</td>
<td>P = 0.766</td>
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Figure 17

Figure 17 SHOWS GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES OF COX GENE
TABLE 7

GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES OF COX2 GENE

<table>
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<td>Chi sq = 1.03</td>
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<td>P value = 0.597</td>
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<td>CC</td>
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FIGURE 18 GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES
RESULTS

Table 5 shows Age, Sex, Comorbidity with mental disorders, Fibromyalgia, Asthma, Sexual and physical abuse, Major life stress, risk factor distribution among patients and control subjects. We obtained a nonsignificant p value with respect to all the confounding variables like Age, Sex, Comorbidity with mental disorders, Fibromyalgia, Asthma, Sexual and physical abuse, Major life stress.

Table 6 & 7 shows Genotype distribution and Allele frequencies of COX2 gene in patients with IBS and control subjects. The Allele frequencies were TT = 30, TC = 14 and CC = 6. This was found to be in Hardy Weinberg equilibrium. $\chi^2$ value is 3.84, P value is .37.

TT genotype was frequent among cases (60%) when compared to controls (50%). TC genotype was common among controls (36%) when compared to cases (28%). CC genotype was common among cases (14%) when compared to controls (12%). There is no difference in T+ genotype among controls (88%) & cases (86%). P value = .766. There was no significant difference in the distribution of CC genotype between cases (14%) and controls (12%). P value = .597.
Discussion
Genetic factors in combination with a number of environmental risk factors are involved in predisposition to Irritable Bowel Syndrome.

Recently, interest has focused on the presence of mucosal inflammation in the pathogenesis that may be particularly valid for diarrhoea-predominant IBS\textsuperscript{202}

5HT\textsubscript{1A} receptor decreases gut motility. As the arachidonic acid metabolite reduces the responsiveness of the 5HT\textsubscript{1A} receptor system which may consequently increase gut motility, any polymorphism associated with arachidonic acid metabolism is being looked for. As studies showed increased amount of PGE\textsubscript{2} in IBS, polymorphism in the COX pathway, if any is being studied. As studies showed increased prevalence of bronchial asthma in IBS patients, the COX2(8473 T → C) gene polymorphism, which is associated with bronchial asthma, was analyzed in this study.

In this study, the effect of polymorphism in the 3-UTR region of the COX2 gene (8473 T → C) is assessed in the occurrence of IBS.

CC genotype which is associated with increased amount of PGE\textsubscript{2} levels in previous studies involving asthma population, does not significantly associate with our IBS population.

As it has been described before, stress activates CRF-CRF\textsubscript{1} signaling pathways in the brain linked with hypothalamic and pontine nuclei which stimulate the sacral parasymathetic nucleus and subsequently the enteric nervous system. The increased CRF/urocortin activates mast cells\textsuperscript{76} & other immune cells which subsequently increases motility, mucus secretion, PGE\textsubscript{2} production\textsuperscript{203}. 
The biopsychosocial model of IBS integrates a number of psychosocial, motility, sensory abnormalities and abnormalities in central nervous system processing of visceral pain as the causes of abdominal pain and altered bowel habits.

As IBS has multifactorial causes, factors other than COX2 gene polymorphism may be involved in this population like the above mentioned stress induced inflammatory pathway or Gene polymorphisms involving IL-10; 5-HTTLPR; α2-Adrenergic Receptors; Cathechol-o-methyl transferase; G-proteins; Mitochondrial DNA SCN5A (Na⁺ channel); Cannabinoid (CB) receptor; Neuropeptide S receptor 1. Further studies may be undertaken in future to investigate the occurrence of the polymorphisms in this population.
Conclusion
CONCLUSION

IBS is the most common disorder encountered by gastroenterologists, and is responsible for reduced quality of life and considerable economic burden on society. Presently there are no known biochemical or structural markers for identifying patients with IBS.

Attention has recently been focused on increased perception of visceral stimuli arising from the gastrointestinal tract wall, a phenomenon referred to as visceral hypersensitivity. Among the sensitising factors acting on nerve terminals at the peripheral level, altered interaction between the mucosal immune system and the afferent nerve terminals which project to the intestine is now receiving increasing attention. Low grade inflammation in the intestinal mucosa has been found in subgroups of patients and may be involved in the pathophysiology of visceral hypersensitivity, in at least some cases of IBS.

As COX2 gene polymorphism is associated with inflammation, this study was designed to investigate polymorphism in the 3'-UTR region of the COX2 gene with occurrence of IBS.

In this study homozygous COX2 TT genotype was more frequent than TC or CC. This study showed that CC polymorphism in the 3’-UTR region of the COX2 gene (8473) which is associated with inflammation was not significantly associated with increased risk for IBS.
Future Prospects of the Study
This study may be further explored in diverse and larger population, as this COX2 gene polymorphism is one of the main polymorphism involving inflammation.
BIBLIOGRAPHY


INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No: 04425305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To

Dr. S. Siva
PG in MD Biochemistry
Madras Medical College, Chennai -3.

Dear Dr. S. Siva

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled “Association of Prostaglandin – endoperoxide synthase 2 gene (PGX2) Polymorphism in Irritable bowel syndrome (IBS) Patients: A case control study” No. 40022011.

The following members of Ethics Committee were present in the meeting held on 17.02.2011 conducted at Madras Medical College, Chennai -3.

1. Prof. S.K. Rajan, MD — Chairperson
2. Prof. A. Sundaram, MD — Member Secretary
3. Prof. R. Sathianathan — Member
   Dean Ic , Madras Medical College, Chennai -3
4. Prof. R. Nandhini, MD — Member
   Director, Institute of Pharmacology, MMC, Ch-3
5. Prof. Pregna B. Dolla MD — Member
   Director, Institute of Biochemistry, MMC, Ch-3
6. Prof. C. Rajendiran. MD — Member
   Director, Institute of Internal Medicine, MMC, Ch-3
7. Prof. Geetha Subramanian, MD,DM — Member
   Prof. & Head , Dept. of Cardiology, MMC, Ch-3
8. Thiru. A. Ulagathan — Layperson
   Administrative Officer, MMC, Chennai -3
9. Thiru. S. Govindasamy, BA, BL — Lawyer
10. Tmt. Arnold Soulika — Social Scientist

We approve the proposal to be conducted in its presented form.

Sd /i. Chairman & Other Members

The institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report

Member, Secretary, Ethics Committee
ஆராவாசி குறிப்பிட்டுச் செய்யும்

ஆராவாசி தலைப்பு:

பாலம் : இல்லை

மேலுதல் : அல்லது செய்வதிற்கு பின் எடுக்கப்பட்டு முடிகிறது

பாறு : அல்லது செய்வதிற்கு பின் எடுக்கப்பட்டு முடிகிறது

சிட்டாது ஆராவாசி விளையாட்டிற்கு அதில் விளையாட்டுக்கு பின்னர் எடுக்கப்பட்டு முடிகிறது.

சிட்டாது என்னும் பொருளிச் செயல்படுத்தலுடன் பின்னர் எடுக்கப்பட்டு முடிகிறது.

சிட்டாது என்னும் பொருளிச் செயல்படுத்தலுடன் பின்னர் எடுக்கப்பட்டு முடிகிறது.

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PROFORMA

NAME: AGE / SEX:    

IP/ OP No: STUDY ID No:  
ADDRESS:  

DIAGNOSIS:  
COMPLAINTS:  
H/O PRESENT ILLNESS:  

PAST HISTORY:  
• DM YRS    THYROID ILLNESS:  
• HT YRS  

TREATMENT HISTORY:  

PERSONAL HISTORY: SMOKING: ALCOHOLISM:  

FAMILY HISTORY:  

GENERAL EXAMINATION:  
Ht: Wt: Pallor / icterus/ Pedal edema  
Vital signs: PR: BP:  

SYSTEMIC EXAMINATION:  
CARDIOVASCULAR SYSTEM:  

RESPIRATORY SYSTEM:  

PER ABDOMEN:  

CNS:  

IMPRESSION:  

INVESTIGATIONS:  