Emerging Infectious Diseases of the 21st Century

I.W. Fong

The Role of Microbes in Common Non-Infectious Diseases

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This book is dedicated to my very supportive wife—Cheryl.

Preface

Our understanding of the pathogenesis and etiology of new and venerable diseases has progressed tremendously in the past 30 years, due to improved technology and advances in genomics and molecular science. However, there are several common diseases where the exact causation still eludes investigators, but intensive research over the past decade has provided clues to implicate a microbial role in pathogenesis and causation.

These medical conditions include common nonlife threatening illnesses that affect the daily function and quality of life, such as irritable bowel syndrome and chronic fatigue syndrome. But also microbial pathogenesis has been postulated for more serious disorders such as multiple sclerosis, diabetes mellitus, Crohn's disease, asthma, and rheumatoid arthritis; to potentially fatal and catastrophic conditions such as Alzheimer's disease, colon cancer, and atherosclerosis that leads to heart attacks and strokes.

A brief outline of these clinical conditions and others will be provided, with in-depth review of current understanding of the mechanisms and pathogenesis. The links to microbial pathogenesis will be reviewed from ongoing research, with exploration of theoretical microbial causes and potential for future clinical research and development of novel therapies.

This new volume in the series "Emerging Infectious Diseases of the 21st Century" should provide fodder for investigators and clinicians faced with these conditions, but more importantly to stimulate interest of new investigators and trainees to take a new and novel approach for investigating the mechanisms and causation of these baffling common disorders.

Toronto, ON, Canada I.W. Fong

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Chapter 1
Irritable Bowel Syndrome and Microbial Pathogenesis

1.1 Background

Irritable bowel syndrome (IBS) is a chronic disorder of greater than 6 months characterized by abdominal pain or discomfort associated with disturbed defecation, loose and more frequent stools at onset, with intermittent constipation and irregularity; and associated with abdominal bloating or distention, flatulence, mucous per rectum and incomplete emptying, in the absence of any definable cause [1]. This is a common disorder with female predominance that is present worldwide, with no racial preference and it is widespread in the population of all countries where it has been studied, including the United States, Europe, China, Japan, South America, and the Indian subcontinent [1]. The estimated prevalence of IBS in North America and elsewhere varies from 3 to 20 % [1]. Approximately 12 % of patients seen in primary care have IBS [2], with an estimated 3.6 million physician visits for IBS annually in the United States [USA] [1, 2].

The impact of this disorder on the healthcare costs is substantial, and the burden on the local economy because of days lost from work can be huge. In the USA it has been estimated that the direct cost of IBS is $1.6 billion and indirect cost a staggering $19.2 billion [1]. It has been calculated that patients with IBS consume over 50 % of the healthcare resources compared to controls without IBS.

1.2 Risk Factors

One of the most important established risk factor for IBS is recent infectious gastroenteritis and prior travel with history of diarrhea within 6 months before [3]. Most cases of postinfectious IBS [PI-IBS] are due to bacterial gastroenteritis but parasites and norovirus related enteritis have been associated with IBS [4].

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Most of the patients with IBS have mild symptoms, about 70 %, and can be managed by their primary physicians, but 25 % have moderate symptoms and about 5 % have severe symptoms [5]. Consultation rates to a specialist vary from 25 to 46 % in the USA, and up to 73 % in Australia where universal healthcare access is available [1]. The diagnostic criteria for IBS have evolved from the Manning and Kruis criteria to the more recent Rome 111 criteria. This latter criteria includes: (1) recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months associated with two or more of the following: (2) improvement with defecation, (3) onset associated with a change in frequency of stool, (4) onset associated with that change in the form or appearance of this stool [1, 5]. Patients with IBS may be afflicted with predominantly diarrhea [IBS-D] or mainly constipation [IBS-C] or of mixed type [IBS-M], besides other concurrent symptoms.

1.3 Pathophysiology

The pathogenesis of IBS is poorly understood and a number of mechanisms have been proposed, including abnormal motility of the intestines, visceral hypersensitivity, central neural dysfunction, low-grade inflammation of the gut mucosa, stress, and psychological disturbances [1, 5]. Abnormal psychological features are present in up to 80 % of patients with IBS and the risk of IBS has been reported to be increased with depression, previous physical and sexual abuse, and extraintestinal somatization symptoms are more common than healthy subjects. The fact that abnormal physical findings or laboratory abnormalities such as leukocytosis, elevated erythrocyte sedimentation rate (ESR), anemia, or blood in the stools, are exclusion factors for the diagnosis of IBS suggests a functional or psychological etiology. However, studies have support for different mechanisms in the pathogenesis of IBS including genetic factors, [tend to run in families], abnormal colonic and small bowel motility, with greater phase contractions following fatty meals and stress, colonic hypersensitivity to balloon distention [visceral hypersensitivity is found in about 60 % of subjects studied]; low-grade inflammation with evidence of increased mast cells and activated T cells above normal in the mucosa of a subset of patients with IBS; certain food intolerance [gluten, fructose]; and central dysregulation with alterations in brain response to visceral stimuli [1, 5, 9].

Conceptual model of the pathobiology of IBS has been proposed to show the interrelationships between early in life factors [genetics and environment], psychosocial factors, physiological disturbance, symptom experience, and behavior outcome [6]. IBS may be the primary manifestation of this clinical complex or be a secondary component of other illnesses such as fibromyalgia, chronic fatigue syndrome, depression, and somatization disorders. Thus, it is often labeled as a functional disorder but it is unclear whether the link with these functional disorders reflects a common etiology, or certain genetic traits, or psychosocial background, and psychobiological constitution.

1.4 Microbial Link to IBS

1.4.1 Epidemiological Association

Gastroenteritis or infective diarrhea is one of the most common diseases in the world and is second only to respiratory tract infections in frequency. Although the annual incidence, morbidity, and mortality from infective diarrhea is greatest in developing countries and in children less than 5 years old, it is quite substantial as well even in countries with excellent sanitation systems. For instance, a large community survey of 9,776 subjects in England in Wales reported an annual incidence of gastroenteritis of 19.4 per 100-person years [7]. Viral infection, mainly norovirus and rotavirus, accounts for about 35 % of these cases, while bacterial infection such as Campylobacter and Salmonella species together accounting for about 13 % of the cases. Numerous studies have found an association with IBS after bouts of infective diarrhea [PI-IBS] over the last two decades with incidence varying from 3.7 to 36 %, averages about 10 % [4]. The variability of the rates of PI-IBS may be due to multiple factors such as etiology of the gastroenteritis, difference in the population at risk, criteria for diagnosis, exposure to antimicrobials and others. For instance, the background rates of IBS in the communities at large may be variable and there may be differences in occurrence of PI-IBS after sporadic gastroenteritis versus outbreaks or even traveler's diarrhea.

Postinfectious IBS was initially described as a clinical entity termed postdysentery colitis in 1950 [8], and subsequently labeled as postdysenteric IBS in 1962 [9]. PI-IBS accounts for about 10–15 % of all cases of IBS but microbes may be involved in the pathogenesis of non-PI-IBS as well, since gut microbiota alterations have been demonstrated even in noninfectious related IBS. Nearly all cohort studies of subjects suffering from gastroenteritis with comparable age- and sex-matched controls have found significant increased risk of PI-IBS in infected patients, with relative risks [RR] varying from 2.1 to 13.9 [see Table 1.1]. Fourteen cohort studies of patients with initial gastroenteritis were reviewed and summarized in Table 1.1 [10–26] from 1999 to 2012. The prevalence of PI-IBS varied from 4.2 to 46 % and rates of IBS in the controls also widely varied from 0 to 14 %. The wide variations in prevalence rates may be related in part to the diagnostic criteria of previous studies for IBS, for the criteria have changed over the years [27]. However, relevant contributory factors may include the inherent risk of the study populations and the inciting events. PI-IBS has been documented from subjects with sporadic bacterial gastroenteritis confirmed by stool cultures, from full blown outbreaks of viral infection [23] or parasitic agents [26], from large community outbreaks from contamination of municipal water with Escherichia coli 0157, and from traveler's diarrhea. There is insufficient data to prove any difference in prevalence rates of PI-IBS between bacterial and viral or parasitic/protozoa infections. However, longitudinal prospective studies from the same group of investigators [using the same diagnostic criteria] have found PI-IBS following norovirus enteritis was more likely to resolve quicker than from bacterial gastroenteritis [17–19, 23]. Following a large municipal outbreak of Escherichia coli 0157 and

Table 1.1 Rates of PI-IBS in cohort studies with controls

Author/year [ref.]	Country	Organism [*n*]	Follow-up (month)	Criteria	Rate of PI-IBS	Rate in controls [*n*]	Relative risk	Attributable rate of IBS
1. Rodriquez, 1999 [10]	Britain [population-based].	Bacterial [318]	12	Physician-based	4.4 %	0.3 % [585, 308]	11.9	4.1 %
2. Ilnyckyi, 2003 [11]	Canada	Travelers' diarrhea [48]	3	Rome-1	4.2 %	1.5 % [61]	2.5 ns	2.7 %
3. Parry, 2003 [12]	Britain	Campylobacter/ Salmonella [108]	6	Rome-11	16.6 %	1.9 % [206]	8.7	14.7 %
4. Wang, 2004 [13]	China	Shigella [210]	12–24	Rome-11	10.2 %	0.8 % [243]	12.7	9.4 %
5a. Ji, 2005 [14]	South Korea	Shigella [101]	12	Rome-1 and -11	14.8 %	5.8 % [102]	2.55	9 %
5b. Kim, 2006 [15]	„	„ [95]	36	„	14.9 %	4.5 % [105]	3.3	10.4 %
6. Mearin, 2005 [16]	Spain	Salmonella [266]	12	Rome-11	11.6 %	1.5 % [335]	7.7	10.4 %
7a. Marshall, 2006 [17]	Canada	*E. coli* 0157/ Campy [904]	24	Rome-1	27.5 %	10.1 % [701]	3.5	17.5 %
7b. Marshall, 2010 [18]	Canada	„ [701]	96	Rome-1	15.4 %	1.9 % [424]	8.1	13.5 %
7c. Thabane, 2010 [19]	„	„ [305]	„	„	10.5 % [children]	2.5 % [162]	4.2	8.0 %
8. Moss-Morris, 2006 [20]	NZ	Campylobacter [592]	3.6	Rome-1 and -11	3m 15 %, 6m 11 %	7–8 % [243]	2.1, 1.3	3 %
9. McKeown, 2006 [21]	Britain	Bacterial [108], GE and Non-GE	3.6	Rome-11	GE-16.7 % [3m, 6m] non-GE-14.8 % [3m]	1.9 % [206]	8.8	14.8 %
						„	7.7	12.9 %
Software Updates. Ink 6.4 % ns							„ 8.3 % [6m]	4.3 ns

10. Stermer, 2006 [21]	Israel	Travelers' diarrhea	6	Rome-1 and -11	13.6 %	2.4 % [287]	5.6	11.2 %
11. Marshall, 2007 [23]	Canada	Norovirus [89]	3–24	Rome-1	23.6 % [3m]	3.4 % [29]	6.9	20.2 %
					12.5 % [6m]	8.3 %	ns	4.1 % ns
12. Soyturk, 2007 [24]	Turkey	Trichinella [72]	6	Rome-11	13.9 %	0 % [27]	13.9	13.9 %
13. Jung, 2009 [25]	South Korea	Shigella [87]	12,36,60	Rome-1 and -11	13.8 % [12m]	1.1 % [89]	12.5	12.7 %
					14.9 % [36m]	4.5 % „	3.3	10.4 %
					20.8 % [60m]	12.2 % [49]	1.7	8.6 %
14. Wensaas, 2012 [26]	Norway	Giardia [817]	36	Rome-11	46 %	14 % [1,128]	3.2	32 %

PI-IBS = postinfectious irritable bowel syndrome; GE = gastroenteritis; non-GE = nongastroenteritis infections

Campylobacter infection even after 8 years there was still a greater prevalence of PI-IBS [15.4 %] compared to rates in the controls [1.9 %] [18]. Other investigators in Korea have found rates of PI-IBS 14.9 % versus 4.5 % in patients and controls 3 years after Shigella dysentery [15], and attributable rates of 8.6 % [rate of PI-IBS in patients minus rate of IBS in controls] after 5 years [25]. In Norway outbreaks of giardiasis have been recently reported to result in persistent PI-IBS for 3 years with an attributable rate of 32 % [26].

Other longitudinal studies without comparable controls have reported on PI-IBS after infectious diarrhea over 5–10 years. In a study of previously healthy subjects with no prior gastrointestinal problems, 41 of 333 [12 %] patients after bouts of gastroenteritis developed persistent gastrointestinal [GI] symptoms for more than 3 months and 28 [68 %] were classified as IBS and 31 [9 %] had persistent symptoms for 5 years [28]. Similarly in a cohort of patients with Salmonella or Campylobacter enteritis followed for 10 years, 56 of 571 [9.7 %] still suffered from PI-IBS [29]. However, this group of patients had high psychiatric comorbidity and somatic symptoms burden. The burden of PI-IBS caused by bacterial enteric pathogens in a community has been estimated in the Netherlands [30]. One year after bacterial gastroenteritis 9 % of the infected individuals developed PI-IBS, which adds 2,300 disability adjusted life years to the total annual disease for the selected pathogens.

1.4.2 Prevalence of IBS

Although the rates of PI-IBS have been variable from study to study it is now established that infectious diarrhea is strongly associated with IBS. However, the attributable rates are similar between countries and regions such as Europe, North America and South America, Middle East, and Asia. Although it is well established that gastroenteritis in the population of developing countries is much more prevalent than well-developed nations [due to poor sanitation and less access to clean water], the prevalence of IBS is not greater in tropical and subtropical countries. The frequency of IBS has been surprisingly low in these countries, 4.2 % in India [31], 4.4 % in Thailand [32], 8.5 % in Bangladesh [33], 5–6 % in the general population of China [34], and 13 % in Pakistan [35]. Whereas the prevalence of IBS in eight Western European countries has been estimated to be greater than most Asian countries with a mean prevalence of 11.5 %, range 6.2–12 % [36]. However, recent studies from Asia suggest higher prevalence of 8.6–9.8 % in more affluent cities like Singapore and Tokyo, and similar trends are found among the better educated and more affluent strata of several Urban Chinese population [37].

1.4.3 Risk Factors for PI-IBS and IBS

Although infectious diarrhea or gastroenteritis relationship with IBS has been extensively studied there has been almost no data on the association or lack of association with other infectious diseases, or the relationship with antibiotic use in general,

which could affect normal gut microbiota. To date only one study has been identified to investigate the relationship of PI-IBS with nongastrointestinal infections compared to controls [21]. In this study both GI and non-GI infections were significantly associated with PI-IBS at 3 months [16.7 % and 14.8 %, respectively] after the acute episode [odds ratio, OR, 6.12] but the nonintestinal infections PI-IBS rate of 8.3 % did not remain significant at 6 months [OR, 4.58]. However, this was very likely secondary to the small sample size of patients with non-GI infections [$N=36$] in this study [21]. There was a mixture of bacterial infections treated with antibiotics such as pneumonia, urinary tract infections, cellulitis, wound infections, etc. Future larger studies are needed with comparable controls to assess this relationship.

The majority of studies assessing for the risk of PI-IBS included patients with bacterial infectious diarrhea such as Campylobacter, Salmonella, Shigella, and *E. coli* 0157 infections or traveler's diarrhea. Some of these studies have shown that the severity of the diarrhea and duration appears to be predisposing factors for PI-IBS [38, 39]. It is rather surprising, however, that Clostridium difficile colitis has not been well studied for the development of IBS and only with one small study found a low risk of 4.3 % PI-IBS 3 months after the acute episodes [40]. Some studies have found that there is a relationship of the development of PI-IBS after the receipt of antibiotics for infectious diarrheas, however, this has not been consistently observed or rigorously analyzed. Moreover, the use of antibiotics in infectious diarrheas may be related to the severity and duration of the acute episodes, which are independent risk factors for PI-IBS. Similarly, one study has reported the relationship between the toxigenicity of Campylobacter strains and PI-IBS [4], but this is likely related with the severity of the disease.

Studies on the role of parasitic gastrointestinal infections and development of PI-IBS have been mixed. Acute giardiasis, especially in local community outbreaks, has been associated with increased risk of PI-IBS even after 3 years [26, 41, 42]. It has been reported that patients with chronic intermittent GI symptoms and the presence of Giardia lamblia cyst in the stools do not respond to specific treatment such as metronidazole but to nonspecific drugs used for symptoms of IBS [43]. This would indicate that the patient's symptoms were not directly due to the protozoa but were related to development of IBS. Similarly, studies in India indicate that Entamoeba histolytica carriage in the stools is not associated with increased risk of IBS [44], but another study from India reported that patients with chronic abdominal pain and frequent bowel disturbances with the presence of *E. histolytica* in the stools do not suffer from chronic amebiasis or the so-called nondysenteric intestinal amebiasis, but rather from IBS [45]. Hence, proper prospective case control studies after acute amoebic dysentery would be more suitable to define the risks of PI-IBS.

There is limited data on the risk of PI-IBS after episodes or outbreaks of viral gastroenteritis. One Canadian study reported increased risk of PI-IBS after a norovirus community outbreak of diarrhea compared to matched controls after 3 months [RR6.9], but the symptoms were more short-lived than with bacterial enteritis PI-IBS [23].

Host factors including younger age, female sex, and psychological comorbidities such as anxiety and depression have been identified as risk factors for PI-IBS and

noninfectious IBS [46, 47]. However, this female sex predominance reported in Western countries has not been observed in a number of Asian studies [37]. In one review and meta-analysis of PI-IBS, risk factors were confirmed to be younger age, prolonged fever, anxiety, and depression [48].

Another review of the topic have listed risk factors for development of PI-IBS in the order of importance as prolonged duration of initial illness, toxicity of the infecting microbe, smoking, mucosal markers of inflammation, female gender, depression, hypochondriasis, and adverse life events in the preceding 3 months [4]. In Asian studies significant risk factors for IBS besides acute recent intestinal infection include food intolerance, genetic factors, and psychological disturbances [34]. IBS comorbidity with other functional gastrointestinal disorders is very high and may be caused by shared pathophysiological mechanisms such as visceral hypersensitivity. Moreover, approximately 50 % of IBS patients seen in primary care or GI clinics have at least one comorbid somatic symptoms, psychiatric disorders, especially major depression and anxiety, and somatoform disorders have been reported in 94 % of IBS patients [47]. Functional disorders observed with IBS subjects include chronic fatigue syndrome [51 %], fibromyalgia [median of 49 %], chronic pelvic pain [50 %], and temporomandibular joint disorder [64 %] [47]. It has been debated as to whether or not IBS should be "lumped" together with other functional somatic syndromes such as chronic fatigue syndrome [CFS]. In one prospective study, 592 patients with acute episodes of Campylobacter gastroenteritis were compared with 243 subjects with episodes of acute infectious mononucleosis [IM]. The odds of developing IBS were significantly greater post-Campylobacter infection then after infectious mononucleosis at both 3 and 6 months follow-up [OR 3.45 and 2.2, respectively]. In contrast, the odds of developing CFS were significantly greater after IM, then after Campylobacter infection at 3 and 6 months [OR 2.77 and 1.48]. The authors concluded that the nature of precipitating infection was important and premorbid levels of distress such as anxiety and depression were more strongly associated with CFS than IBS [49]. The major limitation of the study was the lack of matched healthy control group for comparison.

1.4.4 Genetic Factors

Several studies using various methods have confirmed that IBS symptoms are more common in relatives of patients with IBS suggesting potential genetic component [50]. Five classic twin studies in IBS suggest a modest genetic contribution, the genetic liability estimated to range from 0 to 20 % [50]. Definite disease-susceptibility gene for IBS has yet to be defined, but putative genetic alterations would likely interact with environmental factors. It has been proposed that IBS is a complex genetic disorder as a result of multiple genes of modest effect that exert modifying response to various environmental stimuli or factors. Potential genetic alterations in genes that encode proteins affecting gastrointestinal motility or sensation could be involved; alteration in genes encoding proteins in the immune system

may predispose to PI-IBS; or genetic alterations that affect personality, depression, anxiety, and somatization; and genes encoding digestion [lactase, disaccharidase] that could affect food intolerance.

To date, only one study has assessed genetic risk factor for PI-IBS following the Walkerton waterborne outbreak of gastroenteritis [51]. In this study 79 functional variants of gene products involved in serotoninergic pathways, intestinal epithelial barrier function, and innate immunity were screened for in 228 patients with PI-IBS and 581 controls. Four genetic variants were associated with PI-IBS and two of which were localized in genes encoding the pattern recognition receptor TLR-9; one was in CDH 1, which encodes a tight junction protein, and the other in the gene encoding interleukin [IL]-6, a cytokine. These genetic variants all persisted as independent risk factors for PI-IBS after controlling for clinical risk factor [51]. In IBS candidate gene studies for specific polymorphisms or set of polymorphisms have been performed mostly in small studies on the following genes: serotonin transporter [SLC6A4], 5-HT 2A receptor, norepinephrine transporter [NET], alpha 2a-, alpha 2C-adrenergic receptor [ADRA 2A, ADRA 2C], interleukin-10 [IL 10], transforming growth factor-beta 1 [TGFB], tumor necrosis factor-alpha [TNF-a], B3 subunit of the G protein [GN03], sodium channel 1.5 V [SCN5A], and fatty acid amide hydrolase [FAAH] [50]. Potential association of IBS has been observed with IL-10 gene [52, 53], the TNF-a gene [53], SC N5A gene [54], and FAAH [55], but these studies need to be repeated with larger patient and control samples. The 5-HTT LPR polymorphism is the best studied in IBS patients but a recent meta-analysis did not find a significant association [56]. A recent study in South Korea of 163 patients with IBS and 423 healthy matched controls found the cannabinoid receptor 1 gene [CNR-1] polymorphism was associated with IBS [57]. Cannabinoid receptors are located in the brainstem, gastric and colonic neurons, and functional variants could affect gastrointestinal motility and sensation which may explain IBS symptoms.

The fact that some studies have found increased polymorphisms of genes encoding toll-like receptors [TLR] and genes controlling cytokine response to external stimuli [i.e. microbes] is supported by a recent study showing altered peripheral TLR responses in patients with IBS compared to controls [58]. In this study of 30 IBS patients and 30 controls, peripheral blood demonstrated elevated cytokine levels and TLR activity in IBS patients. There was exaggerated response to TLR-8 agonists for all cytokines; enhanced TLR2-induced TNF-a release, TLR3-induced IL-8 release, and TLR4-induced IL1B and TNF-a release. In addition, plasma levels of cortisol, IL-6, and IL-8 were significantly increased in IBS patients [58].

1.5 Mucosal Injury and Inflammation

Numerous small clinical studies have been performed on patients with IBS or PI-IBS which have demonstrated low grade injury and inflammation of the gut mucosa, and these data indirectly suggest a role of microbes in the mechanism of IBS pathogenesis. In acute gastroenteritis the distribution of inflammation and

mucosal changes vary with the pathogen. In viral infections [rotavirus, norovirus] there are structural changes with villous blunting and intraepithelial lymphocyte infiltration of the upper small bowel associated with increased gut permeability and loss of absorptive surface area [59, 60].

In giardiasis the main pathological changes are in the proximal small bowel with acute lymphocytic infiltration [61]. Whereas, in Salmonella and Campylobacter infection inflammation and ulceration are more prominent in the terminal ileum and proximal colon, and Shigella enteritis more commonly involves the distal colon [62].

A recent review on the histopathology studies performed on patients with IBS has been reported [63], only 16 studies were analyzed with controls and most reports had small number of cases without adequate controls. The areas of mucosal sampling were very variable including the duodenum, jejunum, ascending or descending colon, and rectum. The findings of the individual studies were diverse but the most consistent abnormalities in patients with IBS compared to controls were findings of low grade inflammation with increased mast cells, T-lymphocytes, and B-lymphocytes associated with mucosal cytokine production. In a recent large study of IBS [$n=121$] subjects' blood, IL-1β, TNF-α, IL-6, and IL-8 were elevated in IBS patients compared to controls [64], others have also reported increase in IL-6, IL-1β, and TNF-α mainly in IBS subjects with diarrhea predominant [65].

Several pathology studies on the intestinal mucosa of PI-IBS patients have been performed, but mostly small case series without adequate controls. In one study of PI-IBS following Campylobacter enteritis, intestinal biopsies were obtained in the subgroup of 28 patients and 28 asymptomatic controls after acute infection plus 34 healthy controls [64]. The main significant findings were increased enterochromaffin cells, significantly greater in patients compared to controls [$p=0.02$] and healthy volunteers [$p=0.006$]; increased in T-lymphocytes in the lamina propria of PI-IBS subjects compared to patient controls and volunteers [$p=0.006$, $p=0.05$]. Clinical comorbid factors such as anxiety, depression, and fatigue were also greater in the PI-IBS group versus patient controls. On multivariate analysis, increased enterochromaffin cells [EC] and depression were equally important as predictors of PI-IBS [RR3.8 and 3.2, respectively]. In another study from South Korea, 30 patients with PI-IBS after Shigella dysentery and patients that completely recovered as well as 12 healthy controls underwent mucosal biopsies of the terminal ileum and the rectosigmoid colon [13]. There was significant increased mast cells in the terminal ileum but not the rectosigmoid area of patients with PI-IBS compared to controls [$p<0.01$]. The density of neuron-specific aldolase, substance P, and 5-hydroxytryptamine [5-HT] positively stained nerve fibers were increased [$p<0.05$], appeared in clusters surrounding increased numbers of mast cells in patients with PI-IBS compared to controls. There was also increased expression of IL-1β mRNA in the terminal ileum and rectosigmoid mucosa in PI-IBS patients compared to controls [$p<0.01$]. Similar findings of increased rectal mucosa IL-1β expression have been reported in a smaller number of PI-IBS subjects [$n=8$] versus controls [$n=7$] [66].

How can we interpret these findings? The majority but not all studies have found increased mast cells in the mucosa of the small and large bowel of patients with IBS including those with PI-IBS. Mucosal mast cells are important for wound healing

and host defense against pathogens and they are rich in granules containing histamine and tryptase [67]. These substances are also important as effector chemicals in hypersensitivity reaction. Foreign antigen and IgE bind to specific receptors on the mast cells causing release of inflammatory mediators [67]. Lack of consistency in the findings of increased mast cells in some patients could be related to the subtype of IBS subjects [diarrhea–or constipation–predominant], or the area of mucosa biopsy, or differences could be related to technical aspects and patient selection.

Some of the studies as previously mentioned earlier have found increased expression of peripheral inflammatory cytokines genes in the colorectal mucosa, and increased cytokine expression in blood, predominantly IL-1β, IL-6, and TNF-α in patients with IBS [both postinfectious and noninfectious] compared to controls. These cytokines are usually secreted by monocytes and macrophages to infectious agents but can be stimulated by other foreign antigens and autoimmunity. Although not all studies have demonstrated these findings the absence of increased anti-inflammatory cytokine IL-10 has been constant. However, both decreased and increased IL-12 have been reported from cultured peripheral blood mononuclear cells [PBMC] of IBS patients [68, 69]. Thus, the data is not convincing to suggest that the innate immune activity [Th1] drives the adaptive immunity toward a T-helper-2 [Th2] cell response as has been suggested [68].

Paradoxically most studies have shown that the gut mucosal macrophages are normal or reduced in patients with PI-IBS or IBS [70], with reduced expression of microphage-recruiting chemokines [71], the reverse of what would be expected for ongoing low grade inflammation. However, one study did demonstrate increased level of activated macrophages by calprotectin expression despite reduced total microphages [70].

Several studies have found that T lymphocytes, including CD4+ T cells, are increased in the colonic mucosa of IBS patients, but others have also found normal numbers of T-cells. Increased T cells would indicate activated adaptive immune response. Activated T-cell responses have also been found in the blood of patients with IBS expressing IL-5 and IL-13 [68]. A few studies have also found that the intraepithelial T-lymphocytes in IBS are predominantly CD8+ cells [70, 72, 73]. Cytotoxic CD8+ T cells are able to kill infected or other dysfunctional cells and represent the majority of intraepithelial T cells. There is also augmentation of CD8+ T cells in the lamina propria of the colonic mucosa in IBS patients [67], which indicate that they contribute to the immune reaction in IBS. Immunoglobulin producing B cells in the gut mucosa of IBS patients have been reported to be unaltered or decreased [67]. Reduced number of colonic Ig A + B cells in IBS patients suggests a modified gut immune defense [74].

However, increased amounts of Ig G + B cells have been reported in the blood of patients with IBS [75], and increased antibodies to bacterial antigens [flagellin] have been reported in IBS compared with healthy controls, but greater in postinfectious IBS than IBS of unknown cause [76].

The histopathology studies on patients with IBS have strengthened the evidence for abnormal neuroimmune interaction. Several studies have found increased neuroendocrine cells in the mucosa of PI-IBS patients [13, 64] and increased

concentration of neuron-specific mediators in subjects with IBS compared to healthy controls [13]. Moreover, others have shown that the gut mucosa nerve fibers density was increased in subjects with IBS in clusters around the mast cells [77], which may contribute to increased pain perception and hypersensitivity [78]. Furthermore, the number of sensory nerve fibers along with mast cells and lymphocytes is increased in IBS and express the capsaicin receptor TR Pv1 [79].

1.5.1 Microbiological Data

The intestinal flora has been studied in IBS subjects for many decades, but only since the advent of new molecular and PCR technology that substantial gains have been made in our understanding of the microbiota alterations and pathobiology. Initial studies had concentrated on the theory of small intestinal bacterial overgrowth [SIBO] since the 1990s to explain the symptoms of IBS. Excessive coliform bacterial overgrowth in blind loops and small bowel had long been implicated in chronic diarrhea and some malabsorption states. Most of the studies on SIBO had used surrogate markers such as the hydrogen breath test using lactulose or glucose and less commonly C-14 xylose as substrate. These indirect tests of SIBO have been heavily criticized because of nonspecificity and weakness of the breath tests besides the variation in the interpretation and inconsistency of the results in various studies [80]. A recent review and meta-analysis on abnormal breath test in IBS have been reported [81]. Eleven studies were reviewed, the lactulose breath test was most commonly used, and although abnormal breath tests were more common in IBS subjects compared to healthy controls [OR = 4.46], the conclusion was that this did not necessarily imply SIBO but could reflect abnormal fermentation, timing, and dynamics of the breath tests variability. A recent study also reported that abnormal breath test detects oro-cecal transit and not SIBO [82]. Quantitative culture of the small bowel contents is more invasive and is considered as the gold standard for the assessment of SIBO. A large sample of IBS patients [$n = 162$] and healthy controls had quantitative culture of jejunal aspirate in one study [83]. Using the standard microbial definition of $>10^5$ cfu [colony forming units] of colonic bacteria/ml for SIBO no difference in the two groups was found [4 % each]. However, adopting a modified criteria of SIBO [bacterial counts $\geq 5 \times 10^3$ cfu/ml] showed a significant difference with 43 % of IBS patients with mild increase in bacterial growth versus 12 % of controls, $p = 0.002$. However, there was no correlation between bacterial alterations and symptoms [83].

A subsequent review on SIBO in IBS found the role of testing for bacterial overgrowth in suspected cases to be unclear [84]. Probably the most definitive study on the topic included 675 patients who underwent quantitative culture of duodenal aspirate with various conditions and analyze their association with SIBO [85]. There was no association of SIBO with IBS, but conditions associated with bacterial overgrowth included older age, steatorrhea, narcotic use, small bowel diverticula, pancreatitis, and inflammatory bowel disease. Hence, overall the data do not

support a significant correlation with bacterial overgrowth of the small bowel and IBS. Some studies have implicated methane producing intestinal flora [methanogenic bacteria] to IBS, constipation predominant, and diverticulosis [86], but the data is not very convincing. Methane appears to slow bowel transit and methanogenic flora is more a reflection of chronic constipation [87].

1.5.1.1 Modulation of Gut Microbiota

The gut microbiota is a complex microbial ecosystem which contains about 10^{14} microorganisms [10–100 trillion microbial cells] belonging to more than 2,000 species [88], of which most [60–80 %] remain unculturable. It has only been in the last decade that new molecular methods utilizing new ribosomal RNA and whole genome base technologies, quantitative PCR, and DNA sequence-based studies have led to the understanding of the enormous biodiversity of human microbial endogenous community. The host–microbiome interaction can be beneficial to maintain normal health and homeostasis but alterations in the gut microbiota are linked to diseases and intestinal inflammation [88, 89]. Evolution of the symbiotic relationship between human and their gut flora plays a major role in the development of a mature immune system, and constantly interacts with intestinal immune mechanism, provide nutrients for components of the gut wall and modulate energy metabolism [89].

A novel paradigm-shifting hypothesis has been proposed that the gut microbial community constitutes an organ, which interacts with the host nervous system that innervates the gastrointestinal tract and influences homeostasis, susceptibility to disease, and even behavior [90]. This theory could explain both the gastrointestinal and the extraintestinal symptoms that are commonly associated with IBS, if disturbances of the gut microbiota actually play a role in the pathobiology of this condition. The gut microbiota undergoes substantial changes at the extremes of life [infants and older people], but the adult intestinal microbiota is normally stable over time and similar between individuals [91]. There is recent evidence that the core microbiota of elderly subjects is distinct from that of younger adults with a greater proportion of *Bacteroides* species, and distinct abundance of clostridium groups that remained stable over time [3 months] [92]. This is important when interpreting studies as age-matched controls should be used before accounting for differences in the microbial endogenous community.

Insights into the microbiota of healthy subjects allow for comparison with compromised individuals with different clinical conditions. The majority of the human gut microbiota are predominantly bacteria and 10 bacterial phyla [deep bacterial lineages or divisions] of more than 70 bacterial phyla identified on earth have been found in the intestine [Firmicutes, Bacteroides, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia, cyanobacteria, TM 7, Spirochetes, and Vadin BE97] [93]. There are estimated to be >15,000 species-level bacterial phylotypes and 1,800 genera associated with the human gut [94]. There are also diverse viral phage communities that colonize microbial cells and are reservoirs of genetic material and influence the diversity of the bacterial intestinal population [93].

There is evidence, however, that intersubject variability and differences between stool and mucosal microbial communities pattern exists [variation of biodiversity within an ecosystem] in normal hosts [95].

Studies on intestinal microbiota, comparing healthy controls with IBS patients had used predominantly fecal samples, which are easily obtainable, and less commonly mucosal specimens. Moreover, mucosal specimens between studies have varied in the sites biopsied, and some studies have analyzed differences between subgroups of IBS patients [IBS-diarrhea predominant [IBS-D], IBS-constipation predominant [IBS-C], or IBS-mixed type [IBS-M] compared to healthy controls]. Limitations and difficulty in interpreting the available data from different studies include use of patients on antibiotics in some studies, and different methodologies in techniques in assessing qualitative and quantitative intestinal microbiota. Moreover, the majority of studies included relatively small samples of patients and controls [<30 in each group]. Analysis of intestinal microbiota since 2005 has utilized qualitative profiling and quantitative assays specific to dominant genera and species [96]. Methods have mostly involved 16S ribosomal [r] RNA gene sequencing, quantitative real-time [RT] PCR, PCR-denaturing gradient gel electrophoresis [DGGE], and rarely florescent in situ hybridization [FISH] for qualitative analysis, and more recently phylogenetic microarray analysis [97–111]. A summary of these studies is presented in Table 1.2.

The first 6 studies [1a–1f] were derived from the same cohort of IBS patients performed by Finnish investigators utilizing different complementary tests on the same batch of fecal specimens at different times over several years [97–102]. Nearly all the studies showed some qualitative or quantitative differences in the pattern of intestinal microbiota of IBS patients compared to healthy, age- and sex-matched controls, but of an inconsistent pattern or profile. An initial study performed in Finland reported greater temporal instability of the predominant bacterial population over 6 months in IBS subjects compared to controls [97]. However, limitations of the study included the analysis of patients receiving antibiotics and subsequent repeat analysis of fecal samples with exclusion of patients on antibiotics using the same method [DNA-based PCR-DGGE] did not reveal more instability over time of the pattern of the predominant microbiota [98]. However, higher temporal instability of the predominant bacteria was found in IBS subjects using RNA-based DGGE.

Two of the studies reported lower biodiversity of the bacterial species/phyla in IBS subjects [104, 105], whereas others reported increase in diversity of fecal microbiota [107, 108]. A more consistent finding of the pattern of intestinal microbiota in IBS includes reports of decreased *Bifidobacterium* species [100, 103, 111], but the changes in bacterial profiles and concentrations may be related to the subtype of IBS. Decreased Lactobacillus and Bacteroides species have been reported in IBS-D, [99, 100, 102, 110] but increased Lactobacillus species has been noted in the feces of IBS subjects [subtype not specified] by others [106], and increased Bacteroides species have been found from the rectal mucosa of the IBS group [111], and feces of IB S-M subtype [100]. To date one of the most robust studies with relatively large sample size [62 IBS patients versus 46 controls] applied deep molecular analysis with phylogenetic microarray assays has recently been reported from the

Table 1.2 Molecular analysis of intestinal microbiota in IBS

Author/year [ref]	IBS-subtype [n]	Controls nos.	Sample	Frequency [m]	Findings	Comments
1a. Matto, 2005 [97]	26__IBS-D [12]	25	Feces	0,3,6	Temporal instability of IBS microbiota	Included subjects on antibiotics
	__IBS-C [9]	„	„	„		
	__IBS-M [3]					
b. Maukonen, 2006 [98]	16__IBS-D [7]	16	„	0,6	Temporal instability varied with PCR method; decreased Clostridia species in IBS-C	Subjects on antibiotics excluded; minor perturbations of microbes
	__IBS-C [6]	„	„	„		
	__IBS-M [3]	„	„	„		
c. Malinen, 2005 [99]	27__IBS-D [12]	22	„	0,3,6	Decreased Lactobacilli in IBS-D increased Veillonella in IBS-C	3 Samples suggest difference in Clostridia/Bifidobacteria between IBS gp. vs controls
	__IBS-C [9]	„	„	„		
	__IBS-M [6]	„	„	„		
d. Kassinen, 2007 [100]	24__IBS-D [10]	23	„	„ [pooled]	decr. Lactobacilli/Collinsella in IBS gp incr. Bacteroides/ Allisonella in IBS-M incr. Ruminococcus in IBS-C, decr Bifidobacteria/incr. Streptococci in IBS-D	Same samples as in [99] new method of analysis
	__IBS-C [8]	„	„	„		
	__IBS-M [6]	„	„	„		
e. Lyra, 2009 [101]	20__IBS-D [8]	15	„	0,3,6	Quantities of 14 phylotypes in IBS-D different from control/ other groups	Fecal samples same as a–f
	_IBS-C [8], IBS-M [4]	„	„	„		
f. Krogius-kunikka, 2009 [102].	10__IBS-D	23	Feces	Pooled	incr. Proteobacteria/Firmicutes decr. Actinobacteria/ Bacteroides	Significant difference betw IBS-group vs controls
2. Kerckhoffs, 2009 [103]	41__IBS-D [14]	26	Feces and duod. brush	Once	Twofold decr. in Bifidobacteria vs controls [$p<0.01$]	decr. in feces and duodenum of B. catenulatum, $p<0.001$
	__IBS-C [11]					
	__IBS-M [16]					
3. Noor, 2010 [104]	11__IBS [NS]	22	Feces	Once	Lower diversity of bact. species in IBS/UC-pts. vs controls	Different patterns of Bacteroides spp. Loss in IBS vs UC-pts
	13__UC-pts					

(continued)

Table 1.2 (continued)

Author/year [ref]	IBS-subtype [n]	Controls nos.	Sample	Frequency [m]	Findings	Comments
4. Codling, 2010 [105]	47__IBS [NS]	33	Feces and colon mucosa	„	incr. diversity in microbiota of controls vs IBS-pts	No difference in bacterial communities bet. feces and mucosa
5. Tana, 2010 [106]	26__IBS-D [8] __IBS-C [11]	26	Feces	„	incr. Veillonella/Lactobacilli in IBS-pts vs controls, p=0.04	High conc. of acetic/propionic acids correlated with symptom
6. Ponusamy, 2011 [107]	11__IBS [NS]	8	„	„	incr. diversity of total bacteria in IBS-pts. vs controls	incr. conc. of amino acids and phenolic compounds in IBS-pts
7. Carrol, 2011 [108]	16__IBS-D	21	Feces and recto sigmoid mucosa	8-bx.ea	incr. biodiversity in feces of IBS-pts. vs controls, p=0.008	Compositional difference in fecal and mucosal microbiota between IBS-pts vs controls
8. Saulnier, 2011 [109]	22__IBS-D [1] __IBS-C [13] __IBS-M [7]	22	Feces	71 Samples	incr. gamma Proteobacteria, and Ruminococcus-like microbe in IBS-pts vs controls.	Specific microbiota signatures in pediatric IBS-pts by meta-genomic analysis
9. Rajilic-Stojanovic [110]	62__IBS-D [25] __IBS-C [18] __IBS-M [19]	46	Feces	2 Samples ea.	icr. Firmicutes/Bacteroides ratio in IBS, p=0.0002; Incr. Dorea, Ruminococcus, and Clostridia in IBS-pts, p, 0.005.	Microbiota from IBS-pts different from controls, p 0.0005, by phylogenetic microarray analysis.
10. Parkes, 2012 [111]	47__IBS-D [27] __IBS-C [20]	26	Rectal-mucosa	Once	incr. Bacteroides/Clostridia in IBS-pts vs controls, p=0.001; decr. Bifidobacterium in IBS-D vs IBS-C/controls.	No. stools/day negatively correlated with nos. of Bifidobacteria and Lactobacilli.

IBS = irritable bowel syndrome; IBS-C = IBS-constipation predominant; IBS-D = IBS-diarrhea predominant; IBS-M = IBS-mixed type; HC = healthy controls

Netherlands [110]. Notable findings included twofold increase in the ratio of *Firmicutes*: *Bacteroides* [$p=0.0002$] due mainly to decrease in Bacteroides species in IBS subjects, with corresponding increase [1.5 fold] in numbers of Dorea, Ruminococcus, and Clostridia species [$p<0.005$]; and 1.5 fold decrease in Bifidobacterium and Fecalbacterium species [$p<0.05$], with a fourfold lower average numbers of methanogens, methane producing anaerobic bacteria, [$p=0.003$]. There was significant correlation between bacterial signal intensity of 18 phylogenetic groups and symptoms. Gamma-proteobacteria concentration positively correlated with symptoms, while symptoms were negatively associated with concentration of Bifidobacterium [pain score], Fecalbacterium, Eubacterium, and Ruminococcus species [110].

Metabolic changes resulting from shifting patterns of intestinal microbiota have been postulated to explain the symptoms of IBS. In one study high fecal concentration of acetic and propionic acids correlated with symptoms [106]. In another small study increased amino acids and phenolic compounds correlated with symptoms [107]. In a small study of 10 IBS patients, 13 subjects with ulcerative colitis [UC] and 22 healthy controls, fecal extracts obtained from four specimens per subject over a year, were examined for altered metabolic activity using high resolution nuclear magnetic resonance [NMR] spectroscopy [112]. The pattern of the altered metabolic activity of gut microbiota was predictive of the disease. In UC patients there were increased levels of taurine and cadaverine, while in IBS subjects there were increased concentration of bile acids and decreased branched chain fatty acids compared to controls. This correlated with the difference in composition of the gut microbiota in these groups [112].

In summary, the current microbiological data in patients with IBS compared to healthy controls, despite limitations of sample sizes, differences in methods and results, strongly support a dysbiosis or imbalance of the commensal microbiota of the gut in this condition, but the pathophysiologic mechanisms still remains unclear.

1.6 Animal Models of IBS

Animal models are often useful in determination of the biological mechanisms in many human diseases and sometimes in establishing causality. Previous animal studies had shown that psychological stress alters the intestinal flora, increases cytokine response and intestinal permeability of the lining [113]. Postulated mechanisms by which stress can alter the microbiota of the GI tract include changes in epithelial cell function, motility, mucous secretion, and through the effect of catecholamines which can modify adherence of bacteria to the mucosa. Pathogenic bacteria and parasites have been used to produce postinfectious IBS in rodents [114–118]. Two of these studies suggested that intestinal infection increased visceral hypersensitivity [common in IBS patients], possibly through stimulation of ATP gated receptor [117, 118], and that stress exacerbated the peripheral nociceptive signaling [116].

In Campylobacter induced PI-IBS in rodents, the acute phase within 32 days is characterized by noninflammatory changes of the mucosa of the small bowel consisting of villous widening but no histological changes in the chronic phase [3 months postinfection] [115], but colonic and rectal mucosa showed increase in intraepithelial lymphocytes 3 months postinfection with altered stool form, and lower body weight in an earlier study [114]. Postinflammation but noninfectious models of IBS are induced with chemical agents [trinitrobenzene sulfonic acid the most commonly used agent], and these models are similar in many respects to the postinfectious IBS models [119]. However, none of the models provided clearer insight into the pathogenic mechanisms of IBS and are lacking detailed molecular analysis of the animals' intestinal microbiota. No animal model as yet has been developed to demonstrate that alterations in the bowel flora, as seen in human studies, can produce IBS. Hence, this is a clear area for future investigations.

1.7 Antibiotic and Probiotic Therapy in IBS

Patients with IBS often do not respond to standard treatment with dietary and lifestyle modifications, fiber supplementation, psychotherapy, and pharmacotherapy [120, 121]. Proven efficacy of an antimicrobial agent or probiotic in controlled, randomized clinical trials would strengthen the concept of a role of microbes in the pathogenesis of IBS. Treatment with oral neomycin had shown marginal efficacy [122] and systemic antibiotics had mixed results [123]. The most promising antibiotic is a new, nonabsorbable, oral rifamycin derivative with a broad antibacterial spectrum [rifaximin]. Like other rifamycins this new agent inhibits RNA synthesis by binding to the B-subunit of bacterial DNA-dependent RNA polymerase, and the in vitro activity includes coliforms, gram-positive bacteria, and Fusobacterium [124]. It has been used clinically for traveler's diarrhea and hepatic encephalopathy [orphan drug status by the USA Food and Drug Administration], as it is almost entirely excreted unchanged in the feces and only 0.4 % is absorbed. However, it is not indicated for the treatment of enteric pathogens such as Campylobacter, Shigella, or Salmonella gastroenteritis [124].

Previous small clinical trials of rifaximin in IBS patients had shown promising results with improvement of symptoms [125, 126], and low risk of bacterial resistance has been reported with limited use.

Recently, a single report on the results of two randomized controlled trials of 1,260 patients with IBS-diarrhea predominant was published [127]. Patients were assigned randomly either rifaximin 550 mg or placebo three times daily for 2 weeks and followed for an additional 10 weeks. Significantly more patients receiving rifaximin had relief of global IBS symptoms [40.7 % versus 31.7 %, $p < 0.001$], and overall symptoms of abdominal pain and stool consistency without significant adverse effect [127]. The limitations of these two trials include short-term follow-up, lack of data on fecal microbiota patterns before and after therapy, and the fact that the treated patients had only a modest therapeutic effect.

1.7.1 Probiotics in IBS

Probiotics are defined as dietary supplements of living microorganisms found in the normal flora with low or no pathogenicity, but with a positive effect on the health of the host. Over the past 50 years, probiotics have been used and investigated in several infectious diseases and gastrointestinal disorders, including antibiotic-induced diarrhea, Clostridium difficile colitis, irritable bowel syndrome, necrotizing enterocolitis, and inflammatory bowel diseases [128]. Systematic reviews of large number of patients [>1,000] and meta-analyses of randomized controlled trials of probiotics for the treatment of specific gastrointestinal diseases have recently been reviewed [129]. Some of the believed mechanisms of action of probiotics theoretically are suitable to correct or ameliorate the postulated biologic mechanisms of IBS, such as maintenance of the intestinal mucosal integrity and barrier, and the immunomodulatory effect to suppress mucosal inflammation [128].

There have been several systematic reviews and meta-analyses on the benefit of probiotics in IBS in the past 5 years [130–133]. This may be a reflection of the general interest in the topic or could represent controversy in the results, or the fact that large multicenter trials have not been performed to provide a definitive answer on their therapeutic value. Two meta-analyses published in 2008 each reviewed over 20 clinical trials with a total of >1,000 subjects [children and adults], both came to the same conclusion that probiotics improved clinical outcomes by about 22 % and reduced global IBS symptoms more than placebo [130, 131]. Two later reviews came to similar conclusion that the current data indicate that probiotics overall improved IBS symptoms and risk of persistence, despite several limitations of the studies including study design, dose variation, and the variety of the different probiotics used [132, 133]. Other limitations of these trials included small sample sizes as only 3 of the 22 trials most recently reviewed [133] had sample population >200 subjects. Moreover, the variety and differences of the probiotics studied and varied duration of the treatment from 4 weeks to 8 months has hampered interpretation of the data. It has been recommended that the type of probiotic used in future trials should be tailored to the patients' IBS subtype.

1.8 Summary and Conclusion

IBS which is considered a common functional disorder in the general population is likely a multifactorial disease and although the overall association of infectious gastroenteritis [PI-IBS], presence of disturbances in the gut microbiota pattern in many IBS patients, even without previous infections, modest therapeutic benefits of rifaximin and probiotics, strongly implicate a role of microbial pathogenesis in a high proportion of these patients. However, the relationship between IBS and the microbiome is not simply cause and effect, but is more complex and poorly

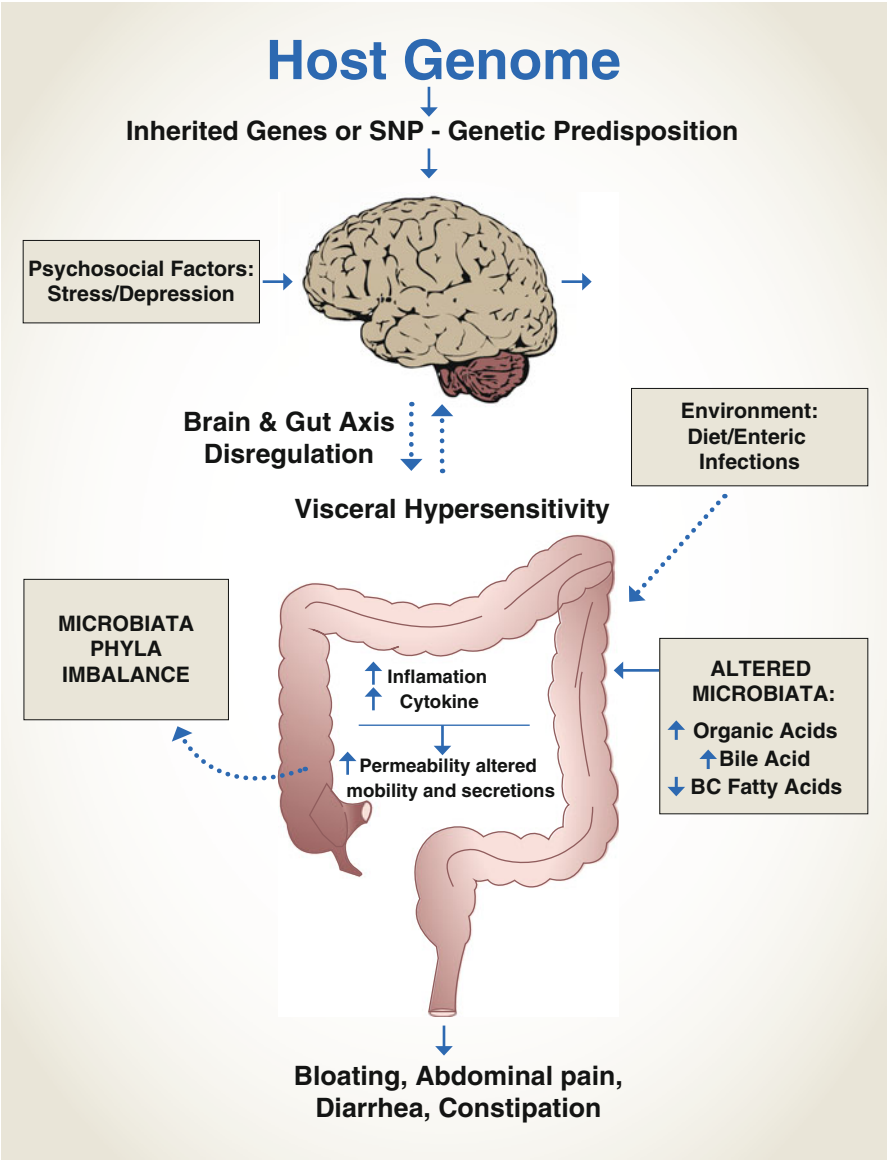

Fig. 1.1 Postulated mechanisms of irritable bowel syndrome showing interaction of various factors that may play a role in the pathogenesis of irritable bowel syndrome. SNP = single-nucleotide polymorphism

understood. It is likely that the biological mechanisms of IBS induction involves genetic host factors, microbiome environment, stress, and psychological factors and possibly dietary influences [see Fig. 1.1].

1.9 Future Directions

Further investigations are needed to clarify the role of imbalance or alterations in the gut microbiota with different subtypes of IBS. Studies with larger sample sizes in each group and with age- and sex-matched controls [preferably on similar diets] should be performed on fecal/mucosal samples a few times over 6–12 months. Microbiological methods should include deep molecular phylogenetic analysis combined with metabolomic analysis [i.e. by NMR spectroscopy] to confirm changes in the microbiome phyla profile with chemical changes in the bowel that would explain the IBS symptoms associated with each subtype.

Therapeutic clinical trials with probiotics need to be standardized for dose, mixture of the composition, and duration. Large multicenter, placebo controlled, randomized trials are needed, preferably with hundreds of patients in each IBS-subtype and controls. Future trials should have extended follow-up of at least 6–12 months or greater and should ideally include fecal analysis before, during, and after treatment to identify specific changes in phylogenetic characteristics of the microbiota and measurement of the associated metabolic changes associated with these alterations by metabolomics of fecal extract. These types of studies are needed to clarify the role of microbiomes in the pathobiology and the use of probiotics in the treatment of IBS.

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Chapter 2
Microbes in Colon Cancer and Inflammatory Bowel Disease

2.1 Introduction

Colorectal cancer [CRC] is the third most common tumor and fourth most common cause of cancer death in the world [1]. It is prevalent in many countries but more common in developed nations. In the United States [US] CRC estimated new cases in 2008 were 77,250 in men and 71,560 in women [1]. The higher incidence of CRC in developed countries has been attributed to dietary and lifestyle habits, and the influence of genetic predisposition.

2.1.1 Risk Factors

The vast majority of CRC occur in the population over age 50 and continue to increase with further aging. The highest incidence and mortality in the US has been found in people of African American ethnicity when compared to other ethnic populations [1]. Genetic factors likely play a strong role, as a family history of CRC in a first-degree relative increases the risk of CRC by two- to threefold, and even second-degree relatives increases the risk by 25–50 % over the general population [1]. Genetic and environmental factors are associated with the development of CRC, see Fig. 2.1 [2]. Three major categories of genes have been implicated in the development of CRC, such as K-ras [retrovirus-associated DNA sequence], tumor suppressive genes, and the mismatch repair genes [1]. Mutations or alterations of the tumor suppressive T53 gene are present in up to 75–85 % of CRC [2, 3]. The transition from normal to malignant colonic mucosa involves a multistep cascade of genetic mutations involving the deleted in colorectal cancer [DCC] gene and the T53 suppressor genes, and the K-ras oncogene mutation leading to tumor formation [47–50 %] [1, 2].

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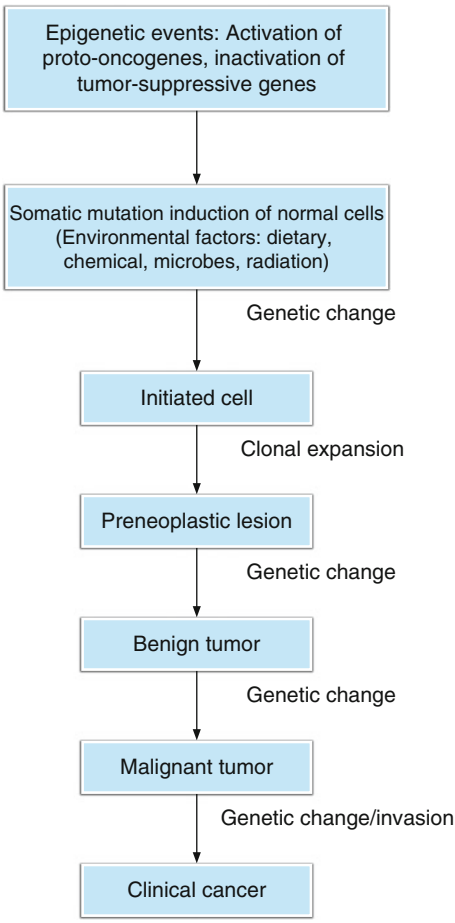

Fig. 2.1 Multistage development of colon cancer

It is generally believed that westernized dietary habits, including increased consumption of red meat and fat with decreased consumption of vegetables and fruits, is largely responsible for the increased rates of CRC in developed nations. There is conflicting data on the protective effect of fruits and vegetables on the development of CRC, while high consumption of these natural products may not directly protect against CRC, low consumption is related to increased risk of CRC [1].

During the progression of normal epithelial cells to CRC there is a succession of clonal expansions and loss of control or inhibition of cell division. Changes in colonic crypt cell proliferation have been shown to proceed and accompany neoplasia. Ingredients in the diet that stimulate cells to divide are vulnerable to effects of carcinogens to promote cancer, and dietary factors that enhance differentiation or apoptosis protect against CRC. The contents of fiber in the human diet consist of

soluble fibers, more readily fermented by colonic bacteria, and insoluble fibers [wheat bran, cellulose, etc.] which remain in the fecal stream as diluents, are believed to be important in the tumorigenesis of CRC. Insoluble fibers create bulk and greater moisture content that decrease transit time in the colon and may have a diluting effect on carcinogens and their constant exposure to epithelial cells [4]. Soluble fibers can be metabolized by the intestinal bacterial flora and these metabolic by-products may influence carcinogenesis. Inflammatory bowel diseases [IBD], such as ulcerative colitis [UC] and Crohn's disease [CD], are well-known risk factors for CRC. The extent and duration of the disease are directly correlated to the risk of CRC. There is greater risk of CRC for pancolitis compared to left-sided colitis [5- to 15-fold increase versus threefold increase], and the risk increases from 2 to 8 % at 10–20 years versus 18 % after 30 years of UC [1]. Obesity has also been shown to be associated with increased risk of CRC and increase in physical activity may be protective [5]. However, the exact mechanisms for carcinogenesis and the association with these conditions are not exactly clear. IBD are putatively linked to CRC through the inflammatory cascade involving proinflammatory cytokines, stimulation of the cyclooxygenase, and prostaglandin pathways. This has led to the potential use of aspirin and other nonsteroidal anti-inflammatory drugs [NSAIDS] as preventative agents for CRC, through their inhibitory effect on the cyclooxygenase pathway [6].

It is unclear at present whether the association of obesity and increased risk of colonic adenomas and CRC can be explained alone on dietary factors, consumption of foods with higher levels of sugar and saturated fats. Visceral adiposity is a stronger risk factor for CRC than increased body mass index alone [7]. There are several elements that are secondarily increased in obesity that have been proposed to participate in the pathogenesis of CRC. Chronic low-grade inflammation with persistent activation of the nuclear transcription factor NK-kB may result in transcription of genes that promote tumorigenesis in visceral adiposity [8]. Hyperinsulinemia, insulin resistance, and insulin growth factor are increased in the metabolic syndrome and obesity, and all of these factors may promote tumorigenesis by increasing colonic cell proliferation and angiogenesis and inhibition of epithelial cell apoptosis, as shown in cell lines and animal models [9].

Lifestyle risk factors for CRC are also associated with obesity and increased risk of cardiovascular disease such as physical inactivity, smoking, and excessive alcohol [9]. Despite the close relationship with physical inactivity and obesity, association remains strong for CRC even after adjustment for age, diet, and obesity [10]. Smoking has been reported to increase the relative risk of CRC in observational studies [10], possibly by inducing genetic alterations in the colonic epithelium [11], but there was insufficient evidence for a causal relationship [9]. Heavy alcohol consumption has been associated with increased relative risk [RR 1.41] of CRC from the pooled analysis of eight cohort studies [12]. This observation may be due to poor nutritious diet in alcoholics, especially to the low intake of folate-containing foods [13].

2.2 Microbes and Colorectal Cancer

There has been increasing evidence over the past two decades or more that the intestinal commensal flora is very important in maintaining a regulated immune homeostasis of the gastrointestinal [GI] tract. Moreover, dysregulation of the normal balance or symbiotic relationship of the microbiota may play a role in the pathogenesis of IBD and colon cancer. Preliminary small case series using routine microbiological methods had suggested differences in bacterial composition and metabolites in feces of patients with CRC compared to healthy controls in 1996 [14]. Probably the most convincing evidence to date of a role of microbes in the pathogenesis of bowel cancer is demonstrable in animal models. There are also precedent, as GI cancers such as gastric cancer and mucosa-associated lymphoma of the stomach are strongly associated or established to be secondary to chronic *Helicobacter pylori* infection in at risk individuals.

2.2.1 Animal Models of Colorectal Cancer

Genetically modified mice with deletion of genes to regulate inflammation or modify innate immune responses are susceptible to multiple neoplasms of the colon in the presence of normal GI flora, but fail to develop tumors in germ-free animals with the same defects. This was demonstrated in T-cell receptor chain and P53 double-knockout mice, with conventional GI flora colonized mice developing ileocecal adenocarcinoma in 70 % of animals at 4 months of age versus none in germ-free mice [15]. Specific murine enteric pathogens that produce epithelial inflammation have also been shown to promote colonic tumors in mice with mutation in tumor suppressive gene, but a fourfold decrease in similar mice without infection [16]. Others have also shown that the specific murine pathogen *Helicobacter hepaticus* will induce colon cancer in 50–60 % of mice deficient in transforming growth factor-beta [TGF-B] signaling pathway [SMAD-3], but not in those without infection [17]. In a pathogenesis study using the same bacteria to induce inflammation and neoplastic changes, it was found that the regulator T-cells [RTC] require interleukin [IL]-10 to inhibit inflammation and early neoplastic changes [18].

Studies in rats, utilizing chemical carcinogens [1,2-dimethyl-hydrazine] to induce early cellular neoplastic changes with aberrant crypt foci, could be influenced by specific species of intestinal flora, and some bacteria might behave as promoters and others as antipromoters in carcinogenesis [19]. For instance, *Bifidobacterium breve* inoculated orally to gnotobiotic rats had lower rates of aberrant crypt foci than with other bacteria after treatment with a carcinogen. Similarly, in male Sprague–Dawley rats injected with azoxymethane [ADM] carcinogen or saline development of colonic tumors fed different fiber diets had different rates of neoplasm associated with different intestinal bacterial population [20]. Rats receiving ADM consuming high cellulose diet and not developing tumors possessed larger amounts of anaerobes in their feces at 10 months [$p < 0.05$].

Investigators utilizing human-flora rats fed high-risk diet [high in fat, sucrose, low in calcium and fiber] compared to rats on low-risk diet [low in fat, high in starch, calcium, and fiber] found significant changes in gut microflora and associated biomarkers of colon cancer [21]. Rats fed high-risk diet had significant altered cecal bacteria, with 2.5-fold increase in beta-glucuronidase activity, increased cecal ammonia concentration, and enhanced genotoxic risk from 7-hydroxy-imidazole quinolone, 3 putative biomarkers of colon cancer. Review of the pathogenic mechanisms in colon cancer about the same time [1997] concluded that CRC was caused by increased mutagenic actions of free radicals produced during oxidation reaction, and that dietary factors and intestinal bacteria produce endogenous metabolites that contribute to free radicals in the colon [22]. It is believed that polyunsaturated fat can be oxidized in the bowel by bacteria to produce mutagens [lipid hydroxyperoxides and malondialdehyde], and that fecal bacteria can generate high flux of reactive oxygen species [superoxide radicals] on the surface of the intestinal mucosa, and inflammatory cells in the colon can produce reactive nitrogen species [nitrogen dioxide]. Theoretically diets rich in antioxidants [i.e. vitamin E] can reduce these harmful effects.

Further studies in gnotobiotic mice treated with chemical carcinogens, 1,2-dimethyl-hydrazine [DMH], to induce tumors have examined the effect of specific bacteria [mono-associated GI colonization] compared with conventional mice treated with DMH [23]. The incidence of colonic adenomas between gnotobiotic and conventional mice after treatment with DMH was similar [74 and 69 %], but the tumors were larger in the conventional mice. The incidence of tumors in gnotobiotic mice with single GI colonization of different bacteria was similar for *Mitsoukella multacida*, *Clostridium butyrican,* and *Bifidobacterium longum* [63–68 %] and significantly lower for colonization with *Lactobacillus acidophilus* [30 %]. Clostridia colonization was associated with larger adenomas and significantly higher concentration of fecal bile acids, whereas *L. acidophilus* was associated with significantly lower levels of bile acids [23]. A subsequent study was performed by the same group of investigators to examine the changes in the immunological environment in gnotobiotic mice with single species of bacterial GI colonization without DMH treatment [24]. These findings suggested that activation of T-cells in the liver and granulocytes in the colonic mucosa may be related to the antineoplastic effect of *L. acidophilus* in this model.

Other investigators have reported that *B. longum*, lactic acid producing intestinal bacteria, fed to male F34C-rats as a probiotic compared to control diet in ADM-treated rats reduced tumor burden and exerted strong antitumor activity [25]. The probiotic inhibited ADM-induced cell proliferation, oncogenic activity, and expression of ras-p21 oncoprotein compared to control diet. Subsequent studies on the impact of probiotics on microbial flora, inflammation, and tumor development have been performed on IL-10 knockout C57BL/6 mice [26]. Twenty-one mice were fed *Lactobacillus salivarius* in milk compared to 10 control mice, fed a modified milk for 16 weeks. Two of 10 [20 %] control animals died of fulminant colitis versus none in the probiotic group. Fifty percent [5 of 10] control mice develop colonic

neoplasms versus just less than 10 % [2 of 21] in the treated group. Fecal coliforms and enterococcus species were significantly decreased in the probiotic versus control mice [$p < 0.05$]. Also at sacrifice there was significantly decreased amount of *Clostridium perfringens* [$p < 0.05$] in the bowel of the treated group. Thus, probiotic significantly reduced inflammation and colon cancer in IL-10 deficient mice [26]. Others have also noted recently that *Bifidobacterium lactis* and resistant starch products had combined effect in protection again CRC in rat model induced by AOM [27].

2.2.2 Mechanisms of Probiotics and Favorable Commensal Bacteria

The protective effect of favorable commensal bacteria and probiotics on modifying carcinogenesis in the bowel has been examined both in vitro using human intestinal cell lines and animal models. In vitro probiotics can ameliorate expression of Cox-2 and prostaglandin E-2 secretion in intestinal epithelial cells, which are considered important in the inflammatory cascade for tumor development [28]. The combination of probiotic and resistant starch may also facilitate apoptotic deletion of carcinogen damage cells, as demonstrated in the rodent model [29]. Apoptosis provides an innate cellular defense against oncogenesis by removing cells with genetic instability or with DNA mutation or damage from carcinogens in the process of carcinogenesis [30]. The probiotic may act via fermentation of resistant starch to produce butyrate and together exert an immunomodulating effect [31]. More recent investigation in both mice and colonic cancer lines supports the paradigm that bacterial fermentation of dietary fiber in the colon generates short-chain fatty acids which protects again some CRC and IBD [32]. Among the bacterial metabolites butyrate appears to be the most important. GPR109A, a G-protein-couple to receptor for nicotinate but recognizes butyrate at low affinity and function as a tumor suppressor in colon [32]. Others, also using experimental models, have concluded that probiotic and favorable commensal bacteria function as "physiologic cancer surveillance" by preventing proliferation of dysplastic cells by induction of apoptosis [33].

Some probiotics may have different mechanisms of antineoplastic effect, which may be species dependent. Recently, studies utilizing human colon cancer cells and xenograft model [CD-1 nude mice] of human colon cancer determined that *Bacillus polymenticus* [commercially available probiotic bacterium] anticancer effect was mediated by inhibition of proto-oncogene ErbB2 and ErbB3 protein expression [34]. The ErbB receptor family consists of four members including EerB1/epidermal growth factor receptor [EG FR/HER1, ErbB2/HER2/, Neu, ErbB3/HER-3, and ErbB4/H ER-4]. ErbB2 is the most oncogenic member of the family and overexpression is observed in many human cancers, including breast, colon, bladder, and lung cancers [35].

2.2.3 *Harmful Effects of Some Commensal Bacteria*

Some commensal enteric bacteria are cancer promoters as shown in animal models of germ-free and gnotobiotic mice colonized with specific bacteria. Infection-associated inflammation has been well established as risk factors for cancers in various organs, i.e., chronic hepatitis B and C predispose to hepatocellular carcinoma. Genetic modified mice that develop greater burden of CRC with specific murine pathogens is through the induction of inflammation and colitis, e.g., *Helicobacter hepaticus* and *Citrobacter rodentium*. However, the human intestinal commensal *Enterococcus faecalis* can induce colitis and colonic tumors in IL-10-knockout mice in mono-microbe associated model, but other commensal and pathogenic bacteria and yeast failed to produce any intestinal pathology [36]. *E. faecalis* can induce chromosomal changes in colonic epithelial cells that may predispose to neoplastic changes [37]. This commensal can cause DNA damage and instability, potentially transforming events and tumorigenesis analogous to radiation-induced effect [37].

Other human bowel commensal universally present, such as *Bacteroides fragilis*, has been shown to induce colonic tumors in multiple intestinal neoplasia [MIN] mice [38]. However, only enterotoxigenic strains of *B. fragilis* [ETBF] induce robust selective colonic signal transducer and activator of transcription-3 [STAT-3] activation of T-helper-type-17-cell response and can trigger colitis and tumors [38]. It was also found that ETBF tumorigenesis in MIN mice is through the contribution of polyamine catabolism [39]. These animal models have also demonstrated the importance of the natural immune defense mechanisms to counteract the harmful effects of some commensal and pathogenic microbes. Immunocompetent regulatory T-cells are important in preventing pathology and remodeling of intestinal mucosa following tumorigenic microbial insults [40]. The anti-inflammatory cytokine pathway is also important to counteract inflammation-induced tumors, as evident in the IL-10 knockout mice model [18]. The innate immune receptor Nod1 also appears to play protective role in the intestine from inflammation-induced tumorigenesis [41].

In vitro experiments have also demonstrated that some species of commensals including Lactobacillus, Streptococcus, and enterococcus species can generate hydrogen peroxide [H_2O_2] [42]. The strong influx of H_2O_2 leads to stimulation of immune cells to produce proinflammatory cytokines which may predispose to IBD, by perpetuating the inflammatory reaction and increasing apoptosis and necrosis [43]. Although apoptosis plays a favorable regulatory role in controlling tumorigenesis, protracted apoptosis in IBD subjects may cause disruption of the epithelial integrity and possibly impair healing of the mucosa that could predispose to neoplasm [43]. Some commensal bacteria may also convert dietary procarcinogens into DNA damaging chemicals, i.e., ethanol and heterocyclic amines, or directly produce carcinogens such as fecapentaenes [43]. Many colonic commensals express alcoholic dehydrogenase [ADH] that can convert sugars to ethanol by the fermentation process. In the presence of excessive alcohol intake even by moderate alcohol consumption, the microbial ADH activity can be reversed and lead to aldehyde production [44]. Aldehyde is a known carcinogen that promotes mutagenesis by

inactivating cellular proteins important in DNA repair. Fecapentaenes are a family of ether-linked polysaturated lipid with potent mutagenic effect [45]. Fecapentaenes are produced by Bacteroides species at detectable concentration in the bowel and may produce oxidative damage to DNA by generation of radicals [44].

Endogenous reactive oxygen radicals that damage DNA are considered an important mechanism for somatic mutations that give rise to cancer [46]. The most important reactive oxygen species are superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. Several of these reactive oxygen species can damage DNA, but H_2O_2 is the only one stable enough to diffuse into cells where hydroxyl radicals can be generated [47]. Abundant hydroxyl radicals production occur in normal feces, especially in diet rich in fat and poor in fiber, and likely produced by certain commensal bacteria, i.e., *E. faecalis* [48–51]. Sulfate reducing bacteria are members of the normal colonic flora that use sulfate as an oxidant for the degradation of organic matter and produce hydrogen sulfide [H_2S]. Biochemical and functional genomic data suggest that H_2S may impair the balance between cell differentiation, proliferation, and apoptosis of the intestinal epithelium [52]. H_2S may be tumor promoting as it is directly or indirectly involved in the signaling and upregulation of genes involved in the mitogenic activated protein kinase [MAPK] signaling [52] and the oncogenic activation of *Ras* pathway [*Ras*/Raf/MEK/ERK] [53]. There is also evidence that H_2S stimulates nitric oxide [NO] production of intestinal epithelial cells [44] that have variable mitogenic and apoptotic elements, and activate several neoplastic-associated genes including vascular endothelial growth factor [VEGF], which plays a role in tumor progression and metastases [52, 54]. Most of the colonic sulfidogenic bacteria are gram-negative bacteria in the delta subdivision of the Proteobacteria phylum, but others such as Desulfotomaculum are grouped with gram-positive bacteria of the Clostridium subdivision [44].

2.3 Human Studies

There are only a few studies of the potential role of microbes in the tumorigenesis of CRC in humans. In a relatively small study of randomly selected subjects, ages 50–65 years, three groups were identified: African Americans [$n=17$], native Africans [$n=18$], and Caucasian Americans [$n=17$] [55]. Comparisons were made for diet, hydrogen and methane breath responses to oral lactulose, culture of fecal samples for 7-α hydroxylating bacteria and *Lactobacillus plantarium*, and mucosal biopsies of colon to measure cell proliferating rates. The aim of the study was to identify factors that predispose African Americans to greater risk of CRC [60 per 100,000] versus native Africans [less than 1 per 100,000]. Similar to Caucasian Americans, the African Americans [AA] consume more meat protein, total fat and saturated fat, cholesterol, vitamins A and C than native Africans [NA], $p<0.05$–0.01 [55]. However, fiber intake was about the same, but hydrogen breath test was higher and methane breath test lower in AA compared to NA. Fecal colony counts of 7-α hydroxylating bacteria were higher and *L. plantarium* lower in AA versus NA.

The colony crypts cell proliferation rates [reflecting propensity for neoplastic changes] were dramatically increased in AA versus NA, $p<0.001$. The conclusion of the investigators was that higher animal products in diet increase potentially toxic hydrogen and by bile salt reducing bacteria that predisposed to CRC [55]. The limitations of this study included small sample size, lack of molecular microbiology to assess differences in ratios or quantization of various phyla of the bowel microbiome, and lack of chemical analysis for putative carcinogens associated with differences in the intestinal microbiota profile.

In a previous study of only 13 male patients with recurrent neoplasia of the colon following surgery compared to 14 healthy males of similar age, H_2S concentrations were significantly higher secondary to sulfate reducing bacteria [56]. However, the design of the study cannot exclude an effect of alteration in bowel microbiota from previous surgery or inflammation. A recent study examined the concentration of mucosal bacteria in 51 patients, by analysis of colonic biopsies taken from adenoma polyps and normal mucosa [57]. There was a 20-fold reduction of mucosa-adherent bacteria from polyps [associated with overproduction of antibacterial molecules α-defensins] compared to normal mucosa. The authors postulated that microflora dysbiosis at the mucosal surface in colonic adenomas may be a potential factor for dysplastic cell proliferation [57]. The cause-and-effect relationship still remains unknown and larger prospective studies are needed. Conversely, a previous clinical study had reported increased mucosal adherence and invasion by *Escherichia coli* in patients with colon cancer [$n=21$] and in Crohn's disease [$n=14$], but not in ulcerative colitis [$n=21$] and controls [$n=24$] [58]. Thus, the data on adherent bacteria in CRC is conflicting.

Two recent studies have reported on the association of Fusobacterium species in human CRC, using quantitative PCR and 16S r DNA sequence analysis [59, 60]. In one study from Boston the composition of the microbiota, using whole genome sequences, was determined from nine specimen pairs of tissues from CRC and normal mucosa [59]. Fusobacterium sequences were significantly enriched in the cancer metagenomes, ranging over 20 % of total bacterial sequences. Further examination of a larger cohort of 95 pairs of specimens of colon cancer and normal colonic DNA, Fusobacterium species were enriched in cancer DNA and tissue, while Bacteroides and Firmicutes phyla were depleted in tissues [59]. In another study reported at the same time from British Columbia, an overabundance of *Fusobacterium nucleotum* sequences in colon tumors was found compared to matched normal adjacent control mucosa in 99 subjects [60]. A Fusobacterium isolate was also cultured from a tumor specimen and abundant Fusobacterium sequences in tumor were positively associated with lymph node metastases.

These two exciting studies strongly link a single species of bacteria, Fusobacterium, with CRC. However, this microbial association does not prove cause and effect and could represent an innocent bystander effect. The fact that Fusobacterium species is part of the normal oral flora and not an abundant constituent of the colonic microbiota [61] favors a pathobiologic role in the development of CRC. A previous report of r RNA sequence in a small number of colorectal tumors and control samples [6 pairs] had found an increased trend of coribacteria in tumors and suggestion of high amounts of Fusobacterium [62].

2.4 Summary of Colorectal Microbial Pathogenesis

Although there is increasing evidence that the bowel microbiota plays a role in the pathogenesis of CRC, this is likely a complex interaction with genetic predisposition, dietary factors, and alteration of the delicate balance between favorable and unfavorable commensal flora. The protective effect of favorable commensals and the mechanisms of cancer promoting flora are summarized in Tables 2.1 and 2.2. Recent evidence demonstrating the association of Fusobacterium with colonic tumors in humans is intriguing but requires further confirmatory studies. At present the animal studies are more convincing of the role of the bowel microbiome in carcinogenesis of CRC than the human data.

Future research is needed to explore the role of microbes in the pathobiology of CRC in both animal models and humans. In the field of animal experimentation rodent models with humanized bowel flora and genetic alterations, utilizing common mutations and polymorphisms associated with human CRC are best used. Other animal models such as primates and pygmy pigs would help strengthen causality role. Animals with humanized bowel flora could be colonized with high concentrations of *Fusobacterium nucleotum* compared to other species in the presence of a high- and

Table 2.1 Cancer-promoting commensals and mechanisms

Microbe	Mechanism	Reference
E. faecalis	DNA damage and instability, H_2O_2, superoxide radicals	[36, 37, 51]
B. fragilis [ETBF]	Inflammation, polyamine catabolism → fecapentaenes tend to mutagenesis	[38, 39, 44]
Lactobacillus, Streptococcus and Enterococcus spp.	H_2O_2 generation, inflammation, and disruption of epithelium	[42, 43]
Proteobacterium phylum [sulfide reducing bacteria]	H_2S upregulation of oncogenes	[52]
Undefined flora	Conversion of procarcinogens to carcinogens by aldehyde and amines	[44]
Fusobacterium spp.	Inflammatory cytokines pathway	[61, 62]

Table 2.2 Anti-cancer commensals and mechanisms

Microbe	Mechanisms	Reference
Bifidobacterium spp.	? Metabolize carcinogens	[19, 20]
B. longum	Decrease cell proliferation	[25]
L. acidophilus	? Alter bile acid metabolism activation of T-cells, granulocytes	[23, 24]
L. salivarius	Reduce inflammatory pathway	[26]
Probiotics	Reduce COX2, PGE-2 expression and inflammation, enhance apoptosis; increase butyrate and immunomodulation, physiologic “cancer surveillance” Inhibition of protooncogene	[28, 29, 31–34]

low-risk diet, as well as the effect of selective probiotics on the development of CRC. Larger prospective studies of the colonic microbiome are needed in high- and low-risk human subjects over several years to map the genome sequence of feces, normal mucosa, polyps, and cancerous tumors.

Preventative large multicenter, randomized trials of hundreds or thousands of high-risk subjects, such as African Americans over 50 years of age and overweight with the first colonic polyp, could be implemented to compare a daily mixture of suitable probiotics versus placebo [e.g., yogurt without probiotics] given for 5–10 years and monitored for recurrence of adenomas or CRC as end point. A less rigorous but acceptable study could randomize patients to daily yogurt with probiotics versus standard diet with no yogurt. These trials although expensive and time–resource consuming should be worthwhile, as CRC is a major disease of the Western world, which is likely to increase in incidence worldwide with the increase in the aging and overweight/obese populations characteristic of most developed countries in recent years. Moreover, as developing nations such as China and India become more affluent societies it is likely that the rates of colorectal cancer in these nations will also increase from adopting westernized customs and diet.

2.5 Microbes in Inflammatory Bowel Diseases

2.5.1 Background

Crohn's disease [CD] and ulcerative colitis [UC] are the two major forms of idiopathic IBD, characterized by chronic or relapsing immune activation and inflammation of the intestines. Although these conditions are not common diseases they are not extremely rare in westernized countries. The incidence of IBD varies with the geographic regions and similar to CRC may be considered a disease of civilization, with the highest incidence in Europe and North America. In North America the incidence rate ranges from 2.2 to 14.6 cases per 100,000 person-years for UC and from 3.1 to 14.6 cases per 100,000 years for CD [63]. IBD is rare in developing countries or regions in tropical and subtropical climate except for Caucasians in Israel, Australia, and South Africa. This may reflect genetic predisposition inherited from their ancestors, arising mainly from Europe. There is evidence, however, of increasing incidence of IBD, especially UC in Japan, South Korea, Singapore, Latin America, Hong Kong, and Northern India, areas of the world previously with low incidence [63]. It is unclear at present whether or not the increasing incidence of IBD in these regions reflects environmental changes or dietary influence with affluence, or genetic mixing of the populations, or theoretically changes in bowel microbiota.

Ethnicity is an important influence on the occurrence of IBD, greater in Jewish populations [two- to fourfold increase] in Europe, North America, and South Africa, with decreasing prevalence in the non-Jewish white, African Americans, Hispanic, and Asian populations [63].

2.5.2 *Pathobiology of IBD*

There is general consensus that IBD occurs in genetically predisposed individuals, with increased risk in family members up to 14- to 15-fold greater in first-degree relatives than the general population [64]. However, in most patients there is an absence of family history and IBD is a familial disease in only 5–10 % of patients [63]. IBD is associated with certain genetic syndromes [i.e. Turner's syndrome] as well as inherited immune deficiency disorders, including Wiskott–Aldrich syndrome, chronic granulomatous disease, hypogammaglobulinemia, selective IgA deficiency, and immune dysregulation [63]. This alone would suggest that the immune response to microbes could be important in the pathogenesis of IBD, besides just genetic predisposition. Recent studies have found association of CD with genetic variants of the nucleotide-binding oligomerization domain-2 [NOD-2] gene, also known as CARD 15 [caspase-recruitment domain 15], of which the gene product [a cytosolic protein] functions as an intracellular sensor for bacteria [64]. It has been estimated that up to 20–30 % of patients with CD may carry abnormal NOD-2/CARD 15-gene. The NOD-2/CARD 15 protein is expressed in monocytes and enterocytes within intestinal crypts and produces an endogenous antimicrobial peptide [defensins] by binding to bacterial peptidoglycan of gram-positive and gram-negative bacteria [64]. The NOD-2/CARD 15 gene mutations have not been associated with UC. However, IBD is a polygenic disorder with multiple clinical subgroups, and with about 100 disease-associated loci on many different chromosomes, of which about one third are shared between UC and CD [63]. An important aspect of the genetic factors that predispose to IBD is their association with innate immunity and autophagy, utilizing immune cells to respond to bacteria, mycobacteria, and viruses [e.g., NOD-2, ATG 16L1, IRGM, JAK-2, STAT-3]; other genes regulate the inflammatory response associated with the regulation of adaptive immunity [i.e., IL-23R, IL-12B, IL-10, PT PN2], through cytokines, leukocyte recruitment, and inflammatory mediator production [63]. Regulatory T cells [RTC] are important in maintaining homeostasis in response to food and microbial antigens. Targeted deletion of certain genes expressed by RTC, which results in colitis with conventional gut flora, includes those that encode IL-10, IL-2, IL-10 R I, TGF beta, TGF BRII, and Fox p3 [64].

It is generally accepted at present that the pathogenesis of IBD is a result of continuous antigenic stimulation by commensal bowel flora that lead to chronic inflammation of the mucosa of the intestines in genetically susceptible individuals [65]. However, although several infectious agents have been implicated in the pathogenesis of IBD, no specific agents have been identified or established to be the cause of CD or UC. The pathogenesis of IBD is characterized by the introduction, maintenance, and intermittent flares of proinflammatory cytokine responses followed by inflammatory changes in the bowel leading to symptoms. The nature of these responses elucidated in the past two decades has been recently reviewed [66]. The initial cytokine responses are controlled by the T-cell differentiating patterns of the disease. In CD the major cytokines are derived from T-helper cell [Th]-1 and

Th-17 CD4 positive T-cell differentiation and generation of interferon gamma [I FN-γ] and interleukin [IL]-17/IL-22 cytokines. In contrast, in UC there is mainly Th-2 cell differentiation with heightened expression of natural killer cells and generation of IL-13 and possible IL-5 [66]. Secondary inflammatory mediators are stimulated by disease-specific cytokines to produce tumor necrosis factor-alpha [TNF-α], IL-1B, IL-6, and tumor necrosis factor-like ligand [T L1A].

There is increasing evidence of the interaction of three fundamental cell biological pathways involved in the pathogenesis of IBD [67]. These include: (1) autophagy, as revealed by the identification of ATG 16LI and IRGM as major genetic risk factors for CD; (2) intracellular bacterial sensing, demonstrated by the importance of NOD-2 in autophagy induction by entry of bacteria intracellularly; and (3) unfolded protein response initiated by endoplasmic reticulum stress, due to accumulation of misfolded proteins [closely linked to autophagy and innate immunity] [67]. These three pathways are increasingly recognized as being important in the pathogenesis of IBD and provide a link between genetic and environmental influence with altered epithelial cell function as the initiating critical event. Autophagy is a process of segregation of the cell's cytoplasmic material within a membrane and its digestion after fusion of the segregated vacuole with a lysosome. Currently, there is cumulative evidence that the intestinal microbes influence host immune development, immune responses, and susceptibility to a variety of diseases, including IBD, CRC, IBS, diabetes mellitus, and obesity [68]. Although the intestinal immunity defends against invading pathogens it maintains a state of immune tolerance [symbiosis] with resident commensal microbiota. Perturbation of the balance is associated with intestinal inflammation as shown by animal models of IBD. A role of microbes in the pathogenesis of IBD has been demonstrated by the strong association between IBD and genes that regulate microbial recognition and innate immune pathology, i.e., NOD-2 gene, genes that control autophagy [ATG 16LI, IRGM], and genes for IL-23-Th 17 pathway that regulate gut immune homeostasis [68]. Intestinal epithelial cell barrier functions are critical for host–microbiota mutualism, and genes regulating mucous secretion [Muc1, Muc2], Paneth cells producing antimicrobial peptides or affect epithelial proliferation and integrity can predispose to IBD [69].

2.5.3 Microbes and Inflammatory Bowel Diseases

The current paradigm in the pathogenesis of IBD involves disturbances in the balance or homeostasis of host genetic factors, barrier function of the gut epithelium, innate and adaptive immunity, and changes in the composition [qualitative and quantitative] of the gut microbiota [Fig. 2.2]. Although dysbiosis of the commensal bacteria, with selected microorganisms have been observed in studies of both CD and UC, it is still unclear whether these perturbations are cause and effect or secondary to changes in the bowel mucosa [70]. The cumulative data will be reviewed in this section.

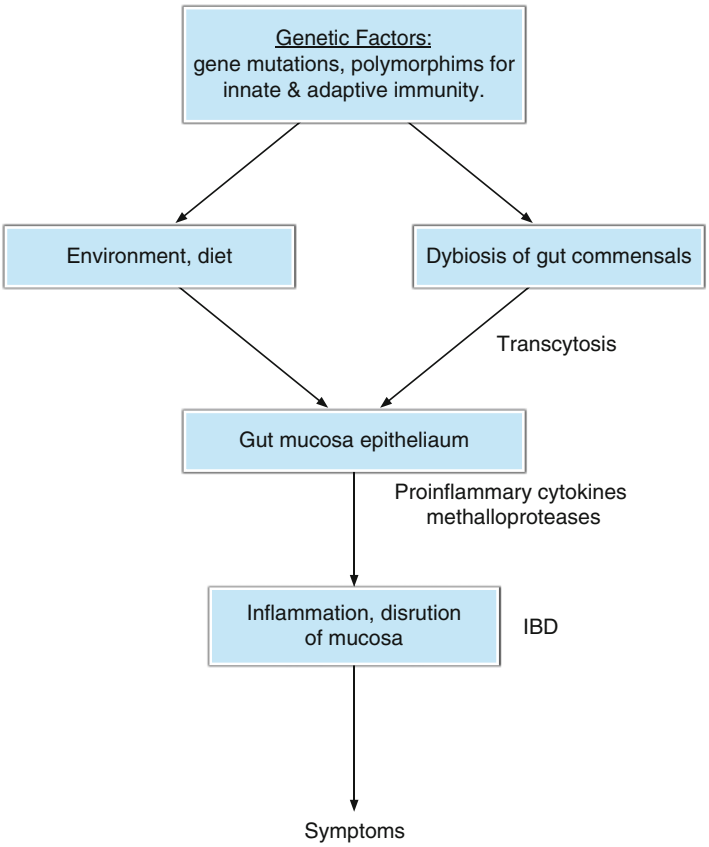

Fig. 2.2 Paradigm of the pathobiology of IBD

Mycobacterium avium subspecies paratuberculosis [MAP] causes a disease in cattle [Johne's disease] that resembles to a great degree the features of CD. It has been postulated for several decades that MAP could be the cause of CD. However, the data is inconsistent and variable and currently MAP is not considered as a strong candidate for the etiology of CD, recently reviewed by Over et al. [71]. MAP infections have been reported in a wide variety of wild and domesticated animals [ruminants and nonruminants], and it can survive in the environment. In a previous systematic review and meta-analysis of the association of CD and MAP, 28 case–control studies were analyzed and although the pooled odds ratio [OR] from studies using PCR in tissue samples showed a strong association with CD compared to healthy controls, similar findings were found in UC [72]. However, in a more recent study of the fecal detection of MAP using IS900 DNA sequences, the results were similar in CD, UC, and healthy subjects, 68 %, 65 %, and 48 %, respectively [73]. The precise mechanisms by which normal commensal bacteria, even in the presence of altered equilibrium, could cause IBD in genetically susceptible subjects still

remain unclear. One of the promising postulates with some supportive evidence involves early state of antigenic overload of the lamina propria due to unregulated transcytosis [translocation] of bacteria across the epithelium, producing innate immune response and chronic inflammation [74]. It has been proposed that dendritic cell function underlies dysregulated T-cell responses in CD, which may be altered by the composition of the intestinal microbiota. In a recent study of 28 CD patients and 10 controls, rectal tissue samples were obtained and analyzed for dendritic cell response and intestinal microbiota composition [75]. IL-6 production by intestinal dendritic cells increased in CD and correlated with the disease activity and composition of the commensal microbiota. Thus, the authors concluded that bacterially driven IL-6 production by dendritic cells may overcome regulatory activity, resulting in uncontrolled inflammation and tissue damage [75].

Some studies have implicated colonization of pathogenic adherent invasive *E .coli* [AIEC], which adhere and invade intestinal epithelial cells [IEC] and survive in macrophages, in the pathogenesis of CD [76–79]. Decreased levels of protective proteases [meprin] that counteract bacterial colonization have been found in the ileum of CD patients and may contribute to increased colonization by AIEC [80]. However, the most promising data on this topic has been from one center and is not very robust. Darfeulle-Michaud et al. [76] analyzed ileum mucosal biopsies from 63 CD patients and 16 healthy controls, as well as colonic specimens from 27 CD and 8 UC patients and 102 normal controls for AIEC. Specimens from ileum were positive for AIEC in 21.7 % of CD lesions and 6.2 % of controls, but in 36.4 % of new lesions [$p = 0.034$] and 22.2 % of healthy mucosa of CD patients. In colonic specimens AIEC were found in 3.7 % of CD and 0 % of UC patients and 1.9 % of healthy controls. If AIEC were an etiologic agent of CD one should expect to detect the organism in a higher proportion of patients, both from lesions of the ileum and colon. However, the ability of AIEC to induce granulomas in vitro [78], and the association of a similar *E. coli* with canine granulomatous colitis, a rare form of specific inflammatory bowel disease of young Boxer dogs, are intriguing and does suggest that AIEC may play a pathogenic role in a subgroup of CD patients. AIEC express an adherence factor that aids in the binding to M cells overlying Peyer's patches and subsequent entry into lymphoid tissue, but it is unclear whether this is causal or secondary to underlying immune deficiencies in CD patients [81]. In an ex vivo study of the mucosa of the terminal ileum and colon of pediatric CD patients compared to the mucosa of healthy controls, there was inappropriate and aberrant response to commensal nonpathogenic bacteria. i.e. Bacteroides species [82]. There is a large diversity of commensal bacteria in the gut and it may be that an overabundance of certain genus or species, predominantly with proteolytic activity, participates in the pathogenesis of IBD by disturbing the mucosal integrity and homeostasis [83].

It has been postulated that a novel or unique strain of Helicobacter bacteria may play a role in UC and investigation in this area is continuing. In a recent study archived and prospectively collected, colonic samples were analyzed by molecular methods [FISH analysis and PCR] for Helicobacter species from UC patients and healthy controls [84]. Helicobacter genus was significantly higher in UC versus controls [32 of 77 versus 11 of 59, $p = 0.004$]. *H. pylori* which has been

well established to cause peptic ulcer and gastric cancer is found in over 90 % of these ulcers, whereas Enterohepatic Helicobacter species are detected in less than 50 % of UC patients. Helicobacter species, however, has been recognized as an important cause of colitis in rodents and primates; some of these bacteria [e.g., *H. hepaticus*, *H. trigontum*, and *H. bilis*] are routinely used in immunocompromised rodent models of IBD [85]. *H. cinaedi* and *H. fennelliae* have been associated with proctitis in homosexual males, but none of the Helicobacter species have been cultured from UC lesions and are not strong candidates for causing UC [85].

In the past few years *Campylobacter concisus* has been implicated in the pathogenesis of CD [86]. The organism has been isolated from children with newly recognized CD and can invade intestinal epithelial cells and disrupt barrier function [87, 88]. Although the detection of *C. concisus* is higher in children with CD [34 of 54, 65 %] versus in healthy controls 11 of 33, 33 % [89], and the bacteria can cause weight loss in immunocompetent BALB/CA mice, it did not produce inflammation of the gut [90]. Thus, the data on *C. concisus* as possible causal agent in the pathogenesis of CD is weak.

2.5.4 Dysbiosis of Intestinal Microbiota in IBD

The leading hypothesis of microbial pathobiology in IBD is related to dysbiosis of the intestinal microbiota, which leads to ineffective control of commensal bacterial invasion, as a result of impaired antibacterial response, resulting in chronic inflammation. For instance, the impairment of clearing intracellular bacteria by the cellular process of autophagy appears to be the main defect in CD [91]. There is cumulative data to support the concepts that defects in the inmate immunity are responsible for the robust proinflammatory response to commensals that resulted in IBD [92]. Recent studies have also confirmed that disease phenotype [CD or UC] and genotype [NOD-2 and ATG16LI risk alleles] are associated with compositional changes of the intestinal microbiota [shift in the relative frequencies of Fecalbacterium and Escherichia taxa [93]. There is increasing evidence that the normal commensal bacteria protect the intestinal epithelium from toxic injury and provide an anti-inflammatory effect [94]. However, disturbances in the immune system or epithelial homeostasis can affect the delicate balance between the gut microbiota and epithelium, which can lead to inflammation. Under these situations the commensal flora appears to enact as a substitute foreign pathogen, which the host response is unable to eradicate and thus results in lifelong inflammation [94]. The immune activation leads to increased concentrations of cytokines, lipid mediators of inflammation, and free radicals with influx of inflammatory cells, the upregulation of matrix metalloproteases from fibroblasts, producing degradation of matrix and eventual ulcerations [95]. Hence, in IBD the homeostatic mechanism that results in coexistence of the host and commensal flora is disrupted, generally by mutations of genes that control inmate and adaptive immunity and epithelial barrier function [96].

Much of the evidence that commensal bacteria can induce gut inflammation is derived from animal models. In many of these models the absence of commensal flora in germ-free conditions resulted in absence of disease or decreased risk even with genetic predisposition [94]. There are over 30 models of IBD in rodents which can be divided into four major groups: (1) colitis that developed spontaneously, (2) chemically induced colitis, (3) animals with defect in epithelial barrier function, and (4) colitis that develops in genetically engineered mice with defects in the immune system or regulating cell function [97]. These studies, however, do not show any specific pattern of the bowel flora component that is important in IBD. Although the propensity of the normal gut flora to induce inflammation is not the same for all species, this can vary with the defect or the model used. For instance, in the HLA-B27 transgenic rats *Bacteroides vulgatus* induces colitis while *E. coli* does not [98], but in the IL-10 deficient mice commensal *E. coli* induces disease but *B. vulgatus* does not [97]. Whether or not this could also apply to humans with IBD and different genetic predisposition or defects is unknown.

Dysbiosis [disturbance of the symbiotic or mutual benefit between microbiota and host] toward selected microorganisms and decreased complexity by the gut flora has been observed in both CD and UC, but it is still unclear whether the dysbiosis causes IBD or is the result of the epithelial pathology [99]. A full understanding of the composition of the gut microbiota, complexity of the diversity, and appreciation of dysbiosis in IBD have only been appreciated in the past several years with the development of culture-independent molecular techniques. Previous studies on mucosal microbiota of patients with IBD and controls using standard culture methods failed to detect differences in composition of mucosal flora but reported marked changes in the concentration of bacteria between normal mucosa [which is almost sterile] and those with IBD [100]. Ileocolectomy induced significant increase in bacterial counts and variety [assessed by standard culture methods] in the neoterminal ileum in CD patients and controls, with greater numbers of *E. coli* and enterococci in CD, but higher quantities of bifidobacteria and ruminococci in controls [101]. Early recurrence of disease was associated with high counts of *E. coli* and Bacteroides with frequent isolation of Fusobacterium.

Recent studies using molecular methods to analyze the intestinal microbiota [mucosa or feces] have in general shown compositional differences between healthy controls and patients with IBD. Table 2.3 summarizes the results of 26 studies performed in the past decade [102–127]. These studies included a total of 462 patients with CD, 308 with UC, and at least 450 healthy controls. The sample size per group varied from 6 to 63 subjects. Three studies [107, 116, 117] did not include healthy controls but were comparing mucosa-associated microbiota from different sites [ileum, colon, and rectum] and from abnormal or ulcerated areas to normal are noninflamed mucosa. A few studies [103, 118, 125] examined the microbiota during active disease and remission, 1 during remission only [119], but the majority of studies did not specify the patients' status and were presumed to be during the active stages of disease. Eight of the studies analyzed fecal samples only, and one study collected samples over a year in UC patients in remission and

Table 2.3 Gut microbiota [by molecular methods] in IBD

Ref./year	CD	UC	Controls	Findings	Method/sample
1. Kleesen [102], 2002	12	12	14	↑ Bacteroides in mucosa of CD [25–55 %], UC [83.3 %] vs controls [0 %]	FISH/ileum colon mucosa
2. Seksik [103], 2003	17 [active and inactive]	–	16	↑ Enterobacteriaceae in CD vs. controls	Dot blot hybr feces
3. Ott [104], 2004	26	31	46	↓ Diversity of microflora in CD by 50 % & UC by 30 % due to loss of anaerobes, $p<0.0001$	RT-PCR, SSCP colon mucosa
4. Mylonaki [105], 2005	33	6	14	↓ Bifidobacteria, ↑ *E. coli* and Clostridia in IBD	FISH/rectal mucosa
5. Lepage [106], 2005	20	11	4	MAM differ from feces but stable from ileum to rectum	TTGE-16S r DNA/feces ileum-rectal mucosa
6. Seksik [107], 2005	15 [75 specimens]	–	–	MAM similar between ulcerated and nonulcerated mucosa, but biodiversity greater in ulcers	TTGE-16S r RNA/ileum-rectal mucosa
7. Manichanh [108], 2006	6	–	6	↓ Complexity of Firmicutes phylum in CD	Genomic DNA/feces
8. Conte [109], 2006	12 [children]	7	7	↑ Aerobes in IBD decreased *B. vulgatus* in UC and CD	PCR, culture ileum-rectal mucosa
9. Martinez-Medina [110], 2006	19	–	15	↑ Clostridia, Ruminococcus and *E. coli* in CD	PCR-DGGE/ileo-colonic mucosa
10. Sokol [111], 2006	14	16	13	↓ Fecal bacteria IBD, ↓ *C. coccoides* in UC, ↓ *C. lepticum* in CD [$p<0.001$]	FISH-flow cytometry/feces.
11. Kotlowski [112], 2007	13	19	15	↑ Enterobacteriaceae B2+D phyla in IBD	RISA, DNA sequence, colon mucosa
12. Baumgart [113], 2007	12	–	7	↑ *E. coli*,↓ Clostridiales in CD	16S rDNA libraries/ileo-colonic mucosa
13. Andoh [114], 2007	–	44	46	Diversity of microbiota vary between UC and controls, ↑ Fusobacterium in active UC	T-RFLP/feces
14. Frank [115], 2007	63	63	63	Depletion of Firmicutes and bacteroidetes in IBD	PCR-broad range/Ileo-colonic mucosa

15. Vasquez [116], 2007	15			Microflora similar for inflamed and noninflamed sites	FISH, TTGE/normal and affected Ileal mucosa
16. Sokol [117], 2007	–	10		Dominant MAM similar for injured and healthy mucosa	TTGE/colo-rectal mucosa
17. Ott [118], 2008	–	13 [remission and relapse]	5	↓ MAM, temporal instability, ↓ bacterial richness with relapse	16S r RNA PCR/colonic mucosa
18. Martinez PCR/ [119], 2008, 1 year	–	16 [remission]	8	Low diversity and temporal instability in UC	16S r RNA feces over
19. Sokol [120], 2008	26	–	21	↓ Firmicutes, *F. prausnitzii* in relapse in CD	FISH/before and 6 months after surgery
20. Nishikawa [121], 2009	–	9	11	↓ Diversity in active UC vs. controls and inactive UC	T-RFLP/colonic mucosa
21. Willing [122], 2010	29	16	35	↓ Faecalbacterium and roseburia in ileal CD, and Incr. Enterobacteriaceae/Ruminococcus	Pyrosquencing/feces and mucosa
22. Mondot [123], 2011	16	–	16	↑ *E. coli*, *E. faecium* and Proteobacteria in CD dysbiosis in CD	RT-PCR, DNA extracted/feces
23. Walker [124], 2011	6	6	5	↓ Firmicutes, diversity in IBD, ↑ Bacteroides [UC, CD] ↑ Enterobacteriaceae in CD	High throughput PCR/paired mucosal specimens [$n=29$]
24. Joossens [125], 2011	68	–	55	↓ *F. prausnitzii*, Bifidobacteria, Clostridia clister and ↑ ruminococcus in CD	DGGE finger printing/feces
25. Andoh [126], 2011	31	31	30	↓ Clostridia in active UC and all CD; ↑ bacteroides in CD	PCR, T-RFLP, feces
26. Lepage [127], 2011	–	8+ disc. twins	17 twins + 10 unrelated	↓ Diversity in UC, unusual aerobic bacteria, lower protective bacteria	16S r DNA, microarray analysis/colon mucosa

CD Crohn's disease, *DGGE* denaturing graded gel electrophoresis, *Disc* discordant, *FISH* fluorescent in situ hybridization, *Hybr* hybridization, *MAM* mucosa-associated microbiota, *PCR* polymerase chain reaction, *RISA* ribosomal intergenic spacer analysis, *RT* real time, *T-RFLP* terminal restriction length polymorphism, *TTGE* temporal temperature gradient gel electrophoresis, *UC* ulcerative colitis

control subjects [119]; 16 studies process mucosal samples only, 1 at the time of an ileum resection and 6 months after surgery [120], and two studies analyzed both feces and mucosal samples [106, 122].

The molecular methods in these studies varied widely and included quantitative real-time PCR [RT-PCR], terminal restriction fragment length polymorphism [T-RFLP], temporal temperature-gradient gel electrophoresis [TTGE], quantitative fluorescent in situ hybridization [FISH], PCR-denaturing gradient gel electrophoresis [PCR-DGGE], ribosomal intergenic spacer analysis [RISA], 16S r DNA-single-strand confirmation polymorphism [SSCP], and 454 pyrotag-sequencing, or combination of two or more methods. Despite the differences in methodologies between studies, which make comparison of findings difficult to interpret, there has been a remarkable similarity in the overall trends. It does appear that mucosa-associated microbiota differ from feces but remained stable from ileum to rectum in the same individuals [106]. Thus, it is important to analyze data of fecal microbiota separately from studies assessing mucosa-associated microbiota [MAM].

Of the 15 studies primarily analyzing MAM 3 did not include samples from healthy controls, but compared ulcerated or inflamed mucosa to nonulcerated healthy mucosa in the same individuals [107, 116, 117]. In two of these studies assessing patients with CD, the MAM were similar between ulcerated and nonulcerated or noninflamed mucosa, which was also similar to the findings in patients with UC [117]. Nearly all the studies comparing MAM of intestinal mucosa of IBD patients compared to controls found significant differences but of variable pattern. Two groups reported increased Bacteroidetes in the mucosal biopsies of both CD and UC patients [greater in the latter group] compared to controls, 18 subjects per group [102, 124]. However, in the largest single study depletion of Bacteroidetes and Firmicutes were found in both CD and UC patients' intestinal mucosa compared to controls, 63 subjects per group [115]. Six studies [105, 109, 110, 112, 113, 124] reported significant increase in *E. coli* or Enterobacteriaceae in the ileal or colonic mucosa of CD patients [$n=104$], and less consistently with UC patients [$n=38$] compared to controls [$n=63$]. Decreased diversity of the microflora of the colonic mucosa has also been reported in CD [by 50 %] and UC [by 30 %] subjects primarily due to loss of the anaerobes [104], and decreased bacterial richness has been associated with relapse in UC [118]; in a discordant twin study UC was associated with decreased biodiversity, different gene expression, unusual aerobic bacteria, and lower protective bacteria [127]. The microbiota of the ileal mucosa before surgical resection and 6 months later demonstrated decreased Firmicutes and *Fecalbacterium prausnitzii* with relapse in a study of 26 CD patients [120]. Moreover, in vitro *F. prausnitzii* demonstrated anti-inflammatory properties and may be protective against active disease.

In fecal samples of 68 CD patients compared to 55 controls there was also evidence of decreased *F. prausnitzii*, bifidobacteria, clostridia, and increased Ruminococcus [125]. In comparison with healthy relatives [$n=85$], CD patients' feces had relatively different microbiota composition [125]. Others have also reported decreased Fecalbacterium and clostridia species in the feces of both CD and UC patients compared to controls [111, 122]. Decreased biodiversity of the fecal flora has also been reported in UC subjects with high concentration of Fusobacterium in

active disease [114], and temporal instability has been noted on analysis of feces over a year [119]. In a more recent study utilizing standard culture methods *Fusobacterium nucleotum* from colonic mucosa has been found to correlate with IBD status from a study of 110 subjects, 22 with IBD. Furthermore, strains of *F. nucleotum* from IBD patients were more invasive than strains from healthy mucosa assessed by Caco-2 cell invasion assay [128].

2.5.5 Probiotics in IBD

The value of probiotics in human IBD has recently been reviewed in 2011 by Meijer and Dieleman [129]. Only randomized, controlled studies reported in English were analyzed, including patients with postoperative pouchitis, and included trials before 2007 through to December 2010. Although the trials since 2007 compared to before this period were more robust, there have been several methodological limitations identified. This included small sample sizes of the cohorts, with only 6 of 22 [27.2 %] trials had a sample size of greater than 100 patients [130–135] and only one study had more than 200 subjects [130]. Other limitations noted by the reviewers were the study of a wide range of probiotic strains or combinations, variation in dose and treatment duration, and inconsistent use of conventional adjuvant medicines or comparators [129]. None of the five small controlled studies in CD showed significant clinical benefit of the probiotics [lactobacillus species used in four studies], but a small study with *Saccharomyces boulardii* in CD patients [15 per group] showed improved intestinal permeability and maintenance of remission [131].

The trials in UC on induction or maintenance of remission with probiotics compared to standard therapy have produced mixed results [129]. The single largest study [$n = 327$], however, found that *E. coli* Nissle 1917 was as effective as mesalamine at maintaining remission for 1 year [130]. Two small studies from the same group of investigators reported that VSL#3 [a commercial mixture containing several Bifidobacterium species, lactobacillus species, and *Streptococcus salivarius* subsp. thermophilus] increases the duration of remission by 9–12 months in patients with pouchitis [132, 133]. A larger study of 117 patients with pouchitis treated for 3 years also found that *Lactobacillus rhamnosus* GG increased the duration of remission [134]. Two relatively large studies in UC patients reported less effective induction or maintenance of remission with VSL#3, but assessment was only for 8–12 weeks [135, 136].

Since the review of probiotics in IBD in 2011 [129], the preliminary results of a trial in UC with a promising symbiotic [combination of a probiotic and prebiotic], consisting of Bifidobacterium and galacto-oligosaccharide, have been reported in a study from Japan [137]. Forty-one patients with mild-to-moderately active UC were randomized to receive the symbiotic versus placebo three times a day for 1 year. There were clinical and colonoscopic indices improvement on the symbiotic, as well as decrease in the amount of myeloperoxidase from colonic lavage, which was used as a surrogate marker of intestinal inflammation. Another recent placebo-controlled

trial of a prebiotic alone [fructo-oligosaccharide] for 4 weeks in CD patients showed no clinical benefit, and there was no change in the fecal flora such as Bifidobacterium or *F. prausnitzii* [138].

2.5.6 Conclusion and Future Directions

The cumulative bulk of evidence over the past decade more strongly support a role of the intestinal microbiota in the pathogenesis of IBD, together with the presence of immune disturbances from genetic predisposition or spontaneous mutations in the genes controlling innate and adaptive immunity. It is unlikely that a single microorganism will be found responsible for the etiology of CD or UC, and more likely that there is a shift in the balance between harmful microbes and protective ones. Although at present, it cannot be definitely stated that dysbiosis of the intestinal flora is the cause of IBD rather than the result, the evidence is more in support of a causative role. The studies to date, however, do not clearly distinguish between the types of IBD based on the bowel microbiota, and predisposition to CD or UC is more likely related to the specific genetic and immunologic disturbances.

Future studies on the pathobiology of IBD should continue to adopt the latest specific and sensitive molecular techniques, but also require larger samples of patients and controls, with analysis of bowel mucosa and fecal specimens multiple times over many months to years. Much larger multicenter, randomized, controlled trials are needed to assess the utility of probiotics for longer duration of time, i.e., 2–4 years. These trials should employ a standardized mixture of probiotics and dosages compared to a standard regimen of comparators. The clinical and colonoscopic indices end points should be well established and accepted by the gastroenterology community. The combination mixture of probiotics should be chosen based on promising preliminary results of clinical trials [i.e., Bifidobacterium symbiotic combination], in vitro biologic studies [such as with *F. prausnitzii*], and animal studies. For instance, in a recent study in mice of *Bifidobacterium bifidum* S 17 was shown to partially protect animals from Th1-driven inflammation [significant reduction of histological score and levels of proinflammatory cytokines] in a chemically induced model of colitis [139].

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Chapter 3
The Role of Microbes in Obesity

3.1 Introduction

Obesity is a major public health concern globally and this condition is epidemic in North America and Western Europe [1]. It has been estimated that two thirds of adults and one third of children in the United States are overweight or obese [2]. The prevalence of obesity and of overweight has increased dramatically in affluent countries in the past three decades. Adult obesity is defined as body mass index [BMI] greater than 30 kg/m^{-2}, and overweight as BMI > 25 kg/m^{-2}. In the United States it had been estimated in 2007–2008 that 32 % of men and 36 % of women were obese, and additionally 40 % of men and 28 % of women were overweight [3]. The prevalence of obesity and overweight has increased by 134 % and 48 %, respectively, from 1980 [4]. Worldwide in 2008 there was an estimated overweight population of 1.46 billion adults or greater, and of these 1 billion were obese, 205 million men and 297 million women [1]. Thus obesity is of pandemic proportions with excess mortality and morbidity from cardiovascular diseases, type 2 diabetes, hypertension, some cancers, and musculoskeletal disorders such as osteoarthritis, causing nearly 3,000,000 deaths each year [5–7].

3.2 Pathophysiology of Obesity

It is most important to understand the pathophysiology of obesity to develop and design novel and more effective treatment for this prevalent condition. It has become clear over the past five decades that dieting to combat obesity is not very effective over the long term. The reasons for the marked increase of pandemic obesity are not fully understood, but likely involves multiple factors besides social and lifestyle

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changes in eating habits, which has been attributable to readily available and affordable sweetened drinks and large serving portions of meals, combined with decreased physical activity and exercise.

Obesity and overweight has long been recognized to run in families. Studies in families have estimated that hereditability may account for approximately 50 % of total body fat mass [8]. Large-scale genome-wide association studies have identified multiple loci, up to 135 candidate genes, linked to BMI or obesity phenotypes [9]. A meta-analysis of the genome-wide association studies has defined 32 loci as more significantly associated with obesity and BMI [10]. However, a few studies have examined the interaction between genetic susceptibility and social/environmental factors [11]. In a recent prospective study of 33,097 subjects, the genetic association with obesity appeared to be more pronounced with greater intake of sugar sweetened beverages [12]. Observational association and interventional studies have provided cumulative evidence that genetic predisposition may modify lifestyle effects [diet and physical activity] on the development of obesity [11]. The current impression is that genetic factors may only account for 20–25 % of the obesity in the population [13]. Genes that contribute to obesity generally involve mutations or abnormalities of one or more of the pathways that regulate the feeding center or satiety, and of those involving energy expenditure and fat storage. There are three monogenic causes of obesity, mutations of melanocortin receptor [MCR]-4 which is the most common, mutation of the leptin gene causing congenital deficiency of leptin which is very rare, and mutation of the leptin receptor gene, which is also very rare [13]. Leptin generally decreases the appetite [anorexigenic].

Although overweight and obesity likely result from the interaction of many factors including genetic, metabolic, behavioral, and environmental effects, there is increasing evidence in the last decade or more that the gut microbiota plays a significant role. Some experts opine that the rapid increase of global obesity and overweight in the past three decades would unlikely to be secondary to biological changes, and suggest that behavioral and lifestyle changes are the main culprits [4]. Some but not all surveys have found that the average calories or energy intake have significantly increased in the past 3–4 decades in the general population, with concomitant decrease in physical activity or energy expenditure [4]. A simplistic view of weight gain leading to overweight and obesity is the following paradigm: Excessive calories or energy consumption minus energy expenditure [decreased from low physical activity] equals positive energy or calorie balance, and thus fat storage. This assumes that all the calories or energy consumed is from food intake and that the energy expenditures are from purposeful physical activity [25 %], nonexercise activity [7 %], thermic effect of food [8 %], and the basal metabolic rate during sleep and arousal [60 %] [13]. The excess calories or energy that are not utilized during daily activities is stored as fat; for each 9.3 cal [K-calories] of excess energy that enter the body approximately 1 g of fat is stored.

The current definition of obesity as defined by BMI does not take into account individuals with large muscle mass. Experts in the field considered that obesity is best defined as 25 % or greater of normal total body fat in men and 35 % or greater in women [13]. However, measurements of total body fat clinically are not readily available, and this is rarely used in practice.

3.2.1 Biology of Adipose Tissues

Fat is stored mainly in the subcutaneous adipose tissue and the intraperitoneal cavity and in obesity fat is also stored in the liver and in other tissues or organs. It was previously believed that the number of adipocytes [fat cells] could substantially increase only in childhood [hyperplastic obesity] and that it in adults obesity was associated mainly in increased size of adipocytes for fat storage [hypertrophic obesity]. Recent studies, however, have shown that new adipocytes can differentiate from fibroblasts-like preadipocytes at any period of life [13]. Thus, adult obesity results from both increased numbers and size of adipocytes.

Control for appetite and satiety are centered in the hypothalamus, the lateral nuclei serving as the feeding center and ventriculomedial nuclei as the satiety center. Signals from the gastrointestinal tract to the hypothalamus are mediated by neurotransmitters and hormones that influence feeding behavior. Increased appetite or feeding is stimulated by orexigenic messengers which include cortisol, melanocyte concentrating hormone, endocannabinoids, ghrelin, neuropeptide γ, Agouti-related protein, endorphins, galanin A and B, and amino acids such as glutamate and γ-aminobutyric acid. Whereas, decreased appetite and reduced feeding is stimulated by leptin, α-melanocyte stimulating hormone, insulin, cholecystokinin, corticotropin releasing hormone, peptide YY, glucagon-like peptide, and cocaine-amphetamine-related transcripts [13].

Feedback signals from adipose tissue regulate food intake since most energy stored in the body consists of fat. There is marked individual variability in the regulation of energy reserve and fat storage. Studies in animals and humans indicate that the hypothalamus senses energy storage through the action of leptin, peptide hormone released from the adipocytes [14]. With increased fat storage increased amount of leptin is produced which act on the hypothalamus to decrease appetite stimulators. Thus leptin is an important means of controlling energy and fat storage as well as the intake of food. However, in obese individuals there is no evidence of deficient leptin production, but it is postulated that leptin receptors or postreceptor signaling may be defective in obese people [13].

Adiponectin, another cytokine or adipokine produced by adipocytes, has insulin-sensitizing and anti-inflammatory properties, and is inversely associated with visceral obesity [15]. The secretion of adiponectin by visceral fat is markedly reduced in obese women [16]. Adipose tissue secretes other cytokines and factors that may be involved in the regulation of metabolic pathways. Obese subjects have altered secretion of tumor necrosis factor [TNF]-α, plasminogen activator inhibitor-1, and interleukin [IL]-6 which can affect lipolysis, insulin insensitivity, and fibrinolysis [8].

3.2.2 Brown Fat and Obesity

It has been recognized for some time that most adipose tissue in adults is in the form of white fat or white adipose tissue [WAT] for storage of fat or energy. But in animals and human neonates, brown fat or brown adipose tissue [BAT] is involved

in energy expenditure as heat and is important for maintenance of body temperature [17]. BAT was considered unimportant in humans beyond early childhood due to rapid involution, until recent studies using FDG-positron emission tomography–computed tomography [PET–CT] showed that BAT is present in adults, but with decreased activity in obesity [18]. BAT tissue influence on energy balance through thermogenesis is mediated by the expression of tissue-specific uncoupling protein-1. It has been postulated that brown fat may affect whole-body metabolism, insulin sensitivity, and influence the tendency for excessive weight gain [18]. In one study by Cypress et al. [18] of 1972 patients, BAT mass and activity was greater in women [7.5 %] compared to men [3.1 %], and was inversely correlated with age [$p<0.001$] and body mass index [$p=0.007$]. Brown fat was mainly located in the cervical, supraclavicular, axillary, and paravertebral areas. The morphology of BAT is significantly different from WAT. The major difference is the abundant presence of mitochondria in BAT compared to the sparsity in WAT, which is a reflection of their difference in function [17]. The cell size and cytoplasm of white adipocytes are larger than brown adipocytes and this could account for the greater capacity for fat storage.

3.3 Gut Microbiota and Obesity

The intestinal microbiota, mainly from the distal intestine, is the home of up to 100 trillion microbial community, which should be considered as an anaerobic bioreactor programmed with an enormous pool of microbial genomes [microbiome] [19]. The gut microbiota consists of predominantly four bacterial phyla or divisions, the gram-negative *Bacteroidetes* and *Proteobacteria*, and the gram-positive *Actinobacteria* and *Firmicutes*, with a highly diverse number of strains or subspecies, 1,100 or more prevalent species [20]. The gut microbiota may be envisioned as a microbial organ within the human or mammalian host [18]. These microbes can degrade a variety of indigestible polysaccharides, including plant-derived pectin, cellulose, hemicellulose and resistant starches, which could be a source of energy and calories for the human host.

Insights of the possible role of microbes in energy balance, weight gain, and fat storage were first observed in animal experiments. Germ-free mice in comparison to conventional mice on the same diet have shed light on many biological effects of the gut microbiota and host, including energy balance [19]. Adult conventional mice consuming less chow than germ-free animals have 40 % more total body fat [21]. This was attributed to the gut microbiota breakdown of indigestible dietary polysaccharides for energy absorption. Subsequent evidence of altered gut microflora associated with obesity was derived from genetic modified murine model with leptin deficiency. Compared to wild-type siblings and their mothers, all fed the same diet, analysis of the distal gut microbiota of leptin deficient mice revealed a major reduction in the quantity of *Bacteroidetes* and a proportional increase in *Firmicutes* [22]. Similar finding with the increased ratio of *Firmicutes*/*Bacteroidetes* was also reported in the large gut of obese humans [23]. In this study of 12 obese males and 2 lean controls, samples of

stools were obtained over a year for analysis with 4 specimens from the obese subjects and 2 from the controls. On dieting with a low-fat or carbohydrate restricted diet *Bacteroidetes* increased [$p<0.001$] and *Firmicutes* decreased. The amount of *Bacteroidetes* correlated with the percentage loss of body weight.

In further studies by the same investigators utilizing the mouse model, it was determined that the obese-associated microbiome had increased capacity for greater energy harvest from diet [24]. Moreover, transmission of the colonizing "obese microbiota" in germ-free mice resulted in greater total body weight and fat than colonization with a "lean microbiota." Human studies, however, on the gut microbiota pattern in obesity have produced mixed results. A few studies have observed an increase in the *Firmicutes*/*Bacteroidetes* ratio in obese subjects [22, 25, 26], but others failed to demonstrate a similar correlation [27–30]. In one of the larger studies with a total of 98 subjects, 30 lean, 35 overweight, and 33 obese volunteers, the ratio of *Firmicutes* to *Bacteroidetes* in feces was the opposite of previous reports, with changes in favor of *Bacteroidetes* in obese and overweight subjects [28]. In this study the main signal in the feces associated with obesity and overweight was the higher concentration of short-chain fatty acids [SCFA] compared to lean subjects, $p=0.024$ and $p=0.019$, respectively [28]. This report suggests that the ratio of *Firmicutes* to *Bacteroidetes* may not be the important alteration of gut microbiota in obesity, but rather the pattern of microflora that leads to greater SCFA metabolism.

In an intriguing study from a group of international investigators recently reported the phylogenetic composition of 39 fecal samples from 6 nationalities compared to previously published datasets [30]. There was no correlation between BMI and ratio of *Firmicutes* to *Bacteroidetes*. However, the metagenomic-derived functional biomarkers identified three marker modules that correlated strongly with the subjects BMI; two of which are ATPase complexes and support the association of the gut microbiota's capacity for energy harvest and obesity [30]. Based on the phylogenetic profile and multidimensional cluster analysis of the gut microbiota, the investigators identified three robust clusters or enterotypes: *Bacteroides* [enterotype I], *Prevotella* [enterotype II], and *Ruminococcus* [enterotype III]. The enterotypes demonstrated variation in phylogenetic and functional aspects: enterotype I generates energy primarily from fermentation of carbohydrates and proteins, encoding enzymes such as galactosidases, hexosaminases, and proteases for these substrates; enterotype II degrades mucin glycoproteins present in the mucosa of the gut; and enterotype III also able to degrade mucins [30].

Another recent study used a unique framework for studying the gut microbiome of healthy subjects, 82 lean or overweight and 42 obese, integrating metagenomic data systems-level network analysis [31]. This report indicated that lean and obese microbiomes differ primarily in their interface with the host and the interaction with host metabolism. A large fraction of the enzymes of the obese-associated microbiomes are involved in the phospho-transferase system [28.6 %] and the nitrate reductase pathway [17.1 %] [31]. The phospho-transferase system has been specifically associated with the *Firmicutes* phylum, used by *Eubacteria* for transporting sugar into cell and has been implicated in carbohydrate uptake [32], and is upregulated in mice following switch to high fat/high sugar diet [33].

A more recent study of 68 obese subjects and 47 lean controls reported that the obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* [$p=0.04$], and depleted with *Bifidobacterium animalis* and *Methanobrevibacter smithii*, $p=0.05$ and 0.03, respectively [34]. Other investigators have also explored the relationship of the gut microbiota with gene polymorphism in obese people [35]. In this study 52 obese subjects were compared to 52 normal weight controls, and the influence of polymorphisms of 2 genes encoding the peroxisome proliferator-activated receptor-gamma [PPAR-γ] was analyzed. PPAR-γ receptors are found in target tissues for insulin, i.e., adipose tissue, skeletal muscle, and liver. Activation of PPAR-γ nuclear receptors regulates fatty acid metabolism, glucose production and utilization, and the maturation of preadipocytes. Obese subjects had lower amounts of *Clostridium perfringens* [$p=0.001$] and *Bacteroides* [$p=0.012$] than controls in their feces, but no difference was found in PPAR-γ, genotype between the two groups [35]. However, among the obese subjects those with PRO/ALa genotype had lower *Bacteroides* than those with PRO/PRO genotype.

3.4 Oral Microbiota and Obesity

In the past 4 years, there have been a few studies assessing the oral microflora and association with obesity. In a study of 313 overweight or obese women and 232 healthy normal weight subjects, the saliva bacterial population was measured by DNA probe analysis [36]. The main percentage difference of 7/40 bacterial species was greater than 2 % in saliva of overweight women. Classification tree analysis of microbial composition showed that 98.4 % of those overweight could be identified by a single bacterial species [*Selenomonas noxia*] at levels >1.05 % of the total salivary bacteria [36]. Thus it was concluded that these salivary bacterial species could be biological indicators of developing obesity and that oral bacteria may participate in the pathobiology of overweight and obesity.

A novel study analyzes the subgingival plaque samples for 40 bacterial species by checkerboard DNA–DNA hybridization in 574 subjects with chronic periodontitis and 121 healthy controls [37]. Periodontitis was significantly greater in overweight [odds ratio [OR] 3.1, 95 % confidence interval [CI] 1.9–4.8] and obese people [OR 5.3, 95 % CI, 2.8–90.5] compared to subjects with normal BMI. After adjusting for age, gender, and smoking status, the OR was 2.3 [95 % CI 1.2–4.5] for obese subjects to exhibit periodontitis. Only *Tannerella forsythia* differed significantly among the BMI groups and significantly increased in obese persons compared to healthy subjects or lean individuals with gingivitis [37]. A smaller but more recent study similarly analyzed the oral subgingival biofilm by the same methods in 29 obese and 58 matched normal weight adolescent subjects with a mean age of 14.7 years [38]. Twenty-three bacterial species were present in approximately threefold higher amounts in obese persons compared to normal weight controls. The sum of bacterial cells in subgingival biofilm was significantly associated with obesity [$p<0.001$] and was not confounded by any of the studied variables, i.e., chronic diseases,

medications, meal frequency, visible plaque index, bleeding on probing, or flow rate of the whole saliva. *Proteobacteria phylum*, *Campylobacter rectus*, and *Neisseria mucosa* were sixfold higher in obese persons [38].

Although these studies are very intriguing and the effects of multiple confounding variables were analyzed, the effect of diet such as the amount of sweetened food or drinks was not addressed in detail and could account for the differences in oral microflora found in these studies.

3.5 Diet and the Effect on Gut Microbiota

Diet is a key factor in the development of obesity and diet may affect the intestinal microflora but this is not well understood. Studies in both animals and humans suggest different diets can modify the relative proportion of various gut microflora [39–41]. Westernized diet with high fat and high sugar, or diets rich in vegetables and fruits have been shown to significantly alter the microbiome composition of the intestines at different phylogenetic level [40].

In genetically modified rodents [obesity-prone or obesity-resistant phenotypes], high fat diet resulted in a decrease in *Bacteroidetes* and increase in *Firmicutes* [mainly *Clostridiales*] and *Proteobacteria* phyla in the presence or absence of obesity [42, 43]. In mice with humanized gut flora a change in diet resulted in a shift in composition of the microbiota just within 24 h, along with changes in the metabolic pathways in the microbiome [33]. Thus obesity by itself is not responsible for the changes in composition of the bowel microbiota but is diet related. High carbohydrate and high fat diet result in similar alteration of the gut microbiome in mice [44].

Although genetic modified [RELM βKO]-mice resistant to obesity on high fat diet have similar changes in bowel microbiota as wild-type mice, they appeared to remain lean not by altered food intake or fat absorption but by increased energy expenditure [43]. Metagenomic analysis of the gut microflora, on standard chow and high-fat diet, has also provided some insights into the mechanism of the gut microbiome on weight and obesity. Genes for amino acid and carbohydrate metabolism decrease in abundance from switch to high fat diet, whereas genes or signal transduction [two component response regulator system], and membrane transport [mostly ABC transporters] increase [43]. ABC transporters control transport of a variety of nutrients such as lipids, sugars, peptides, and metals. Genes involved in import and assimilation of sugars were more abundant as well on high fat diet. Similar to the murine obesity models, the human obese gut microbiome is enriched in phosphor-transferase systems involved in microbial processing of carbohydrates [31]. In the study of obese and lean twin pairs there were 383 genes that were significantly different between obese and lean gut microbiome [$p<0.05$], 273 enriched and 110 depleted in the obese microbiome. In contrast only 49 genes were enriched or depleted between all twin pairs [31]. Seventy-five percent of obesity enriched genes were from *Actinobacteria* and 25 % from *Firmicutes*, whereas 42 % of the lean enriched genes were from *Bacteroidetes* compared to 0 % of the obesity

enriched genes. The main hypothesis of the increase in body weight with changes in the gut microbiota is associated with an increased capacity of the bowel microflora to extract nutrients from the diet not normally well absorbed, and inducing metabolic changes in the host including increased fatty acid oxidation in muscle and increased fat storage in the liver and subcutaneous and visceral adipose tissues [21, 24]. However, other mechanisms may also be implicated.

3.6 Gut Microbiota and Inflammation

Obesity and metabolic disorders, arising from excessive weight such as diabetes type 2 and atherosclerosis, are associated with chronic "low-grade" inflammation [45]. Diet-induced obese mice displays a constant low-grade increase in blood endotoxin from endogenous bowel gram-negative bacteria lysis, which releases the membrane lipopolysaccharide [LPS] for absorption. LPS through LPS-receptor acts via toll-like receptor-4 [TLR4] to stimulate the inflammatory pathway through pro-inflammatory mediators such as tumor necrosis factor [TNF]-α and interleukin [IL]-6, which are associated with a low-grade inflammation and insulin resistance [46, 47]. Mice deficient in the TLR4 adopter protein CD14 [LPS receptor] do not develop diet-induced obesity and insulin resistance [46]. Continuous subcutaneous, low dose infusion of LPS in these mice with high fat diet resulted in increased adiposity, excessive weight, and insulin resistance [46, 48]. Furthermore modulation of the gut microflora by diet modification with oligofructoses [prebiotic] or antibiotic treatment reduced weight, inflammation, and glucose intolerance in obese prone mice on high fat diet [49, 50]. Whereas high fat diet decreases bifidobacteria in mice, adding the prebiotic to the diet selectively increases bifidobacteria, suggesting a role of this species to control obesity [50].

Obesity resulting from consumption of high calories and high fat diet leads to hyperphagia [overeating] which may be partly related to changes in the gut microbiota. Germ-free mice on high fat diet failed to gain weight or insulin resistance and are not hyperphagic compared to conventional mice. In Sprague–Dawley obese-prone rats, the obesigenic effect of high fat diet associated with hyperphagia and excessive weight is attributed to alteration in plasma leptin and activation of the vagal pathway in response to intestinal lipids [51]. Further studies in this model demonstrated that high fat diet caused an increase in the relative proportion of *Bacteroidales* and *Clostridiales* orders regardless of phenotype, but increase in *Enterobacteriales* was seen only in gut microbiota of obesity prone rats [52]. There was a strong link between gut inflammation and obesity. The sequence of events following feeding high fat diet was first altered gut microbiota leading to increase in luminal LPS, and an increase in TLR4 activation in the epithelium associated with decreased intestinal alkaline phosphatase activity, leading to the altered tight junction permeability, and subsequent increase in inflammation [52]. Thus high fat feeding appears to disturb food intake regulation via intestinal epithelial inflammation which may be a trigger for hyperphagia and obesity.

3.6.1 Gut Microbiota on Energy Extraction and Balance

The large intestine has limited digestive capability and the indigestible carbohydrates and proteins received by the colon represent only 10–30 % of the total ingested calories or energy [40]. Microbiota of the distal gut ferments indigestible starches, sugars, cellulosic and noncellulosic polysaccharides, and mucin into SCFA and gases. The type and quantity of SCFA varies with the diet, age, and composition of the microbial community, and the major SCFAs produced are acetate, propionate, and butyrate [40]. In healthy adults on a westernized diet about 100–200 mM SCFA are produced per day in the colon, of which about 90–95 % are absorbed [53]. The absorbed SCFAs are utilized by the colonocytes as nutrients and transferred to other peripheral tissues or organs for metabolism. Butyrate is mainly utilized by the colonocytes for metabolism and development, whereas a major proportion of propionate serves as substrate for gluconeogenesis and cholesterol synthesis in the liver [54]. Acetate is the main circulating SCFA in the blood and is a source of energy for the peripheral tissues, and in the liver it is used for lipogenesis and cholesterol synthesis [54]. The reservoir and concentration of SCFAs in the human intestine has recently been linked to obesity which is correlated with the gut microbiome [53, 54]. In adults a greater concentration of total SCFAs especially propionate is found in the feces of obese subjects compared to lean controls [28], and this has also been reported in children [55].

The gut microbiota may affect energy balance not only through the process of colonic absorption of monosaccharides and SCFAs but by a complex mechanism through regulating gene expression. In addition SCFA produced by the gut microbiota activate gut hormone production [peptide Y Y], which slows the intestinal transit, allowing more complete absorption of intestinal nutrients including the fatty acids themselves [41]. Microbes of the intestines may also regulate energy metabolism by reducing the expression of fasting-induced adipocyte factor [Fiaf] in gut epithelial cells. Fiaf, a member of the angiopoietin-like family of proteins, is a circulating lipoprotein lipase inhibitor. Suppression of Fiaf results in degradation of lipoproteins and microbiota-induced deposition of triglycerides in adipose tissue [21]. However, other studies have indicated that the intestinal mucosa is not a major source of Fiaf in mice [56], and does not support the hypothesis that gut microbes influence accumulation of body fat via Fiaf gene expression [41].

It has also been proposed that the intestinal microbial fermentation results in increase SCFA and plays a role in energy balance via stimulation of adipocytes to induce production of leptin and adiponectin [40]. Leptin increased production acts on the hypothalamus to decrease appetite and adiponectin stimulates glucose utilization and fatty acid oxidation by inactivating AMP-activation protein kinase [57]. These counter regulating mechanisms would normally prevent obesity in the individuals and in germ-free mice on high fat diet. In obesity the level of adiponectin decreases resulting in deactivation of AMP-activated protein kinase and leading to reduced fatty acid oxidation and increased accumulation of free fatty acids in the liver [58].

Recently the gut microbiota has been proposed to affect the endocannabinoids system which is also involved in energy balance [59]. The endocannabinoids receptor CB 1 [family of G-protein-coupled receptors] contributes to appetite regulation in conjunction with leptin and through other functions independent of food intake [41]. It has been hypothesized that the obese-prone gut microbiota leads to increase in plasma LPS levels, resulting in low-grade inflammation and greater endocannabinoids system tone, which results in dysregulation of adipogenesis [59]. A combination of a westernized high fat diet rich in *n*-6 polyunsaturated fatty acids and microbiota-mediated effects has been speculated to influence the endocannabinoid system in the metabolic syndrome [41].

3.7 Viral Infection Implicated in Obesity

Some studies in adults and children have found greater seroconversion to certain viruses in obese subjects compared to lean controls. Adenovirus-36 is the most widely studied and this topic has recently been reviewed by Esposito et al. [60]. Adenoviruses are common causes of respiratory tract infections, gastroenteritis, and conjunctivitis in young children and young adults. There are over 50 serotypes of adenovirus and serotype 36 is the most widely studied in animal models and humans for the association with obesity. An animal virus, canine distemper virus, was first shown to induce obesity in an experimental murine model in 1982 [61]. Subsequently Dhurandhar et al. [62] in 2000 demonstrated that chicken and mice infected with adenovirus 36 [AD36] showed marked increase in weight with significant fat accumulation not seen in animals infected with avian adenovirus. Similar findings were reported in rats [63] and in nonhuman primates [64]. There was a significant association between spontaneous occurring AD36 antibodies and weight gain in 15 rhesus monkeys over 18 months [64]. Furthermore, 28 weeks after infection with AD36 three male marmosets showed a threefold increased weight gain with greater fat accumulation than three uninfected controls [64]. Transmissibility of adenovirus-induced obesity was also demonstrated in the chicken model by the same group of investigators [65] and it raised the possibility of transmissible obesity in childhood.

Several studies have been performed in humans to assess AD-36 antibodies and the association with obesity with varying results. Two studies in adults showed an association between obesity and AD36 antibodies [66–68], but three others failed to confirm the association [69–71]. However, all three studies in children consistently found significant association with AD36 antibodies and obesity [72–74].

The possible mechanism of AD36 infection and link to adiposity has been postulated to be a direct effect on adipose tissues by upregulation of one of the genes essential in preadipocyte differentiation [64, 75]. Although AD36 DNA has been found repeatedly in the fat cells of infected animals, it has not been detected in visceral

adipose tissue of 31 severely obese patients undergoing surgery [70]. Other proposed mechanism of the virus includes reduction of leptin expression and secretion with resulting increased appetite and glucose uptake [76], besides an indirect effect of chronic inflammation on lipid metabolism [77].

3.8 Conclusion

The current cumulative evidence supports a role of changes in the gut microbiome which are influenced by diet and interaction with the host genome, to produce changes in energy homeostasis that leads to increased lipogenesis and fat accumulation. Although relatively large population studies have found an association with increased gingival bacteria [associated with periodontitis] and obesity, this effect may also do to the influence of dietary factors. However, it is possible that changes in oral-dental and gut microbiota may contribute to obesity by increasing circulating LPS to produce sustained chronic low-grade inflammation [through IL-6 upregulation], resulting in increased lipogenesis and insulin resistance. This mechanism has also been postulated for the association of periodontal disease and atherosclerotic heart disease [78]. Although animal experiments do support a role of adenovirus 36 in obesity the relationships in humans only seem promising in children. The interactions of these various microbes and potential pathogenic mechanisms in human obesity are summarized in Fig. 3.1. Although microbial pathogenesis may play a role in overweight and obesity it is almost certainly not the main driver of the global obesity pandemic.

3.8.1 Future Directions

It is quite evident that larger population-based studies are needed to define the role of microbes in the pathogenesis of obesity. Large prospective observational studies on cohorts matched for known risk factors for obesity including diet and physical activity are needed. Future studies should also address the issue of multiple microbial influence on the same groups of subjects, such as analysis of gut and gingival microbiota at the same time.

Large randomized trials should be performed in conjunction with counseling on diet and physical exercise on the value of prebiotics and probiotics to optimize the bowel microbiota along with the measurements of fecal and blood short-chain fatty acids. With respect to the role of adenovirus 36, larger population-based longitudinal studies over several years are needed to confirm the role of this virus in childhood obesity. Preliminary studies, however, should also assess the detection of the virus by PCR intermittently over time from the nasopharynx and feces, as sero-prevalence data alone are not very convincing.

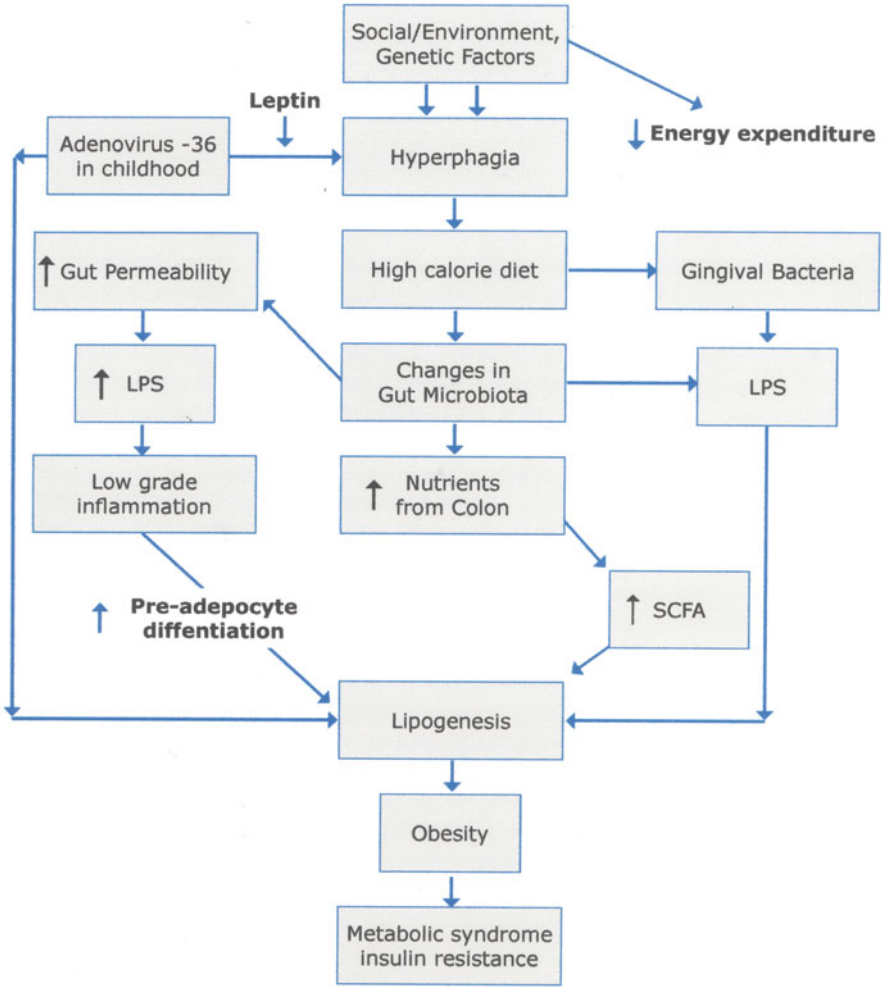

Fig. 3.1 Pathogenic mechanisms in obesity and microbes

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Chapter 4
Microbes in the Pathogenesis of Diabetes Mellitus

4.1 Introduction

Diabetes Mellitus [DM], now considered a group of metabolic disorders rather than a single disease entity, was first described in India about 600 BC [1]. Up till the 1960s, diabetes was still considered a relatively uncommon disease occurring predominantly in developed nations [1]. In 2007 it was estimated that diabetes occurred in over 25 million people or more than 7 % of the population in the United States [US], with a rate of about 1 million new cases every year [2]. A recent estimate by the WHO indicates that greater than 347 million people have diabetes worldwide [3]. The number of people with diabetes more than doubled since 1980 globally, from 153,000,000 to 347,000,000 in 2008 [4]. The diabetes pandemic [largely from type II DM] may be driven by the obesity pandemic and the increasing aging population. The largest increase in prevalence of diabetes in the past three decades was found in Oceania [islands of the central and South Pacific], followed by South Asia, Latin America and the Caribbean, central Asia, North Africa, and the Middle East [4]. The prevalence of diabetes globally in adults >25 years was estimated to be about 10 %.

The complications of diabetes result in substantial morbidity and mortality, with an estimate of 3.4 million people died worldwide from diabetes in 2010, with more than 80 % occurring in low- and middle-income countries [3]. Diabetes increases the risk of heart disease and stroke and 50 % of diabetic patients die of cardiovascular disease and stroke [5]. DM is an important cause of blindness and is among the leading causes of kidney failure, peripheral vascular disease, and peripheral neuropathy, which frequently leads to chronic ulcers, foot infections, and eventually lower limb amputations. Lower limb amputations are ten times more common in people with diabetes than nondiabetic subjects [6].

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4.2 Pathogenesis of Insulin-Dependent Diabetes Type 1

Insulin-dependent diabetes mellitus [IDDM] 1 is considered a chronic autoimmune disease causing destruction of the insulin producing pancreatic islet beta cells, resulting in insulin deficiency. Although it occurs in all races of people it occurs most frequently from people of Northern European descent. The pathogenesis of IDDM-1 is quite different from that of DM2, in which both decrease insulin release [not on an autoimmune basis] and insulin resistance are important factors in the latter [7]. Although inherited susceptibility and environmental factors play pivotal roles in both diseases, genome-wide association studies indicate that type I and type II DM genetic loci do not overlap, although interleukin-1 [IL-1] mediated inflammation may play a role in both conditions [8]. The main gene associated with IDDM predisposition is on the major histocompatibility complex [MHC] loci on chromosome 6, in the region of the HLA immune-recognition molecules [9]. Polymorphisms of multiple genes influence the risk of IDDM [HLA-Dq-α, HLA-Dq-β, HLA-DR, preproinsulin, PTP n 22 gene, and CT2A-4], and additional genes and loci recognized by whole genome analysis, such as KIAA0035 encoding a lectin [7]. Although genes in both MHC and elsewhere in the genome influence the risk of IDDM only the HLA alleles have a major influence.

There are a number of autoantigens within the pancreatic islet cells associated with IDDM such as pancreatic sialoglycoconjugate, proinsulin, insulin, glutamic acid decarboxylase, insulinoma-associated protein 2, zinc transporter of islet beta cells, beta-cell sulfatides, 37-kDa and 52-kDa islet cell antigens [7, 9]. Although autoantibodies to the enzyme glutamate decarboxylase may predict the disease, the presence of autoantibodies reactive to the 37-kDa antigen is a better predictor of IDDM [9]. Islet cell cytoplasmic autoantibodies and insulin/proinsulin autoantibodies appear years before the onset of hyperglycemia. Islet cell cytoplasmic autoantibodies are found in 70–80 % of newly diagnosed IDDM patients and only 0.5 % of normal controls, and insulin autoantibodies are present in about 50 % of newly diagnosed IDDM subjects before any insulin treatment [9].

The initial pathogenic event leading to IDDM in susceptible host has been proposed to involve one of two processes, both ultimately resulting in autoimmune reactive damage to the pancreatic islet beta cells [9]. The first event could be a viral coxsackie infection with viral proteins that shares amino acid sequence with a beta cell protein [i.e., molecular mimicry between coxsackie viral protein and glutamate decarboxylase] that result in influx of cytotoxic CD8-lymphocytes. Alternatively, an infection or environmental insult could produce inflammation of pancreatic islet cells with generation of inflammatory cytokines, activation of endothelial cells and adhesion molecules, with increased leukocyte infiltration, further release and exposure of the islet cell antigens to macrophages and lymphocytes then perpetrate a chronic autoimmune reaction [9].

4.3 Microbes in IDDM-1 Pathogenesis

The concept that a microbial pathogen can play a role in the etiology of IDDM, especially in childhood, has been proposed for several decades. Much of the data to support the hypothesis has been generated in animal models, but human studies have been conflicting. Interest in this area has waxed and waned over the years, but renewed interest has appeared in the medical literature of recent. There are now three different theories that have been proposed by which microbial pathogenesis may influence the development of IDDM-1. The first and foremost is the paradigm of childhood viral infection inducing pancreatic islet cell inflammation and subsequent chronic low-grade autoimmune reaction. Second, the completely opposite term the hygiene hypothesis that relates to improved sanitation and decreased exposure to microbial pathogens in early life may lead to increased risk of allergic and autoimmune disorders in developed nations, including autoimmune diabetes [10]. Third, in more recent years it has been proposed that specific patterns of gut microbiota can interact with the innate immune system to influence the development of IDDM in genetically susceptible individuals.

4.3.1 Infection as an Etiological Factor in IDDM-1

The concept that IDDM may be of an infectious etiology was first proposed in 1927, when it was reported that cyclical peaks of diabetes incidence were preceded by previous outbreaks of mumps [11]. Subsequently, IDDM was reported in children after rubella and coxsackie virus infections [12, 13], and coxsackie virus was subsequently isolated from a child with acute onset diabetic ketoacidosis and the virus was shown to induce diabetes in mice [14]. The hypothesis of an environmental external trigger, such as a virus infection inducing diabetes, was consistent with the fact that hereditability alone could not fully explain the concordance rate of 50 % in human monozygotic twins with IDDM [15]. Human studies also indicated that there was a long prediabetic period in most cases of IDDM-1, and this differs from the acute viral-induced DM in earlier experimental models [16]. In previous animal experiments with pancreatic islet beta cell tropic viruses, i.e., encephalomyocarditis virus, mengovirus, and coxsackie virus, there was direct beta cell lysis and rapid induction of DM without evidence of autoimmunity [17–19].

A low grade persistent infection in the pancreas would be more consistent with a prolonged human prodromal period in IDDM. A few experimental studies, however, have shown persistent infection in beta cells of mice with reovirus1 and rubella virus with the development of autoimmune insulitis [20, 21]. Autoimmune insulitis in humans may involve the overexpression of MHC class I molecules by beta cells [22]. In vitro infection of human and rat beta cells with reovirus has demonstrated

upregulation of MHC class I molecules' expression on cell surfaces [23]. Although the glucose intolerance in the mice model of reovirus-induced diabetes appears to be immune related, there was no overt diabetes but only transient glucose intolerance [20, 24]. The most persuasive animal experiments of viral-induced autoimmune DM were demonstrated with inoculation of DR BB rat substrain with Kilham's rat virus, which reproducibly produced insulitis and diabetes [25]. The DM was caused by self-reactive inflammatory cells that appeared in the pancreatic islets and not by viral cell lysis or immune reaction to viral antigens bound to cell membrane [26].

4.3.2 Specific Viruses in IDDM1

Coxsackie B virus had been most extensively investigated for its role in the etiology of IDDM. A review of this topic in 1996 by Yoon and Kominek [27] summarized the results of seroepidemiological studies with the correlation between a recent coxsackie B virus infection [<3 months] and the onset of IDDM. Sixteen of the studies showed a positive correlation between coxsackie B virus infection and IDDM but seven studies found no or reverse correlation. Some studies reported genetic susceptibility to IDDM and antibodies to coxsackie B virus as being linked to HLA haplotypes, but no consistent pattern was found although HLA–DR 3 and HLA–DR 4 haplotypes were more commonly associated [27].

Coxsackie B virus has been isolated or the antigen detected from the pancreatic islet beta cells of two children with recent onset IDDM [14, 28]. The potential of coxsackie B virus to produce DM in mice varies with the strain of mice, genetic background, and the strain of virus tested [27]. In one study using 37 coxsackie B isolates only 25 % of mice exhibited abnormal glucose tolerance when sequentially infected with coxsackie B3, B4, and B5 viruses [29]. Thus the diabetogenic potential of coxsackie B virus strains in nature may be limited. Although several studies demonstrated that coxsackie B virus can induce beta islet cell necrosis and lysis in some mice models, others found the virus can initiate or enhance an autoimmune reaction to glutamic acid decarboxylase [29–31]. An indirect autoimmune damage to beta cells in a coxsackie virus-mediated DM has been described more recently [32]. However, the diabetogenic potential of coxsackie B virus depends on an inadequate antiviral defense or can be triggered by cytomegalovirus infection [33].

Production of DM in nonhuman primates with coxsackie B4 virus has been attained in pasta monkeys but not in others such as rhesus, cynomolgus, and cebrus [34]. These studies suggest that genetic factors are critical for glucose homeostasis in monkeys infected with coxsackie virus, and that cumulative insults to the beta cells are important for development of DM.

Studies of other viruses as possible triggers for IDDM are less robust and include cytomegalovirus [CMV], rotavirus, rubella, and mumps [35]. Although there have been limited reports of association with CMV infection and autoimmune DM [36, 37], others have failed to confirm this association [35]. However, animal models do

support the concept that CMV infection could trigger IDDM by activation of macrophages and proliferation of autoreactive cells, or through enhancement of islet cells autoantibodies [38, 39].

4.4 The Hygiene Theory of IDDM

Paradoxically microbial agents which can induce autoimmune disease experimentally and clinically can also suppress autoimmune and allergic diseases in certain settings [10]. Epidemiological studies in developed countries have shown an increasing trend of many allergic diseases, including IDDM, asthma, etc., in the past 60 years which has been paralleled with an increase in sanitation and decrease in incidence of infections. The decreased risk of exposure to microbial pathogens is especially striking for intestinal organisms including parasites. This is attributable to multiple factors including access to clean water, modern sanitation, vaccination, and possible antimicrobial agents.

There is a marked discrepancy in the distribution and incidence of many autoimmune and allergic diseases geographically in the world, with greater incidence in temperate developed countries compared to less developed nations in tropical and subtropical continents. This North–South gradient typified by the higher incidence of multiple sclerosis and IDDM in Europe compared to Africa [10] could be explained by climatic differences and exposure to sun, genetic, and environmental factors, including exposure to microbes. Interaction of genetic and environmental factors is considered the key components in the pathogenesis of these allergic and autoimmune diseases, but the exact mechanisms are not fully understood.

There is epidemiological evidence even within countries of the northern hemisphere that the exposure risk to microbial pathogens in childhood can influence the development of IDDM. A case-controlled study in Yorkshire [England] found a correlation with type I DM and the attendance at day care centers, and number of infections before 1 year of age [40]. IDDM was lower in children attending day care centers with more frequent infections than children not attending day care centers and not exposed to older siblings [who presumably increase the risk for infections].

The best evidence to support the hygiene hypothesis and development of autoimmune DM is derived from animal models. In an experimental rodent model using BB rats or nonobese diabetic [NOD] mice, caesarian delivery and isolation against microbial pathogens increases the incidence of DM from 40 to 80 % [10, 41]. Diabetes is also prevented in NOD mice by infecting young mice with various microbes including mycobacteria, viruses, and parasites [42–47]. The findings that BCG vaccination and lymphocytes from infected animals can prevent IDDM in uninfected mice implicate T-cells in the mechanism of DM type I [48, 49].

The protective effect of infectious agents against IDDM has been proposed to involve the following mechanisms: 1) homeostatic competition, infection induced lymphocytes competes with other lymphocytes involved in autoimmune pathogenesis; 2) and bystander suppression, immune response to infection suppresses the autoimmune process [35]. In the murine model protection against IDDM by bacterial

components involves Th2 cytokines within the islets [50], and this can be abrogated by antibodies against IL-4 and IL-10 [51]. However, the protective effect is more complex as mycobacteria can protective effect is more complex as mycobacteria can protect against DM even in IL-4/IL-10 deficient mice [52]. The bystander suppressive effect of infections are likely mediated by IL-10, transforming growth factor β (TGF-β) and other factors produced by regulatory T-cells, that inhibit Th1 and Th2 responses [53].

Toll-like receptors [TLRs], which are receptors for various microbes, are important in the immune response to infectious agents and immune stimulation of mononuclear cells via TLR could downregulate allergic and autoimmune reactions [10]. Thus, TLRs are believed to contribute to the protective effect of infection on the development of DM [35].

4.5 Intestinal Microbiota in the Development of IDDM

There is recent evidence that interaction between the gut microbiota and the host innate immunity may contribute to development of IDDM. The hygiene hypothesis of diabetes and the role of the gut microbiota in the pathogenesis of IDDM appear to be closely linked. There is increasing evidence to support the concept that changes in exposure to certain infectious agents and the composition or pattern of the intestinal microbiota in the early years of life can influence the development of IDDM [10].

In NOD mice kept under specific pathogen-free [SPF] conditions the development of spontaneous DM was accelerated compared to conventionally housed NOD mice [54]. There is also cumulative evidence that the gut immune system plays a part in the pathogenesis of IDDM, and the development of the immune system largely depends on the interaction with the bowel microbiota. Based on rodent models and to a lesser degree in humans, studies indicate that the pathogenesis of IDDM involves a complex interplay between aberrant gut microbiota, "leaky" intestinal mucosa barrier, and altered immune responsiveness [55]. In biobreeding diabetes-prone [BB–DP] rats, differences in bacterial composition of the gut microflora were observed in rats that eventually develop DM compared to those that did not [56]. This difference was observed long before development of overt IDDM. Rats resistant to development of IDDM at a later age showed lower amounts of *Bacteroides* species in the bowel flora. Moreover altering the intestinal microbiota with antibiotic decreased the development and delayed the onset of DM [56]. Other investigators have also found that lactobacillus species were negatively correlated with type I DM development in rats, and that administration of *Lactobacillus johnsonii*, isolated from BB-diabetes resistant rats, inhibits the onset of IDDM in the diabetic-prone rats [BB–DP] [57]. Therefore, supporting the concept that the gut microbiota is important in the pathogenesis of IDDM.

The innate immunity is critical in the development of the host defense and the intestinal homeostasis [58]. Thus it is postulated that certain innate immunity signaling pathways in the intestine might account for the role of the gut microbiota in the pathogenesis of IDDM. Recent experiments in NOD mice with or without

the myeloid differentiation primary response gene 88 [My D88] elucidated some important mechanisms [58]. MyD88 is a key intracellular component of multiple TLR-mediated signaling pathways [except for TLR 3]. In their elegant experiments, Wen et al. [59] discovered some important findings: (1) genetic alterations of MyD88 protected against IDDM; (2) MyD88 deficient NOD mice showed decreased reactivity to diabetes-associated peptide and decreased interferon-γ [IFN-γ] by pancreatic lymph nodes [PLN]-derived lymphocytes compared to wild-type animals; (3) transfer of CD4+ T-cells harboring a diabetogenic TCR-receptor BD 2.5 failed to proliferate in the PLN of MyD88 deficient mice, compared to the MyD88 competent NOD animals; (4) this was not a systemic dysfunction of autoimmune T cells as no differences were observed in T cells from the spleen or mesenteric lymph nodes; (5) and genetic ablation of single TLR [TLR 2, 3 or 4] was not sufficient to attenuate the incidence or progression of IDDM in NOD mice. Hence, it was considered that abnormal sensing of certain commensal microbes at the gut barrier may play a role in the development of clinical DM through the host failure to prevent development of autoimmune T cells. The investigators then designed further experiments to determine whether the resistance to IDDM in MyD88 deficient mice was related to certain microbiota-derived signals [59]. It was found that the absence of MyD88 was linked to a change in bowel flora pattern, in the ratio of *Firmicutes*/*Bacteroidetes* mainly due to increase in load of *Lactobacillaceae*, a *Firmicute*, and that antibiotic treatment could normalize the ratio. Furthermore, the microbiota of MyDD88 deficient mice could protect germ-free NOD mice from IDDM. These experiments indicate that host recognition of the gut microbiota is essential in the prevention and progression of IDDM through interaction of a MyD88-independent signaling pathway yet to be discovered.

In more recent studies in BB-DP and diabetes-resistant [BB-DR] rats, it was found that IDDM induction could be prevented or circumvented by gut flora-mediated Th17 differentiation, by feeding the probiotic *L. johnsonii* [60]. Previously Roesch et al. [61] had shown that feces of BB–DR rats contained much higher populations of probiotic-like bacteria such as *Lactobacillus* and *Bifidobacterium*, and BB–DP rats had greater numbers of *Bacteroides*, *Eubacterium*, and *Ruminococcus* [61]. However, the differences in pattern of bowel microbiota between diabetes-prone versus diabetes-resistant rats are more complex, as a total of 24 bacterial species, and hundreds of bacterial taxa, not classified to genus level, were found to differ in abundance.

Studies on human gut microbiota and development of IDDM are very limited. In a small study from Finland, stool samples were collected every 3 months from eight children, four with development of autoimmune IDDM and four healthy matched control children with similar age and HLA genotype [62]. Three stool samples were collected at different times, the first sample at 4–8 months of age before development of autoantibodies, and the third sample within a few months of development of two autoantibodies [all four cases], and eventually IDDM with time. Feces were analyzed by high-throughput, culture independent methods to identify bacteria that correlated with the development of the disorder. The two main indicators of development of autoimmune diabetes were the instability of the autoimmune microbiome and a high ratio of *Firmicutes* to *Bacteroidetes* in cases

[observed in the first 6 months after birth] compared with a low ratio in controls [62]. These preliminary results suggest that there is an autoimmune microbiome for IDDM, which tend to have more classified members, i.e., *Bacteroides ovatus*, which represented 24 % of the total increase in the phylum *Bacteroidetes* in DM, but decreased diversity and reduced stability when compared with the healthy microbiome. Studies in murine models of autoimmune DM also suggest that host differences in the capacity to sense intestinal microbes and specific composition of the gut microbiota modulate susceptibility to IDDM [59].

4.6 Other Microbes Implicated in IDDM-1

Other microbial exposures in early life implicated as possible triggers for autoimmune DM, especially in Europe, include *Mycobacterium avium* subspecies paratuberculosis [MAP], presumably from dairy milk ingestion as the organism can survive pasteurization [63]. There is some recent evidence that antibodies recognizing MAP epitopes cross-react with pancreatic islet beta cell antigen ZnT8 in type 1 diabetes patients [64]. There have been a few small case-controlled studies from Italy that found greater prevalence of antibodies or DNA by PCR of MAP in pediatric and adult IDDM but not in type II DM [64–66]. Specific immunoassay was also used to confirm the association of MAP with type I DM [47.3 %], but not type II DM [7.7 %] compared to healthy controls [12.6 %] [67]. Polymorphism of the gene encoding the membrane transporter [S LC11A1] that is expressed in endosomes of antigen presenting cells, implicated in the immunopathogenesis of IDDM, has also been found to be associated with the presence of MAP DNA in blood and type I DM [68].

4.7 Type 2 Diabetes and Microbial Pathogenesis

Type 2 diabetes is the predominant form of DM accounting for at least 90 % of cases and largely responsible for the global pandemic of DM. The rise in prevalence is predicted to be much greater in developing than developed countries by 2030, 69 % versus 20 % [69]. The increase in incidence of type II DM is linked to lifestyle changes, resulting in the rise in overweight and obese populations [70]. The main pathogenic mechanism of type II DM is the chronic excess of calories/energy with overweight and obesity, insufficient supply of insulin by islet beta cells, and insulin resistance in genetically susceptible individuals [71, 72]. It is estimated that hereditability of type II DM is greater than 50 %, and the greatest risks are in subjects with at least two affected siblings irrespective of the parental status [72]. Islet beta-cell deficiency or dysfunction mechanisms are complex and varied and include loss of beta-cell mass, islet amyloid polypeptide deposit, and glucotoxic/glucolipotoxic factors associated with hyperglycemia further accelerating pancreatic islet beta-cell failure [71, 72].

The potential role of microbial pathogenesis in type II DM would largely be similar to the mechanisms in obesity as previously described in Chap. 3. Gastrointestinal microbes could enhance fuel consumption and excess storage with chronic stress on pancreatic islet cells leading to secondary insulin deficiency. Moreover, raised concentrations of inflammatory cytokines and nonesterified fatty acids, due to decreased secretion of adiponectin and or bowel microbes, can lead to insulin resistance. Hence gut microbiota of susceptible host may influence the development of type II DM by increasing energy harvest from diet, changes in the host gene expression, energy expenditure and storage, and alteration in intestinal epithelial permeability with increased chronic low-grade endotoxemia, inflammation, and insulin resistance [73]. In the mice model gut microbiota modulation with antibiotic improved glucose tolerance in both diet-induced obese and insulin-resistant diabetes mice [74]. This improvement in glucose tolerance was associated with reduction in plasma LPS and increase in adiponectin. Therefore it supported the proposal that modulation of gut microbiota alters the expression of intestinal and hepatic genes involved in inflammation and metabolism and changes the hormonal, inflammatory, and metabolic status of the host [74]. This concept is further supported by recent findings in obese diabetic patients compared to obese healthy volunteers, and in obese mice, where there is elevation of IgG levels against specific bacterial antigens [75]. In addition, in a prospective longitudinal study of 3,280 participants without diabetes or obesity at baseline, 16Sr DNA of bacterial gene was measured at baseline and its relationship with incident diabetes and obesity over 9 years was assessed [76]. Bacterial 16Sr DNA was shown to be an independent marker of DM and supports the theory that bacteria are involved in the onset of type II DM in humans.

Limited studies on the gut microbiota and type II DM patients, not included in larger cohorts of obese subjects, have been performed on a few individuals. These studies used quantitative PCR to assess bacterial DNAs or 16Sr RNAs. In one study of fecal bacteria composition from 36 male adults, 18 with type II DM, the proportions of phylum *Firmicutes* and class *Clostridia* were significantly reduced in the diabetic group compared to the control group, $p=0.03$ [77]. In addition, the ratio of Bacteroidetes to Firmicutes, as well as the ratio of *Bacteroides–prevotella* group to *C. cocoides–E. rectale* group correlated positively and significantly with plasma glucose concentration [$p=0.04$], but not with body mass index. The class *Betaproteobacteria* was also highly enriched in diabetic compared to nondiabetic subjects [$p=0.02$] and positively correlated with plasma glucose, $p=0.04$ [77].

In another similar study of 16 type II diabetic patients and 12 healthy controls, the species diversity profiles were not significantly different [78]. However, sequencing results showed that the bacterial composition of the feces in the diabetic group was different from the healthy group. *Bacteroides vulgatus* and *Bifidobacterium* were lowly represented in the microbiota of the diabetic patients, and a decrease in *Bifidobacterium* was significant [78]. Together these two studies suggest that the gut microbiota of type II DM have some changes with the occurrence and development of diabetes.

4.7.1 Other Microbes Linked to Type 2 Diabetes

Few studies have assessed the association of type 2 diabetes and the metabolic syndrome with *Helicobacter pylori* infection. In a large cross-sectional national survey, The Third National Health and Nutrition Examination Survey [NHANES 111], there were no consistent associations of *H. pylori* infection with diabetes prevalence or insulin resistance in American men age 40–74 years [79]. However, in diabetic men *H. pylori* infection was associated with the prevalence of coronary heart disease. However, in a more recent prospective study of senior Latino subjects, >60 years and diabetes free at the onset, 782 individuals were followed for 10 years and development of incident diabetes type 2 was correlated with antibodies to various bacterial, viral, and parasitic microorganisms [80]. Only subjects who were seropositive to *H. pylori* at enrollment were 2.7 times more likely to develop diabetes than seronegative individuals, hazard ratio 2.69 [95 % confidence interval 1.10–6.60]. Subsequent cross-sectional analyses using data from 7,417 participants in the NHANES 111 [>18 years] found significant association with *H. pylori* seropositivity and glycated hemoglobin [Hb A1c] levels after controlling for confounding factors, $p<0.01$ [81]. In this study there was synergistic interaction between *H. pylori* infection and higher BMI with increased levels of Hb A1c.

4.8 Summary

There is good biological and plausible data, largely supported by animal experiments in rodents, that microbes play a role in both type I and type 2 diabetes pathogenesis. However, this is not yet proven in humans and more studies are needed to establish a microbial pathobiological role in DM. The cumulative evidence does suggest that some viruses, especially coxsackie B virus, may predispose to type I DM in genetically susceptible children, and that certain admixture of gut microbiota can influence the risk for IDDM by altering the innate immune response of the host.

The role of the gut microbiota on type II DM is through a somewhat different mechanism, but is likely driven by increased energy harvest and storage as proposed for obesity. However, there is also supporting evidence for increased inflammatory cytokines, possibly through "leaky" intestinal barrier, mediated by the host response to gut microbiota causing insulin resistance.

4.9 Future Directions

Large-scale studies need to be performed in early childhood in children with strong family history of IDDM prospectively for several years [7–10 years], randomized to probiotic/prebiotic, included in yogurt as part of the daily diet that will influence the bowel flora to a nonautoimmune microbiota compared to standard diet.

Similarly genetically susceptible individuals for type II DM could be randomized to a similar form of daily probiotic therapy, to enhance *Bifidobacterium* and possibly *B. vulgatus* concentration in the gut microflora, compared to standard diet for several years in middle-aged adults. The main difficulty in interpretation of a trial of this nature will be to differentiate the beneficial effect of the probiotics as being due to antidiabetic effect from an antiobesity effect, particularly if there is significant weight loss in the intervention group.

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Chapter 5
Asthma and Microbes: A New Paradigm

5.1 Introduction

Asthma is a heterogeneous disorder with interaction between genetic predisposition, atopy, and environmental factors, including environmental allergens, air pollution, and respiratory infections. The current definition of asthma combines clinical–pathological presence of chronic inflammatory airway with hyperresponsiveness and episodic airway obstruction with variable degrees of reversibility with treatment or spontaneously [1]. Asthma is worldwide and is of pandemic levels for the past 30 years, with >300 million people afflicted globally [2]. Although asthma is considered more common in affluent and developed countries, approximately half are in developing countries [3], which account for more than two-thirds of the world's population. It is estimated that asthma accounts for 25,000 annual deaths, and that by 2025 the number of asthmatics will increase by more than 100 million new cases [2, 4]. In the United States 8.4 % of the population has asthma with substantial annual morbidity of 500,000 hospitalization and 1.9 million emergency visits, at an annual cost of $56 billion [5].

5.2 Pathogenesis of Asthma

Traditionally asthma has been considered a condition as a result of a complex interaction between multiple genetic influences and environmental stimuli. Studies of twins in families of subjects with asthma show an inheritable pattern but the current data indicate that asthma is likely transmitted by multiple genes [6]. Different genes may lead to the same phenotype in separate individuals [locus heterogeneity], and multiple genes acting in the same individual [polygenic inheritance] may lead in the expression of the asthma phenotype [6]. Some genes influence the development of asthma and others may influence the severity of the disease or responses to treatment. Genome-wide association studies have recently identified the number of genes that are

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important in the development of asthma. Two key genes, IL-33 and IL-1 receptor-like 1 [IL1RL1], act in this signal transduction pathway [7]. IL-33 encodes a cytokine released on damages cells and IL1RL1 encodes part of the receptor complex. Functional studies in humans and mouse models of allergic airway disease indicate a key role of IL-33 signaling in driving the Th2 inflammation, which is pivotal in allergic asthma [7]. The IL-33/IL1RL1 pathway can activate innate immune cells to produce cytokines such as IL-5 and IL-13 in the lungs [8, 19].

In a previous review of asthma genetics in 2006 [10], there were 118 genes implicated or associated with asthma or atopy related phenotypes, 25 genes were considered true susceptibility genes, but there were 10 elite groups of genes associated with asthma or atopy in more than 10 studies. This elite group of genes, include those encoding IL-4, IL-13, ADRB2, TNF, HLA–DRB, FCER1B, IL4RA, CD14, HLA-DQB1, and ADAM 33 [10]. Asthma susceptibility genes may be classified according to four main functions: Innate immunity and immunoregulation [e.g., CD14, HLA genes, and TLR4]; Th2-cell differentiation/activity [e.g., IL4/IL4R, IL 13, and FCER1B]; epithelial biology and mucosal immunity [e.g., CCL genes and FLG]; and lung function, airway remodeling, and asthma severity [e.g., ADRB2 and TNF] [11, 12]. IL-13 has been one of the best studied candidate gene for asthma and allergy, and has been consistently implicated in genome-wide based studies [13, 14].

The pathogenesis of asthma may also involve epigenetics, chemical reactions that may switch parts of the genome on and off, and thus may be one of the mechanisms for interaction of the genome with the environment [6]. For example, environmental factors may cause changes in gene expression through noncoding changes to the DNA, i.e., DNA methylation [15]. There are several observations supporting a pathogenic role of epigenetics, including the complexity of gene–environment interactions. The concordance rate of only 50 % in monozygotic asthmatic twins supports nongenetic factors [16]. Common epigenetic mechanisms, such as prenatal exposure to tobacco smoke or air pollutants altering DNA methylation, may result in increased or decreased gene expressions [121]. For example, expression of ADAM 33 in bronchial epithelial and fibroblasts cells has been shown to be controlled by epigenetic mechanisms [12]. Currently epigenetic analyses in respiratory diseases have been limited to certain candidate genes, i.e., FOXP3 involved in regulation of T-cell function and no large-scale studies have been published [17].

5.2.1 Pathological Aspects of Asthma

The hallmark of asthma from a pathological perspective is the presence of inflammation with smooth muscle contraction and largely reversible airway obstruction. The inflammation and exaggerated airway responsiveness is associated with mucus hypersecretion, often with increased eosinophils locally and systemically. At the cellular level the changes consist of airway remodeling characterized by smooth muscle hyperplasia, subepithelial cell fibrosis, goblet cell hyperplasia, and neovascularization [18]. The airway inflammation which is a major component of

asthma involves influx of mast cells, neutrophils, Th-2 cells, and eosinophils. On exposure to allergen or infectious agent the physiological changes that occur are driven by Th-2 inflammation, with increased Th-2 cytokines [IL-4, IL-5, IL-9, and IL-13], and decreased anti-inflammatory cytokines, i.e., IL-10 [18]. The heightened Th-2 response causes activation of natural killer cells, dendritic cells and eosinophils, which result in eosinophilia, increased matrix metalloprotease activity, increase in serum IgE levels, and promotion of smooth muscle hypercontraction. IL-13 is central to the progression of asthma and is produced in response to toll-like receptor-4 [TLR-4] signaling, and many micro-RNAs are directly or indirectly involved in the production and upregulation [18].

Asthma may be classified as two forms, based on history and skin test reactivity to common inhaled allergens, atopic asthma in over 80 % of cases and nonatopic or intrinsic asthma in about 10 % [19]. Nonatopic asthma is usually late or adult onset with normal serum IgE levels, and more commonly associated with nasal polyps and aspirin sensitivity. However, there is data to suggest that IgE-mediated mechanism in the airway is involved and staphylococcal enterotoxins have been implicated [19]. The nonatopic form of asthma may be the commonest form in children of developing countries and has been related to exposures to environmental dirt, bacterial infection, and psychosocial distress of poverty [20].

5.3 Infection and Asthma

Microbes have been recognized to be important in the exacerbation of asthma with precipitations of severe attacks for many decades. The pathogenic mechanisms could be either from respiratory infections worsening bronchial inflammation or allergic reaction to environmental molds as in allergic bronchopulmonary aspergillosis. However, in recent years there has been cumulative evidence that repeated lower respiratory viral infections in early childhood may be responsible for later development of asthma. Conversely, lack of environmental exposures to common microbes in early life may be responsible for the asthma epidemic in developed countries, as part of the "hygiene hypothesis" of allergic diseases.

5.3.1 Asthma Exacerbations and Infection

Respiratory viruses account for 50–60 % of asthma exacerbations in all age groups [21, 22]. Rhinoviruses [RV], common cause of the "common cold," are the most frequent culprit and the genotypes C, [RV–C] may cause more severe exacerbation than other respiratory viruses [23]. Although RV–C infections are associated with asthma, recurrent wheezing, and bronchiolitis in children admitted to hospital with respiratory tract infection, no clinical differences were found from the RV–A genotype in one study [24]. In temperate regions infections with RV or other respiratory

viruses are associated with increased emergency room admissions for asthma exacerbations, and "asthma epidemics" have been associated with children returning to school in September [21]. In animal models RVs have been shown to exacerbate the airway inflammation induced by airway allergen challenge [25], and this is likely similar for other respiratory viruses as well.

Bacterial infections were until recently not considered important in the pathogenesis of asthma. However, there is evidence that asthmatics have increased susceptibility to bacterial respiratory infections. In children pneumococcal carriage is more common in asthmatics than nonasthmatics, and adult asthmatics have increased risk of invasive pneumococcal infections [26, 27]. There is also increasing evidence that *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* may play a role in promoting airway inflammation that could contribute to the onset and course of asthma [28]. Neonates colonized in the hypopharynx with *Streptococcus pneumoniae* and *Haemophilus influenzae* and *Moraxella catarrhalis* are at increased risk for recurrent wheeze and asthma in early life [29].

Prospective studies in Europe have also found increased frequency of *C. pneumoniae* detection by RT-PCR from asthmatics compared to their normal spouses from October to December, 22 % versus 9 % [30]. Bronchial lavages of children with asthma were also found to have slightly greater prevalence of *C. pneumoniae* [40 % by PCR and 20 % by cultures] compared to nonasthmatics with various respiratory disorders [35.7 %]. However, blood culture positivity for *C. pneumoniae* from asthmatics [40.5 %], other respiratory disorders [29 %] versus matched nonrespiratory controls [11 %] was significantly greater, $p < 0.01$ [31].

The role of viruses and atypical bacteria in exacerbation of asthma for hospitalized children was prospectively evaluated in Europe [France] over a 9-month period in 15 hospitals. Viruses were detected in 38 % [enterovirus 15.8 %, RV 12 %, and respiratory syncytial virus [RSV] in 7 %], and atypical bacteria in 10 % [32]. Persistent clinical symptoms were more frequently associated with atypical bacterial infections. A similar prospective study was performed in the Southern hemisphere [Argentina] over a year [33]. Two hundred nine patients were assessed and a potential causative agent was detected in 78 % of the children. The most frequently detected viruses were RSV [40 %] and RV [24.5 %], with *M. pneumoniae* and *C. pneumoniae* in only 4.5 % and 2 % of the cases, respectively [33]. The impact of acute respiratory infections with viruses and atypical bacteria, on severity and resolution of symptoms, in children with nonhospitalized exacerbation of asthma was also assessed in Australia [34]. Respiratory viruses were detected in 54 % of 78 nasopharyngeal aspirates but there was no difference in clinical outcome between those with or without proven infection.

Currently the overall data from multiples studies indicate that viruses are much more important and frequent than atypical bacteria in precipitating exacerbations of asthma. There is no good evidence that this is related to any specific respiratory virus. The relative frequency of the various respiratory viruses reported may vary according to age group, season, and geography, as well as methods of detection. A large diversity of viruses have been found in the respiratory tracts of adults with and without asthma using the Virochip, a DNA microarray-based

detection method [35]. These include a set of 5 divergent isolates that formed a distinct genetic subgroup, besides >20 different serotypes of RVs and multiple serotypes of human coronaviruses.

The exact mechanism by which upper respiratory tract viral infection induces asthma attacks is not fully understood. In established asthma respiratory viral infections attract bronchial inflammatory cells, alter airway receptor expression on smooth muscle cells, and modulate neuroimmune mechanisms, leading to bronchospasm and exacerbation of disease [36]. Multiple mechanisms are probably involved including loss of function or downregulation of M2 muscarinic receptors on the airway parasympathetic nerves, which normally inhibit vagal-mediated bronchospasm [37]. Interferons produced in response to viral infections downregulate the expression of the M2 receptor gene, and eosinophils recruited by allergens release major basic proteins, which bind to the M2 receptors and block their function [37]. Thus a multitude of respiratory viruses that can cause upper and lower respiratory tract infections and a few atypical bacteria can cause exacerbation of asthma.

5.3.2 Viruses in Early Life as a Cause of Asthma

Lower respiratory viral infections commonly cause wheezing in young children <2 years of age, and there is increasing evidence that repeated viral bronchiolitis in early life predispose to asthma in later childhood. Development of new onset of asthma has been correlated with a variety of respiratory viruses, most commonly RSV bronchiolitis in infancy and RV infection in older children [39]. Wheezing illness in infancy is most commonly caused by marked RSV bronchiolitis [70 % of episodes], and less frequently with parainfluenza virus, influenza, and metapneumoviruses [39]. Although RSV infection is almost universal in young children only about 40 % develop clinically overt disease and some develop severe bronchiolitis with recurrent infection and wheezing. Risk factors for severe lower respiratory tract infection include younger age, small lungs size, passive smoke exposure, and virus-induced immune responses [39].

In the Tucson Children's Respiratory Study 880 children were followed from birth for over a decade, and development of lower respiratory tract infection in the first 3 years of life was later correlated with diagnoses of asthma or recurrent wheezing at ages 6 and 11 years [40]. Although RSV bronchiolitis increase the risk of frequent [>3 episodes per year] or infrequent [<3 episodes per year of wheezing], the risk gradually decreased by age 13. The results of this study may be explained by a more recent study assessing recurrent wheezing and development of asthma in a birth cohort followed for 13 years, and the relationship to perennial allergen exposure and sensitization [41]. Chronic asthma developed in atopic children in the first 3 years of life to repeated exposures to allergens, and determined by continuing allergic airway inflammation characterized by airway-hyperresponsiveness and impairment of lung function at a later age school age. Whereas children with nonatopic wheezing phenotype lose their symptoms over school age and retain normal

pulmonary function at a later age [41]. Other studies have suggested that RSV bronchiolitis predispose to recurrent wheezing and asthma in the genetically predisposed children [42, 43]. A large epidemiological study from Tennessee [USA] found that children born approximately 120 days before the peak RSV season were at the highest risk for hospital admission with wheezing, and at a higher risk for later development of asthma [44]. However, the association of severe RSV infection in infancy and later development of asthma overlapped with genetic determinants in a study of 8,280 twin-pairs [45]. The investigators of this study concluded that severe RSV infection may not be the cause of asthma, but there was a genetic predisposition to both RSV bronchiolitis and asthma. The best evidence to date in support of a causal association between RSV infection and asthma is the result of the long-term study over 18 years in Swedish children. Children who had RSV bronchiolitis as infants compared to matched controls had a greater prevalence of asthma at 18 years old, 39 % versus 9 % [46]. The affected children also demonstrated increase in perennial allergens sensitization [41 % versus 14 %] and recurrent episodes of wheezing, 30 % versus 1 %. In a previous study children with past bronchiolitis had an enhanced IL-4 response to RSV and cat allergen [Th-2 response], whereas controls had an equally strong interferon-gamma [IFN-γ] response [Th-1 type] to RSV antigens [47].

Controlled studies with prophylactic therapy with anti-RSV monoclonal antibody [palivizumab] in infancy also support a causal role of RSV in asthma. In one study of 191-palivizumab treated and 230 untreated premature babies, the rate of recurrent wheeze was 50 % lower at 24 months in the treated babies [48]. The investigators later showed that RSV prophylaxis treatment had a similar protection against recurrent wheeze in older children age 2–5 years. However, the protective effect of recurrent wheezing was primarily in children without a family history of asthma or atopy, and no effect on subsequent wheezing was demonstrated in 90 children with the family history of asthma or atopy [49].

Human rhinoviruses [RVs] are recognized as a common cause of the "common cold" and exacerbation of asthma, but these viruses can also cause lower respiratory tract disease leading to hospitalization in children with or without asthma [50]. There is increasing evidence that RV infections can lead to the development of asthma [50], and bronchial epithelial cells of asthmatics have a deficient innate immune response to RV infection [51]. In a recent prospective longitudinal study of 285 children with high risk for allergic diseases and asthma followed from birth, allergic sensitization leads to increased risk of RV-induced wheezing but not RSV infection up to 6 years [52]. However, viral wheeze did not lead to subsequent allergic sensitization. In an earlier study sensitization to common aeroallergens nearly doubled the risk for asthma at 6 years, and viral respiratory tract infection with wheezing quadrupled the risk for asthma over this time [53]. The combined effect appeared to be synergistic as the risk of asthma was ninefold greater with both wheezing episodes from respiratory infections and allergic sensitization. Recent evidence also suggests that RV infection induces bronchial epithelial production of a number of growth factors and other mediators [chemokines and cytokines] that could contribute to the development and progression of airway remodeling present in asthma [54].

5.3.3 *Mechanisms of Virus-Related Asthma*

A variety of mechanisms have been proposed to explain increased susceptibility of asthmatics for certain respiratory pathogens. These include impaired innate immunity in asthma and atopic diseases, including deficient epithelial cell function, mucus hypersecretion, decrease in interferon responses, and impaired alveolar macrophage function [55]. However, it is unclear how repeated viral bronchiolitis in early childhood could lead to asthma in later years.

Viral bronchitis or bronchiolitis precipitation of acute attacks of wheezing or exacerbation of asthma seems to involve local inflammation of the epithelial lining and enhanced airway bronchoconstriction. Experimentally induced viral infections in animals and human volunteers indicate that viruses can enhance airway hyperresponsiveness [56]. Virus-induced airway hyperresponsiveness is multifactorial and involves changes in neural control of the airways, impaired inactivation of tachykinins, and effects on nitric oxide production [57]. There is further evidence that atopic or allergic subjects with increased IgE and eosinophilic airway inflammation have greater risk of virus-induced wheezing than nonallergic individuals [58]. Although several studies suggest that there is an interaction between response to viral infection and aeroallergens in asthma the mechanisms are unclear. Viral infections could damage the epithelial airway barrier to enhance absorption of aeroallergens and thus enhance airway inflammation [59]. Generation of various cytokines and chemokines by viral infection may upregulate cellular recruitment to enhance allergen-induced inflammation and airway hyperresponsiveness. Experimental RV infection can enhance lower airway histamine responses and eosinophils recruitment to allergen challenge [60]. The possibility that allergic inflammation might intensify the host response to viral respiratory infections or impair viral clearance has been suggested by some studies but not confirmed by others [39].

The evidence that the strongest predictor of subsequent asthma is the presence of both atopy and severe lower viral respiratory tract infections in infancy suggests that there is a virus–allergen interaction at least in some asthmatics. This simply could imply that there is a common predisposition or susceptibility to both asthma and viral infections [61]. It has been hypothesized that severe, recurrent lower viral expiratory infections hinder proper lung growth and development at a very vulnerable age, leading to changes in the airway structure that promote asthma [62]. There is experimental evidence that RSV and RV in vivo and in vitro increase the synthesis of factors that can modulate lung growth, development, and repair [63, 64].

A central role in the pathogenesis of asthma is the allergen-specific IgE, the production of which is mediated by the Th-2 cytokines, IL-4 and IL-13 [38]. IgE receptors on mast cells and basophils bind to allergens, resulting in release of mediators such as histamine which produce airway inflammation and hyperreactivity. It has been proposed that the immune dysfunction in infancy could predispose to recurrent viral lower respiratory tract infection and be involved in the pathogenesis of asthma. In a community-based cohort of children, with a family history of atopy, relative deficiency of circulating plasmacytoid dendritic cells in infancy was correlated

with increased frequency and severity of viral respiratory infections, wheezing, and diagnoses of asthma [65]. The usual response to viral respiratory infections by a mature immune system is to stimulate proliferation of cytolytic T cells and the Th1-CD4 helper T cells. This results in release of IFN-γ, upregulation of IL-12, and stimulation of macrophages [66], typical for the Th1 response. However, there is experimental evidence that severe RSV infection in neonates could determine the pattern of T-cell-mediated disease during later life. Primary RSV infection in neonatal mice was associated with reduced and delayed IFN-γ responses, and subsequent reinfection resulted in increased inflammatory cell recruitment, with a shift to increased Th2-cytokine and increased eosinophils, typical for allergy and asthma [67]. A similar animal model also confirmed that viral respiratory infection in early life results in overproduction of the Th2-cytokines, IL-13, with increased risk for respiratory allergies, and changes in the airway structure conducive for asthma [68]. In clinical studies of acute exacerbation of asthma induced by viral infection, the peripheral blood mononuclear cells demonstrate increased expression of IFN-responsive genes and increased expression of Th2-chemokines, indicating enhanced allergic inflammation linked to the innate antiviral response [69, 70].

5.4 The Hygiene Hypothesis of Asthma

In 1989 Strahan first observed that the risk of allergic rhinitis [hay fever] was inversely linked to the size of the family and hygiene [71]. He proposed that increased infection in childhood had a protective role in allergic rhinitis and this concept was labeled the "hygiene hypothesis". However, it has since been noted by others that repeated viral lower respiratory tract infections in the first 3 years of life appear to be associated with asthma at 7 years of age, whereas recurrent upper respiratory tract infection in early life reduced the risk of asthma at school age [72]. Multiple epidemiological studies from Europe and elsewhere have shown that people living on farms from childbirth through to adults, especially with exposure to livestock or consumption of unpasteurized milk, have significantly lower prevalence of asthma and allergic rhinitis than the general population [73]. This is presumably due to greater environmental exposure to higher burden of multiple pathogens and microbes. The timing and duration of exposure to unhygienic environment appears to be critical, as the reduction in risk of allergic disease is greatest for those exposed prenatally and continuously until adulthood [73]. The maternal exposure to animal sheds and unpasteurized cow's milk has also been shown to influence the production of IgE antibodies in the cord blood of neonates [74]. Furthermore, children living on farms are exposed to higher bacterial components, endotoxin and muramic acid, from abundant mattress dust compared to nonfarm children. Exposure to the microbial laden mattress dust has been associated with lower frequency of wheezing, asthma, and hay fever [75–77]. Similar findings were reported in a case-controlled study from Chile, where daycare attendance and regular farm animal contact were inversely related to childhood asthma [78]. In a case-controlled study from Brazil the

effect of crowding in the home environment was also associated with the development of asthma, crowding in the home was associated with a 60 % reduction in incidence of asthma, yet there was a 2.5-fold increase in incidence of lower respiratory tract infections [79].

The microbial burden and exposures in homes, even in Urban areas, may vary with overcrowding, presence of pets, and social status. In a longitudinal birth cohort study from Boston with enrollment of approximately 500 children, followed until school age, dust samples were collected and analyzed for bacterial biomarkers [80]. Multiple exposures to both gram-negative and gram-positive bacteria were associated with decreased asthma, and school age, endotoxin exposure remained protective for allergic disease adjusted for early life endotoxin. In a preliminary study utilizing molecular methods [PCR–the nature ring gradient gel analysis] to analyze home dust, differences in the dust bacterial community were associated with asthma outcomes in young children, including wheezing and specific IgE [81]. The bacterial community structure of the house dust was significantly impacted by the presence of dogs or cats in the homes. Experimental findings in pregnant mice with asthmatic phenotype indicate that prenatal exposure with farm-derived bacteria operate by means of epigenetic mechanisms, altering the activity of genes without changing their structure to protect against transmaternal asthma [82].

Not all studies, however, support the hygiene hypothesis in the pathogenesis of asthma. In a longitudinal study of birth cohort, 3,963 newborn children were prospectively followed for 8 years in the Netherlands [83]. Early daycare provided no protection against asthma or allergic sensitization at the age of 8 years. A prospective study of allergic prone children from birth to 6 years [$n = 620$] was assessed for the effect of early childhood infections and immunizations on the development of asthma in Melbourne, Australia [84]. Recurrent gastroenteritis in early childhood was associated with greater risk of asthma, and Sabin polio vaccination in the second year of life was associated with a decreased risk.

In Africa studies have shown that allergic diseases have shown a steady increase over the past 10 years or more. Allergic diseases, IgE, and skin reactivity to allergens increase with increasing affluence and greater gross national income of the countries [85]. Association between helminthic infections and allergies was contradictory but rural living was associated with a decreased risk of allergic diseases [85].

5.4.1 Microbial Colonization and Asthma

In a cross-sectional study of 7,412 children [3–19 years of age], participants of the National Health and Examination Survey [NHANES], *Helicobacter pylori* seropositivity was inversely associated with asthma in children [86]. Colonization of the gastrointestinal tract may be a reflection of sanitation and exposure burden of microbes and this could support the hygiene hypothesis. The same investigators had previously reported that colonization with *H. pylori* [Cag A+] in adults was inversely

associated with the presence of asthma or allergic rhinitis in a study of 7,663 subjects [87]. The combined infectious burden of *H. pylori*, *Toxoplasmosis gondii*, hepatitis A, herpes simplex 1, *C. pneumoniae*, Epstein–Barr virus, and *Cytomegalovirus* [determined by presence of antibodies] was associated with lower risk of atopy, asthma, and allergic rhinitis in 1,249 adults in Europe [88]. However, in other cross-sectional studies in adults there was no association of *H. pylori* serological status and asthma or atopy [89]. Although in an experimental mouse model of allergic asthma, *H. pylori* infection could prevent asthma development through the induction of regulatory T cells [90].

The relationship between intrauterine bacterial colonization at delivery and development of asthma 15–17 years later in 460 children has been reported from Finland [91]. In vitro growth of pathogenic anaerobic bacteria, and streptococcus species from the maternal womb at birth was associated with significant increased risk of asthma diagnosis compared to those with negative bacterial cultures. There are several limitations of this study, however, including the small sample size and failure to provide information on other potential risk factors, such as household environments and previous lower respiratory tract infection in infancy. This study may be worthwhile repeating in a larger population with molecular methods to assess the microbial environment of the vagina, uterine cavity, and the household dust, especially in women with a family history of asthma or atopic diseases.

Can oral microbial pathogens influence development of allergic diseases? This topic was reviewed in 2011, with the conclusion that it is biologically plausible that oral bacteria through immune mechanisms could influence the risk of allergic diseases, but the data was insufficient to draw any conclusions [92]. In a childhood birth cohort prospective study from Copenhagen, aspirates of the hypopharynx were obtained from asymptomatic 1-month-old infants of asthmatic mothers for bacterial culture and subsequently correlated with later development of asthma at 5 years of age [29]. Colonization of the hypopharynx with *S. pneumoniae*, *H. influenzae*, or *Moraxella catarrhalis* or with a combination of these organisms was at increased risk for recurrent wheeze and asthma in early life. A subsequent study has shown that polymorphism of the IL-17 gene was associated with childhood asthma and bacterial colonization of the hypopharynx in bronchiolitis [93]. Thus bacterial colonization of the hypopharynx in neonates and childhood asthma may be linked to a genetic predisposition. However, there is no evidence that antibiotics use in early life decreases or increases the risk for later development of asthma [94]. It has been postulated that bacterial colonization of the airway could be related to development of IgE against bacterial antigen, implicating a role for bacterial-specific type-2 immunity in the pathogenesis of asthma. Titers of IgE against *H. influenzae* and *S. pneumoniae* and *Staphylococcus aureus* were measured in 1,380 teenagers and correlated with asthma and immunophenotypes [95]. IgE titers against *S. aureus-derived* enterotoxins were highest among atopic subjects and were associated with increased risk of asthma. However, high IgE titers against *H. influenzae* and *S. pneumoniae* were associated with a decreased risk of asthma. The investigators postulated that lower availability of soluble forms of *H. influenzae* and *S. pneumoniae* antigens reduces the cross-link with IgE receptors systemically, but the availability of

these antigens at the mucosal level to antigen presenting cells and type-2 memory cells could lead to mucosal secretion of IL-4/IL-13 producing an atopic response [95].

S. aureus enterotoxin [a super-antigen]-specific IgE antibodies have been associated with asthma severity and the various phenotypes in other studies [96]. In a review and meta-analysis of ten studies, patients with asthma or allergic rhinitis were more likely than controls to have serum-specific IgE to *S. aureus* enterotoxins [97]. In a more recent case-controlled study *S. aureus* enterotoxin IgE antibodies, but not IgE against inhalant allergens [grass pollen and house dust mite], were risk factors for asthma severity [98].

The intestinal microbiota appears to be important in development of the host innate immunity and may play a role in the pathogenesis of other allergic and autoimmune diseases. A recent study investigated the relationship between fecal microbiota composition, mode, and place of delivery with atopic diseases [99]. Fecal samples were collected from neonates at age 1 month [$n = 1{,}176$] to determine microbiota composition, and blood samples were collected at ages 1, 2, and 6–7 years to determine specific IgE levels. Colonization by *Clostridium difficile* at 1 month of age was associated with wheezing and eczema throughout the first 6–7 years of life and with asthma at age 6–7 years. Vaginal delivery at home was associated with decreased risk of eczema, food allergy, and asthma in comparison to vaginal delivery in hospital [99].

Previous but smaller prospective birth cohort of 117 children had found that *Bacteroides fragilis* fecal colonization at the age 3 weeks was an indicator of possible asthma in later life, asthma predictive index was positive in 64 % versus 34 % in those without *B. fragilis*, $p < 0.05$ [100]. In another study of 76 infants at high risk for atopic diseases, intestinal microbiota were analyzed at 3 weeks and 3 months of age and subsequently correlated with skin reactivity at 12 months [101]. Atopic children had more clostridia and fewer bifidobacteria, reduced ratio of bifidobacteria to clostridia than nonatopic infants, $p = 0.03$. Moreover, experimental studies in mice have shown that alterations of the gut microbiota can play an important role in regulating immune responses in the lungs to inhaled antigens [102]. Antibiotic-induced perturbations of the gut microbiota can produce allergic airway response that is mediated by IL-13 and CD4 T cells [102].

There is also evidence that the normal bronchial tree is not sterile and contains a mean of 2,000 bacterial genomes per square centimeter of surface area [103]. Pathogenic *Proteobacteria* [especially Haemophilus species] were significantly increased in asthmatic children and adults than controls; conversely, *Bacteroidetes*, particularly *Prevotella* species, were more frequent in controls than in asthmatics [103]. It remains unclear from the results of this cross-sectional study whether the asthmatic airway predisposed to specific bacteria or vice versa. A subsequent study in 65 adults with suboptimally controlled asthma and 10 healthy controls assessed the bacterial burden of bronchial epithelium by 16S ribosomal RNA amplicon concentration [104]. Bacterial diversity and concentrations were significantly higher among asthmatic patients than controls. The relative abundance of particular phenotypes including members of *Comamonadaceae*, *Sphingomonadaceae*, *Oxalobacteraceae*, and other bacterial families were highly correlated with the

degree of bronchial hyperresponsiveness [104]. These two studies suggest that the microbiome of the airways may contribute to the pathogenesis of asthma. However, another recent study using molecular methods found the bacterial communities of the lungs are indistinguishable from the upper airways, but the microbial biomass was 2–4 logs lower in healthy adults. Thus there is no unique lung microbiota or microbiome.

5.5 Microbes and Asthma at the Cellular Level

At the cellular and molecular level the mechanisms of viral lower respiratory tract infections and asthma, and bacterial colonization of the airway, or environmental exposure with development of asthma may be different. Eosinophils, the key effector cells of atopic asthma, are considered the potential link between viral infections and asthma [106]. Recruitment of eosinophils in the airway is the main trigger for inflammation and bronchoconstriction on exposure to aeroallergens, and may play a role in the antiviral immunity to eradicate viruses and to decrease invasion of epithelial cells [106]. Neutrophils, which are considered more important in bacterial infection as part of the innate immune response, appear to be important in the pathogenesis of nonatopic intrinsic asthma. It is estimated that about 20 % asthmatic patients have neutrophilic airway inflammation which is associated with bacterial persistence, such as *H. influenzae*, in the airway and steroid resistance [107].

Host factors are likely important in the association of viral infections in infancy proceeding development of asthma. Polymorphism in genes controlling innate immunity, antiviral and Th1 and Th2 immune responses are associated with both asthma and severe respiratory tract infections in early childhood [108]. However, it is unclear whether or not these genetic predispositions are present in only a fraction of children who develop asthma after severe recurrent lower respiratory infections. There is experimental evidence that viral respiratory infection in atopic children may initiate an atopy-dependent cascade that amplifies and sustains airway inflammation initiated by antiviral immunity by utilizing underlying atopic-associated mechanisms [70]. Recent evidence that anti-IgE monthly prophylactic therapy can significantly reduce asthma exacerbations in children, particularly during the common cold season [109], supports a mechanistic interaction of the viral Th1-type response and the allergic Th2-type response which result in increased IgE. This has been attributed to a defective type-1 response to rhinoviruses in atopic asthma, with reduced IFN-γ and shift toward a type-2 phenotype [108].

The relationship between commensal microbiota colonization in early life and later development of asthma may partly be explained by recent experimental findings. Previous investigations have demonstrated that inflammasome activation and downstream cytokines play a role in innate and adaptive antiviral immune defense in vivo [111, 112]. Recent studies in mice demonstrate the importance of gastrointestinal commensal microbiota in regulating immunity, through establishment of Th1, CTL, and IgA responses in the respiratory mucosa following influenza infection [113].

The data indicate that commensal microbiota responsible for conferring an immunogenic environment in the lungs is either gram-positive bacteria of the gut and possible commensal of the nasal mucosa. These results provide a link between commensal microbiota and inflammasome-dependent cytokine activation [113].

5.6 Alternative Hypotheses Linking Microbes and Asthma

Although the hygiene hypothesis is currently in vogue to explain the rising incidence of asthma in prosperous countries, there are other theories. It has recently been postulated that changes in diet, which is different in developed countries from developing nations, and associated changes in the gut microbiota are driving the increasing incidence of autoimmune and allergic diseases in developed countries [114]. Diet itself has considerable effect on the composition of the gut microbiota, and experiments in mice show changes in their microbial composition, metabolic pathways, and gene expression just after 1 day on the Western diet [115]. The Western diet causes an increase in bacteria of the *Firmicutes* phylum and a decrease in those of the *Bacteroidetes* phylum. The gut microbiota of children in Africa is greatly different from those in Europe, and this is attributed primarily to dietary differences [115]. The examples provided by the authors to support the hypothesis of diet rather than hygiene affecting the incidence of asthma are the relatively low prevalence of asthma in Japan compared to Australia and the United States [115]. Japan has high a degree of sanitation and urbanization but much different diet that would influence the gut microbiota/microbiome. In addition, the urban poor in the USA with greater frequency of infection, crowding, and likely less sanitary habits still have a high incidence of asthma.

Vitamin D deficiency could be the common factor linking asthma, allergy, and respiratory infections [116]. Vitamin D is important for effective function of the innate and adaptive immunity. Vitamin D is associated with a dose-dependent reduction in the transcription of Th1-cytokines such as IL-2 and IFN-γ and increase in the Th2-cytokines, IL-4, IL-5, and IL-10, in peripheral blood mononuclear cells culture [116]. Thus vitamin D has a key role in the Th1-Th2 balance. Deficiency of vitamin D is common in children and adults of temperate regions of the world, and is associated with higher risk of upper and lower respiratory infections, especially in children. Only 10 % of vitamin D is acquired from ingested food and 90 % from synthesis after sunlight exposure. Therefore, the higher prevalence of vitamin D deficiency seen in countries of the Northern hemisphere could explain the greater incidence of asthma in prosperous countries compared to poorer nations, which are predominantly located in tropical and subtropical regions of the globe. The link between vitamin D deficiency and respiratory infections is particularly relevant in children who develop asthma after recurrent respiratory tract infection in early childhood. This seasonality of influenza and RSV-induced bronchiolitis has been linked to the greater prevalence of vitamin D deficiency in the winter [117, 118]. In a recent prospective birth cohort study of 156 healthy neonates, low concentration

of vitamin D from cord blood was associated with increased risk of RSV bronchiolitis in the first year of life [119].

Several epidemiological studies have suggested that vitamin D deficiency is associated with increased risk of asthma and allergic diseases [120–124]. In a study from Costa Rica, where sunlight exposure should be uniform throughout the year, insufficient levels of vitamin D were associated with higher eosinophil count and IgE levels and increased airway hyperresponsiveness [124], while higher vitamin D levels were associated with lower risk of asthma exacerbation and hospitalization. These results were confirmed in another study by the same group of investigators in a cohort of 1,024 children, and it was suggested that vitamin D may protect against respiratory infections and symptoms of asthma by reducing bronchial inflammation [125]. This postulate was confirmed by a small double-blind randomized study that showed vitamin D supplementation reduced the risk of asthma exacerbation by respiratory tract infections from fall to spring [126].

However, not all studies have found an association between vitamin D deficiency and asthma and atopy. In fact, some reports suggest that vitamin D supplementation can increase the risk of asthma and atopic diseases [127]. A birth cohort study from Finland found that subjects given regular vitamin D supplements in the first year of life had a somewhat higher risk of asthma, atopy, and allergic rhinitis as adults compared to controls, not previously given supplements [128]. Similarly, a Swedish study reported that high vitamin D intake in infants correlated with greater risk of eczema at 6 years of age [129].

5.7 Probiotics for Allergic Diseases

The concept that commensal microbiota of the gut plays an important role in the pathogenesis of asthma and allergic diseases suggests that probiotics should be useful. Atopic eczema is the earliest manifestation of allergic diseases in children, and often precedes development of atopic asthma. In mice feeding of *Lactobacillus casei* strain Shirota was effective in inhibiting IgE production to a commonly used allergen [130]. The value of probiotics in allergic diseases has been assessed in several randomized, controlled trials, but mainly involving subjects with atopic eczema, allergic dermatitis, and allergic rhinitis. The main limitations of these trials have included small sample sizes, heterogeneity, and lack of a standardized acceptable probiotic mixture. This topic was last reviewed by Yao et al. who came to the conclusion that there was insufficient evidence to recommend probiotics for allergic diseases, including asthma [131]. The authors noted that the preventative studies failed to show a significant reduction in atopic sensitization, and there have been reports of probiotics administered during the perinatal period being associated with greater risk of later development of wheezing or asthma [131].

5.8 Conclusion

It is generally accepted that viral and some bacterial respiratory tract infections can exacerbate asthma. However, it is still not clear or well established that microbes can cause asthma, but it is biologically plausible that microbes could play a significant role in the pathogenesis of asthma and there is accumulating supportive evidence over the past 10 years. A diagrammatic paradigm of the role of microbes in the pathogenesis of asthma is shown in Fig. 5.1. It is quite possible that microbes influence the development of asthma in different ways, depending on the underlying genetic predisposition and that asthma is truly a heterogeneous group of disorders with different pathogenic mechanisms but with similar clinical manifestations.

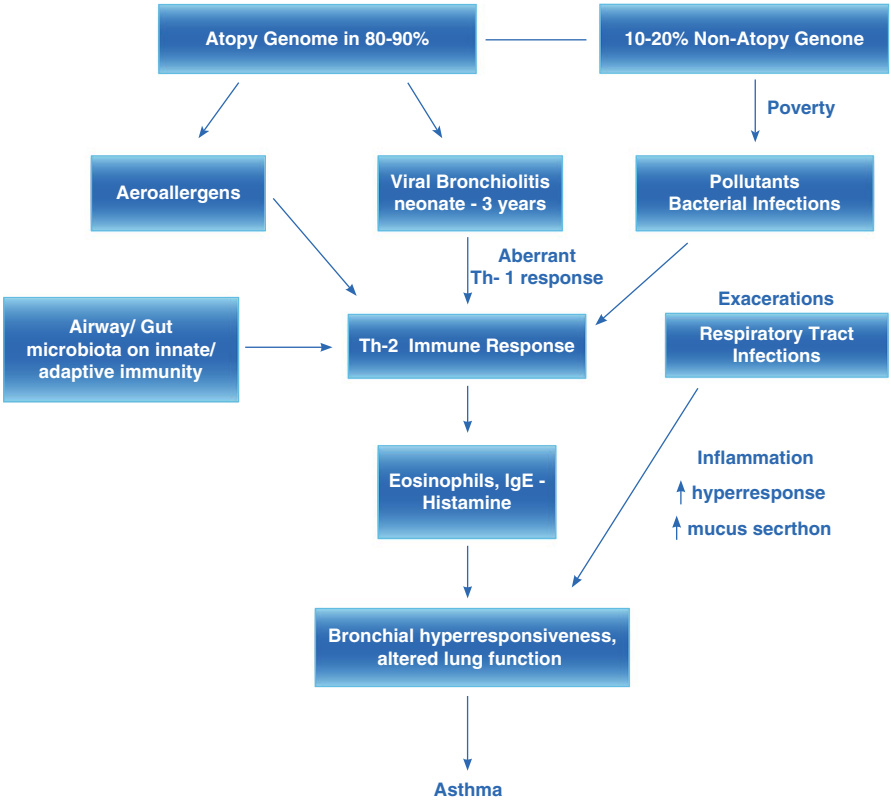

Fig. 5.1 Paradigm of microbes in asthma pathogenesis

5.9 Future Directions

Further studies to elucidate the role of microbes in the causation of asthma are definitely needed and should combine the various aspects in the same high-risk birth cohorts. For example, studies starting in the antenatal period through school age should assess by modern molecular methods the environmental microbial composition, i.e., house dust, the microbiota of the maternal feces, vagina and uterine cavity at delivery, and in the newborn samples from the upper airway or hypopharynx and fecal specimens all should be tested together; plus documentation of subsequent lower respiratory tract infections up to 3 years of age. Then analyze and correlate these various factors with development of school-age asthma.

Before embarking on any large randomized controlled trials in early childhood of the value of probiotics in prevention of asthma several elements should be met. There should be agreement among a committee of experts on the composition of the probiotics chosen to be studied. Pilot studies should be performed on a small sample of subjects to determine whether or not the probiotic supplements produced the desired changes in the gut or upper airway microbiota composition. Endpoints should include skin test reactivity to aeroallergens, atopic manifestations such as eczema, development of wheezing and asthma, and the persistence in subsequent years in school.

Another prospective, longitudinal study in high-risk birth cohorts that is warranted is the assessment of diet on fecal and oro-pharyngeal microbiota, levels of vitamin D and respiratory infections over 8–10 years or more, and later development of asthma in a large sample of children.

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Chapter 6
Chronic Fatigue Syndrome: Searching for a Microbial Etiology

6.1 Introduction

Chronic fatigue syndrome [CFS] is a perplexing symptom complex of unknown cause, affecting 0.2–0.4 % of the population in epidemiological studies [1]. It is more common in women, accounting for 75 % of the cases, and the mean age of onset is between 29 and 35 years. This condition has also been called myalgic encephalomyelitis or myalgic encephalopathy [1], and likely has existed for over a century under a different synonym, such as chronic brucellosis syndrome, and more recently has been labeled by some medical practitioners as chronic Lyme disease. In 1959 the name epidemic neuromyasthenia was proposed by Henderson and Shelokov [2], and at that time there was limited medical interest in this condition. They observed at that time that patients experience CFS-like syndrome after episodes of influenza or flu-like illness or infectious hepatitis, and postulated an infectious etiology or initiator. But unlike influenza, outbreaks of CFS occurred spontaneously in several states of the United States and isolation in the absence of intervening cases [3]. It was felt by the investigators in the 1950s that CFS was a real illness of infectious origin and was likely caused by an unknown virus [4].

CFS is characterized by a combination of serious symptoms with prominent chronic fatigue, headaches, sleep disturbance, sore throat, tender lymph glands, myalgia and arthralgia, feverish sensation or low-grade fever, cognitive disturbance, and other neurological symptoms. Fibromyalgia, also a common functional disorder of unknown cause, overlaps with CFS, but the generalized aches and pains of the muscles are more prominent than chronic fatigue [5]. While most cases of CFS are seen in adults under -50 years of age, it has been noted occasionally in children and adolescents [6]. The definition of CFS as defined by the Centers for Disease Control and Prevention [7] includes two major criteria that must be met: (1) new onset of persistent or relapsing, debilitating fatigue or easy fatigability, not relieved by bed rest, that last at least 6 months and reduce the patient's daily activity below 50 %; and (2) by examination and investigations must exclude known medical conditions

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and major psychiatric conditions [schizophrenia, bipolar disorder, and major depression] that can cause similar symptoms. The inclusion of the case definition of CFS required two major definitions and eight of eleven minor symptoms criteria, or six minor symptoms criteria and two of three physical examination criteria, such as cervical or axillary lymphadenopathy, nonexudative pharyngitis, and low-grade fever. In 1994 CDC and the International Study Group revised the definition of CFS [8]. Besides new onset disabling fatigue for at least 6 months, four additional symptoms should be present, such as muscle and joint pains, sore throat, headaches, unrefreshing sleep, and cognitive dysfunction. Further modification of the definition of CFS/ME was advanced in 2007 by the National Institute of Health and Clinical Excellence [NICE] guidance in the United Kingdom [9]. This new criteria specified that the symptoms should be persistent for at least 4 months, and besides chronic fatigue only one or more minor symptoms need to be present. Adding more controversy to the definition is an independent international group of practitioners redefining CFS as myalgic encephalitis [ME], on the assumption that it is a neurological disease, and dropping the 6 months duration requirement with no specific duration, plus three other symptoms besides postexertional exhaustion [10]. To date, the scientific community has not adopted the latest definition, and the CDC revised definition of 1994 is the most universally accepted definition of CFS.

The prognosis for full recovery from CFS in longitudinal studies has been poor, with only about 5 % of patients reporting full recovery at 1–5 years, and with 40 %, showing some improvement in the same time [11]. Thus, the economic impact from unemployability and disability insurance is very substantial. However, these data were derived from specialty centers where the more severe cases of CFS were seen or referred by family physicians.

6.2 Is Chronic Fatigue Syndrome a Psychosocial Disorder?

Despite intensive medical research over the past 30 years, no reproducible organic basis has been established as the etiologic explanation for CFS. There are several similar or related conditions associated with multiple symptoms, suffering, and disability with no demonstrable abnormalities of organ structure or function, termed functional somatic syndromes. These syndromes include multiple chemical sensitivity, the sick building syndrome, chronic whiplash, chronic Lyme disease, candidiasis hypersensitivity, the Gulf War syndrome, and others; CFS, fibromyalgia, and irritable bowel syndrome [IBS] were added to the list in a previous review of 1999 [12]. It was noted by the authors that somatic functional disorders share many similar features: high rates of co-occurrence, similar epidemiological characteristics, and higher than expected prevalence of psychiatric comorbidity.

Several prospective studies have found a high rate of premorbid psychosocial factors for the onset of CFS, such as depression, anxiety, and psychological distress [13–15]. In a prospective birth cohort study from 1946 in the UK of 5,000 individuals followed until development of CFS, individuals with prior psychiatric disorders,

especially anxiety and depression, were two and a half times more likely to develop CFS [16]. Patients with functional somatic syndromes, including CFS, have a higher prevalence of somatization and medically unexplained symptoms unrelated to their functional somatic syndrome, which often predate their main disability, suggesting pre-existing tendency to experience and report bodily distress [12]. CFS shears other features typical of other functional somatic syndromes, such as chronic intractable symptoms refractory to treatment and palliative measures, and the patient's belief that he or she has a serious disease, exacerbated by a self-perpetuating cycle [12]. Several prospective and cross-sectional studies have shown that CFS patients have greater incidence of overactive perfectionism-premorbid personality compared to controls [17]. However, one large birth cohort study of data collected from 1970 found childhood experience of sedentary lifestyle and a limiting illness were predictors of CFS [18]. It has been suggested that predisposing genetics, personality factors, activity patterns [too much or too little], and distress may be interlinked as predisposition for CFS. In a Swedish twin cohort of about 20,000 subjects, personality traits of emotional instability, experience of psychological distress, and premorbid levels of perceived stress significantly predicted onset of CFS [19]. Although genes may play a role in the predisposition to CFS and emotional stability, no specific genes have been identified, and a review of the evidence concluded that environmental influence is a more prominent factor [20].

6.3 Pathobiology of Chronic Fatigue Syndrome

The pathogenesis of CFS is not well understood, and most earlier studies had addressed the psychosocial factors. Disturbances in various physiological states of the body have been proposed and investigated for the root cause of CFS. These include persistent chronic inflammation, immune system activation, autonomic dysfunction, dysfunction of the hypothalamic–pituitary–adrenal [HPA] axis, and neuroendocrine dysregulation----have all been suggested as causes of CFS. Studies on the biomarkers of chronic fatigue associated with CFS/ME, and other diseases associated with fatigue had recently been reviewed by Klimas et al. [21]. Moderate to severe fatigue has been reported in 50–70 % of cases with immune-mediated inflammatory diseases, such as Sjogren syndrome or inflammatory bowel disease [22, 23]. Fatigue in these disorders have been associated with increased blood levels of proinflammatory cytokines, interleukin-1 [IL-1], interferon alfa [IFN-α], tumor necrosis factor alpha [TNFα], and IL-6 in both humans and some animal models [21]. However, in a cohort of patients with rheumatoid arthritis the strongest correlate of fatigue was depression [$p < 0.001$] and anxiety [$p < 0.001$] [24].

Several studies in patients with CFS have found evidence of low-grade inflammation as indicated by mild elevation of the C-reactive protein [CRP] compared to healthy controls [21]. The largest population-based study performed by CDC researchers of 21,165 residents found CRP levels >3 mg/L was significantly higher in subjects with CFS [34.4 %] compared to healthy controls [20.7 %], odd ratio

[OR] = 2 [25]. However, this was similar to fatigued individuals not fulfilling the criteria for CFS, and was not significantly different after adjustment for other factors, such as body mass index and depressed status, etc. In the general population study of 63,000 people, the mean CRP concentration was 1.53 mg/L, but 34 % of the population had levels of greater than 2 mg/L and 97 % had levels less than 10 mg/L [26]. Thus, CRP levels >2–3 mg/L are commonly found in the population and maybe associated with underlying atherosclerosis, and in patients with symptomatic inflammatory diseases the CRP levels are usually greater than 8–10 mg/L.

The presence of increased plasma proinflammatory cytokines at rest or after exercise, or from stimulated peripheral blood mononuclear cells [PBMC] in CFS patients compared to healthy controls has been reported, but not all studies have confirmed these findings [21, 27]. Also natural killer cells quantity and function have been reported to be low in a few studies in CFS subjects compared to healthy controls [28, 29]. Similar findings have also been reported in the Gulf War illness and may be a reflection of the stress-mediated activation response of the immune system [30]. The stress hormone, neuropeptide Y, has been reported to be elevated in CFS patients and it appears to correlate with the severity of symptoms such as fatigue [31]. Limited studies have also shown that with moderate exercise there is increase in gene expression in CFS patients, greater than controls, for genes essential for sympathetic nervous system processes, such as sensory and adrenergic receptors, and genes that can detect increases in muscle produced metabolites [32, 33]. These postexercise increases correlated with symptoms of fatigue and pain in 71 % of CFS patients, but 29 % had decreased or unaltered gene expression [33].

Dysfunction of the HPA axis has also been reported in CFS and is considered clinically relevant according to a recent review [34]. The dysfunction in CFS includes mild hypocortisolism with attenuated variation of cortisone, enhanced negative feedback, and blunted HPA axis responsiveness. Hypoactive HPA axis has also been associated with a number of neuroimmune disorders, and dysfunction of this axis has been correlated with severe fatigue in breast cancer survivors [35]. Investigations over the past decade have established that multiple factors can moderate changes in the HPA axis. These include lower level activity, depression, and early life stress, which are associated with reduced cortisone levels, whereas psychotropic drugs and cognitive behavior therapy can increase cortisone levels [34, 36]. Stress management has been shown to improve diurnal cortisol slope, lower IL-2 levels and resulted with less fatigue in CFS patients, with the greatest benefit in those with elevated IL-6 levels [36]. Interestingly, the glucocorticoid receptor gene NR3C1 polymorphism has been implicated as a possible mechanism for CFS symptoms, through alteration of the HPA axis regulation [37].

A few studies have assessed the metabolic cellular functions in CFS patients, by examination of the cerebrospinal fluid or circulating blood cells for metabolite profiling. Quantitative proteomic analysis of the cerebrospinal fluid, by liquid chromatography with high-resolution mass spectrometry, has been performed in patients with CFS and Lyme disease compared to healthy controls [38]. The cerebrospinal fluid protein profiles were unique and could distinguish the patient groups from each other and from the controls. A prior smaller pilot study had detected unique

proteome profile of the spinal fluid of CFS patients compared to healthy controls, but 20 unique proteins were also shared with patients diagnosed with Gulf War illness [39]. It has also been reported that there is increased ventricular fluid lactic acid in patients with CFS as determined by special neuroimaging [40]. In one study evidence of mitochondrial dysfunction in 71 CFS subjects compared to 55 normal controls was reported, by measuring adenosine triphosphate [ATP] in circulating blood leucocytes [41].

Analysis of gene expression of the regulatory machinery for cell signal transduction and metabolism has also been performed in CFS patients to understand the mechanism of the disorder. In a study of 111 female subjects, there were associations of 17 transcripts related to cellular processes involving cell signaling, ion transport, and immune function [42]. The most significant finding in CFS was increased Sestrin 1 [SESN1], supporting the involvement of oxidative stress in this condition. Kerr et al. [42] had previously reported on the presence of CFS "signature genes" from a 44-gene classifier set that could discriminate between CFS and healthy controls subjects with a 95 % sensitivity [42]. Only 5 of the 44 genes were related to immune function. A subsequent study by the same group using a variety of methods and blinded sample sets, found that the 44 gene classifier was not as sensitive and accurate in identifying patients with CFS [43]. Furthermore, other investigators could not identify any biomarker of gene expression in circulating leukocytes of monozygotic twins discordant for CFS [44]. In a more recent study, metabolic, adrenergic, and immune gene expression [m RNA] were measured in patients with CFS [$n=22$], and multiple sclerosis [$n=20$], versus healthy controls [$n=23$] at rest and after exercise [45]. Patients with CFS had greater postexercise increases in pain and fatigue, associated with greater mRNA increases than controls in purinergic type 2×4 receptor, CD14, and all adrenergic receptors above baseline, $p=0.04$–0.05. Similarly, multiple sclerosis patients demonstrated significantly greater postexercise increases than controls in β-1 and β-2 adrenergic receptor expression [$p<0.001$], and greater decreases in toll-like receptor 4 [TLR4]. Thus, both CFS and multiple sclerosis patients showed abnormal increases in adrenergic receptors but the postexercise increase in metabolite detecting receptors was unique to CFS, and in multiple sclerosis greater fatigue was correlated with blunted immune marker expression [45].

6.3.1 The Central Sensitizing Theory

In the late 1990s it was hypothesized that CFS was distinguished by hypersensitivity of the central nervous system [CNS] to a variety of stimuli or inputs, and this was termed central sensitization [46]. The proposed mechanisms of central sensitization involve altered sensory processing of the brain with malfunctioning of the inhibitory processes that could explain widespread pain in CFS and fibromyalgia [47, 48]. In central sensitization the altered sensory processing results in an overactive pain neuromatrix with increased activity of the pain centers of the brain, i.e., the insula, anterior cingulate, and prefrontal cortex [49]. Long-term potentiation of neuronal

synapses of the anterior cingulate cortex and decreased gamma-aminobutyric acid [GABA]–neurotransmission may also be important in the overactive brain neuromatrix and altered sensory processing [46].

Peripheral mechanisms may also take part in central sensitization as well. Various stressors such as injury and infections upregulate and increase inflammatory cytokines, with subsequent activation of the spinal cord neuroglia with increased expression of cyclooxygenase-2 and prostaglandin E2 in the CNS [50]. The increased sensitivity to variable stimuli, such as mechanical, chemical, sound, cold or heat, and light, results in decreased tolerance. The clinical picture of central sensitization is typical of the symptoms experienced in CFS and has been described in a variety of unexplained disorders, including chronic low back pain, chronic tension headaches, temporomandibular disorders, chronic whiplash syndrome, fibromyalgia, and others [46].

There is cumulative evidence that central sensitization is a core feature of CFS. Generalized hyperalgesia has been described in ten studies of patients with CFS to various sensory stimuli, but five of these were from the same group of investigators [46]. Normally pain threshold increases during physical activity as a response to release of endogenous opioids, growth factors, and inhibitory mechanisms by the CNS [51]. However, studies in CFS patients have found dysfunction or lack of endogenous nociceptive inhibition during exercise, with lower pain threshold postexercise compared to controls and subjects with chronic low back pain [52, 53]. Reduction of serotonin transporters involved in descending inhibitory action of the pain center has been found in CFS patients compared to healthy controls by positron emission tomography [54]. Substance P, a neurotransmitter peptide of pain from the periphery to the CNS, is markedly elevated in the cerebrospinal fluid of subjects with primary fibromyalgia but not in CFS patients without widespread pain [55].

6.4 Infections and Chronic Fatigue Syndrome

The observation that most patients with CFS report preceding viral-like prodrome and symptoms consistent with an infection, and reports of geographic and temporal clusters of cases have suggested a microbial causative agent. Moreover, the pattern of changes on analysis of cytokine networks and immune regulation in CFS are consistent with several processes active in latent viral infection [27]. In 1985 two reports stimulated a flurry of interest in the Epstein–Barr virus [EBV] as a cause of CFS [56, 57]. This condition was then referred to as chronic EBV infection or chronic mononucleosis syndrome. However, further studies found lack of correlation between EBV antibody titers and clinical status, and antibody titers to early antigens were present in many healthy subjects for 2–4 years after acute infection [58]. Also there was no clinical benefit found in subjects with CFS treated with acyclovir which is active in vitro against EBV [59]. Chronic active EBV infection is a separate entity associated with high morbidity and some mortality, characterized by fever, lymphadenopathy, hepatosplenomegaly, anemia, thrombocytopenia,

leukopenia, and less commonly persistent hepatitis, interstitial pneumonia and uveitis, and may be a seen in apparently immunocompetent and immunosuppressed subjects [60, 61].

However, acute EBV infection may be a precipitator of CFS and many patients suffering from this illness often have debilitating fatigue for several weeks to months. In a recent prospective cohort of 246 patients with acute infectious mononucleosis, 7.8 % met the criteria for CFS at 6 months [62]. Logistic progression revealed that anxiety, depression, somatization, and perfectionism were associated with new onset CFS. In another prospective study of acute infectious mononucleosis in adolescence [$n = 301$], followed for 2 years, at 6 months after the diagnosis 39 [12.9 %] patients met the criteria for CFS [63]. Physical activity levels before, during, and after infection did not predict the development of CFS but those with severe fatigue and daytime sleepiness soon after infection were more likely to develop CFS. The female gender and greater fatigue severity at the time of the acute infection were the primary risk factors for CFS, as reported in an earlier report by the same group of investigators [64].

In a prospective cohort study from Australia, 253 patients with acute infections from EBV, Q-fever, or Ross River virus were followed over 12 months [65]. At 6 months 12 % of patients developed CFS but at a similar incidence after each infection. Therefore, this indicated that there was no specificity of EBV to predispose to CFS. In a retrospective review of 873 patients with CFS referred to a tertiary center, initial infection was reported in 77 % and 75.3 % were females [66]. This study highlights the importance and frequency of an infectious illness as an initiator or precipitator of CFS and the greater propensity of females to become afflicted. The main limitation of this study, however, is that cases being referred to this center may be more severe and introduces a selection bias, and a population-based study would be more unbiased and better reflect cases of CFS seen by community physicians. A previous study of 548 patients with CFS used viral serology only for diagnosis which is not ideal, could not support the role of active infection with common viruses such as herpes simplex type I and 2, EBV, cytomegalovirus [CMV], rubella, adenovirus, coxsackievirus-B, and human herpes virus 6 [HHV6] [67].

Enteroviruses, especially coxsackie B virus, are common causes of acute respiratory and intestinal infection, with tropism for the central nervous system, cardiac and skeletal muscle. Moreover, there is evidence that enterovirus can persist in humans [68]. Cohort studies for the seroprevalence rates of enterovirus in CFS have reported mixed results [69–71]. However, the presence of enterovirus sequences in muscle of patients with CFS had suggested a role in the causation of weakness and fatigue [72–74]. The findings of enteroviral RNA in muscle of patients with CFS may not be specific as this has been reported in other neuromuscular disorders, even without active inflammation at a similar rate, 26.4 % and 19.8 % [75]. The presence of enterovirus sequences in inflammatory muscle diseases such as dermatomyositis or polymyositis, which are considered autoimmune diseases, suggests that this represents an epiphenomenon although absent in healthy subjects [76, 77]. One study has reported the finding of enteroviral sequences and blood of CFS patients [78], but others failed to confirm this finding [79, 80]. In more recent studies by Chia and his

group, enterovirus RNA were detected from blood leukocytes in about 35 % of 518 CFS patients [81]. However, the importance of enteroviral RNA in CFS still remains undefined. Small pilot studies of antiviral agents, ribavirin, combination of alpha and gamma interferon, and more recently pleconaril [specific activity for enterovirus and rhinovirus] have been reported by the same group in uncontrolled trials with various degrees of symptomatic improvement, but often with later relapse [81, 82]. These reports are difficult to interpret due to the small sample sizes and lack of controls and the symptoms and course of CFS are often variable and fluctuating. Hence, these reports are not conducive for further larger randomized, placebo, controlled trials with these agents.

Some studies have investigated the role of parvovirus B19 in CFS. Patients with CFS have been found to have raised IgG antibodies to parvovirus B19 NS1 protein [41.5 %] compared to healthy blood donor controls [7 %] [83]. Also viral DNA was detected in 11 of 200 CFS patients and none of 200 healthy controls. It was postulated that impaired cellular immunity may contribute to inadequate control of parvovirus B19, resulting in chronic arthralgia in some cases of CFS. In a more recent study, 210 patients were followed after parvovirus B19 infection to determine the correlation of prolonged viremia with the presence of persistent symptoms [84]. In this study there was no difference in the rate of blood DNA positivity in patients with or without persistent symptoms, but complement levels were persistently decreased in a greater proportion of patients with prolonged symptoms.

The unique feature of the herpesviruses with lifelong latency after primary infection and with intermittent reactivation after certain immunological conditions led to the suspicion that the herpes virus member was involved in the pathogenesis of CFS. EBV association with CFS has been discussed earlier in this chapter. Investigations have also been performed on the association of CMV and HHV-6 with CFS. IgM antibodies to nonstructural genes of CMV have been detected in 16 of 34 CFS patients and 0 of 59 controls, 40 of whom were CMV IgG positive [85]. The presence of IgM antibodies to early antigen products was considered indicative of active CMV infection. In a more recent study by the same group of investigators, using a more sensitive immunoassay only 51 of 517 CFS patients [11.8 %] had detectable IgM antibodies but all were positive for CMV IgG [86]. Results of this study would suggest that only 10 % of patients with CFS were precipitated by acute CMV infection.

HHV-6, the cause of a mild childhood exanthem, exanthem subitum or roseola infantum, has been associated with reactivation in immunosuppression that can lead to bone marrow suppression, encephalitis, or neurological dysfunction [87]. Frequent HHV-6 reactivation has been reported in a study of 35 CFS patients by detection of raised anti-HHV-6 IgM and the presence of the antigen in PBMC cultures [88]. In an earlier study of 259 patients with symptoms consistent with CFS [before the case definition was developed], 70 % showed active HHV-6 replication versus 20 % of controls by culture of lymphocytes [89]. Another study using similar culture methods detected active HHV-6 infection in 30 of 52 [57.7 %] CFS patients and 6 of 51 [11.7 %] matched controls [90]. The role of HHV-6 infection and its association with CFS was previously reviewed in 2006 by Komaroff [91].

The conclusion of this review was that studies showing a slightly positive association with CFS and HHV-6 relied primarily on serological methods [$n=48$], whereas other studies utilizing PCR of serum or plasma, IgM of early antigen, and primary cell culture techniques [$n=717$] found a stronger positive correlation between the two. In vitro ampligen [poly[I].poly[C12U]] has demonstrated antiviral activity against HHV-6 [92]. A preliminary randomized, placebo-controlled trial of this agent in 92 CFS patients showed significantly greater improvement in global performance after 24 weeks treatment than placebo [93]. Of the 39 patients undergoing primary PBMC culture, 69 % demonstrated evidence of HHV-6 reactivation. Unfortunately no long-term follow-up result was provided as to the proportion in each group that was able to return to functional daily activity and the rate of later relapse. Furthermore, no confirmatory trial on a larger sample size has been published [to the author's knowledge] since the initial report in 1994 [93]. Valganciclovir also has activity against HHV-6 and EBV, and a small preliminary study reported resolution of symptoms in 9 of 12 patients with CFS [94]. Absence of further larger trials on this readily available oral agent in the past 6–7 years or reports in the medical literature on this topic is not an encouraging sign. However, a recent review on the role of viruses in CFS stated that a double-blind, randomized trial [not yet published] failed to show a significant benefit of valganciclovir [95]. A previous uncontrolled retrospective study of valganciclovir or valacyclovir for long-term treatment in 142 CFS patients reported an improvement in energy, particularly in those with evidence with active replication of HHV-6, CMV, and EBV [96]. In patients with only herpesviruses infection, 79 of 106 responded to this therapy with return to near-normal functional life. The publication of the results of a large randomized, controlled trial is therefore eagerly awaited.

In 2009 a report in Science attracted major international attention of the scientific community, when it was reported that detection of a murine retrovirus, XMRV, was found in the lymphocytes of 68 of 101 [67 %] CFS patients [97]. This gammaretrovirus, xenotropic murine leukemia related-virus, was also implicated in the pathogenesis of prostate cancer. Subsequent studies by other researchers and laboratories failed to confirm these findings in patients with CFS, and investigations concluded that the previous results in humans were likely the result of laboratory contamination [98].

6.4.1 Interpretation of Current Data on Microbes in Chronic Fatigue Syndrome

Although several epidemiological and biological studies lend support for a role of microbes in the pathobiology of CFS, the available data do not strongly support a causal role of any specific microbial agent. The evidence does suggest that certain individuals, especially females, with underlying genetic predisposition and psychological premorbid profile, are at risk for development of postinfectious fatigue which may persist to become CFS. However, no specific genetic marker has been identified [99]. CFS is a heterogeneous disorder and it has been suggested that

genetic subtypes exist and that precipitation of the illness with different triggers may depend on the genetic subtype. A recent study of 68 CFS and 14 depressed patients, compared to 29 normal controls, defined 8 genomic subtypes of CFS from the expression level of 88 genes [100]. There was evidence of subtype-specific relationships for EBV and enteroviruses, probably the two most common triggers for CFS. Gene expression in patients with endogenous depression was similar to normal controls and different from CFS, suggesting that endogenous depression and CFS are biologically distinct [100]. This is at variance with the common opinion that depression and CFS are co-associated disorders with manifestations of aberrant inflammatory, oxidative, and nitrosative pathways [101].

Despite the observation that multiple studies have found evidence of reactivation of one or more herpesviruses in a high proportion of CFS patients, a more recent well conducted but small study could not confirm these findings [102]. The hypothesis that active or reactivation of persistent latent viruses is responsible for CFS is thus very questionable and controversial. Moreover, CFS has occurred after bacterial infections such as brucellosis and Q fever, and more recently has been described after parasitic infection such as giardiasis [103]. The mechanisms by which preceding infections could lead to CFS are speculative and are not well understood. Various postulates have been proposed and a more recent hypothesis suggests that a viral infection can induce deficient cell stress response and thereby impairs stress tolerance and makes tissues vulnerable to damage [104]. Others have proposed that infection or peripheral inflammation in certain risk individuals results in aberrant immunological physiology of the brain that leads to symptoms of CFS [105].

An animal model of CFS would assist to unravel the relationship of this condition and various infections, as well as provide better understanding of the pathogenesis. However, it would be difficult to produce a suitable animal model, as there are no objective findings in CFS. Despite this obstacle Japanese researchers have described a mouse model of CFS, which has the main feature of this condition reduced physical activity [106]. Although this is not a perfect or ideal model of CFS, it may elucidate some of the mechanism of CFS pathogenesis. This CFS model was induced by six injections of killed *Brucella abortus* antigen in BALB/C mice. Reduced daily activity was associated with decreased hippocampal brain-derived neurotrophic factor mRNA expression, hippocampus apoptosis, and atrophy [106]. A further study by these investigators demonstrated resveratrol, a polyphenolic activator of sirtuin 1, by repressing apoptosis and promoting neurogenesis could improve physical activity in the animal by more than 20 % [107]. A notable difference between CFS and this model is the presence of structural and functional changes in the hippocampus of mice, not reported in patients of CFS by neuroimaging. There are, however, some reports of significant changes in the brain of CFS patients by special neuroimaging techniques. Reduced absolute cortical blood flow of middle cerebral arteries in patients with CFS compared to healthy controls had been reported in one study [108]. The volume of the gray matter has been estimated to be reduced in CFS patients compared to controls [109], and subjective mental fatigue was correlated with decreased gray matter volume of the right prefrontal cortex [110]. Significant greater activity in several cortical and subcoritcal regions of the brain on functional neuroimaging has been reported during

fatiguing cognitive task in CFS patients ($n=11$) versus controls ($n=9$) [111]. Despite these reports which involve limited number of subjects with CFS, further studies are needed in larger number of patients and healthy controls, subjects with anxiety and depression, matched for age and gender, in order to determine the specificity and consistency of these findings.

A rat model of CFS has also been described which was produced by prolonged physical activity and stress by the forced swim test [112]. In this model there were structural changes in the spleen and thymus, and accompanied by increased levels of TNF-α in the serum, and enhanced oxido-nitrosative brain stress. Interestingly these changes could be attenuated by probiotics, *Lactobacillus acidophilus* [112]. It has recently been suggested that CFS may be related to changes in the gut microflora. In a recent study of 108 CFS patients on 177 controls, there was a significant increase in D-lactic acid producing enterococcus and Streptococcus species [$p<0.01$] in the CFS group [113]. The authors postulated that the cognitive impairment which is commonly present in CFS may be secondary to D-lactic acidosis. To strengthen their hypothesis, the investigators should have demonstrated significantly increased D-lactic acid in the blood of CFS patients compared to the controls. Irritable bowel syndrome [IBS] is commonly associated with CFS and is believed to be related to changes in the normal bowel microbiota after gastroenteritis. In a large study of 4,388 CFS patients, matched with two groups attending for IBS or for another reason at UK primary care centers, prospectively collected General Practice Research Database, it was found that viral infections were more commonly a risk factor for CFS compared to IBS [OR, 2.8], while gastroenteritis was a greater risk factor for IBS [OR, 2.4] [114]. While these findings are intriguing the results need to be reduplicated by multiple independent investigators to verify their validity and reproducibility, especially for the animal models.

Furthermore, it is unclear as to the duration of the abnormalities detected after the initiating trigger in these models. To be directly correlated with the course of human CFS we should expect chronic persistent abnormalities.

6.5 Conclusion

CFS is a common chronic persistent disabling disorder still without an established etiology. The toll on individuals and families can be devastating and the economic burden is tremendous, estimated to be $7 billion annually in the United States [115]. Most but not all studies on the pathogenesis have reported mild increases in immune and inflammatory biomarkers, which could be consistent with a chronic low-grade infection. However, these findings of increased stress biomarkers may not be specific, as similar findings have been reported in anxiety and other psychogenic-related stress. Based on the overall review of the literature I have outlined a diagram for the pathobiology of CFS in Fig. 6.1.

Although there are many studies showing a direct correlation with an acute infectious illness, predominantly viral, and the onset of CFS the evidence does not

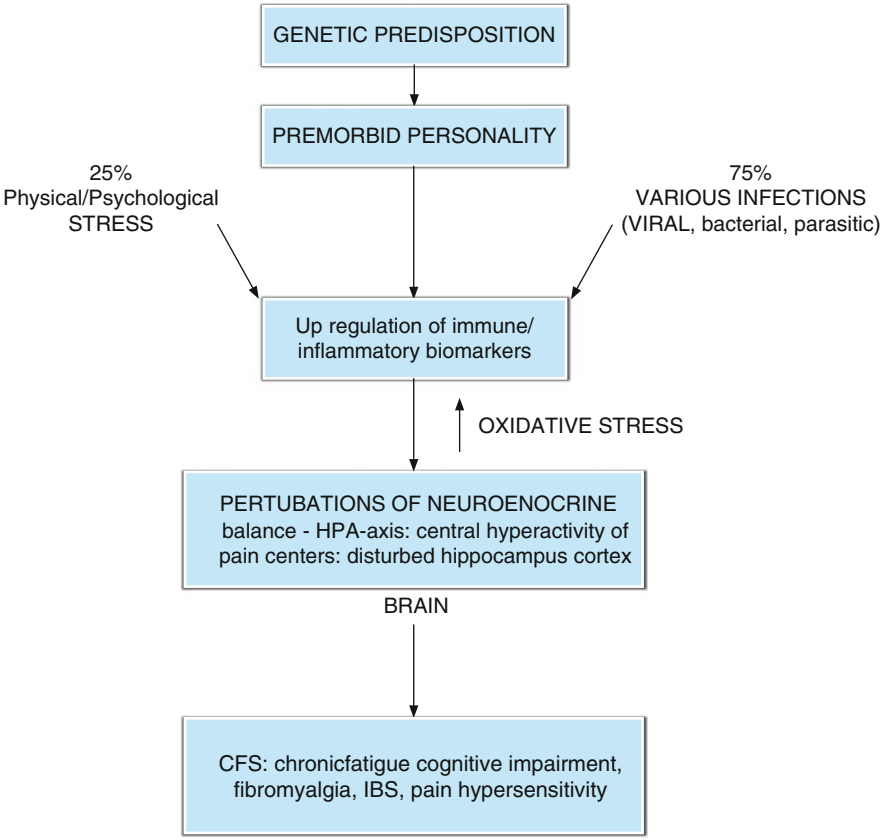

Fig. 6.1 Outline of the pathobiology of CFS

support a direct causal relationship by any specific microbe as seen with chronic infections, such as chronic hepatitis viruses C and B, and human immunodeficiency virus [HIV]. It is possible, but not very likely, that CFS is caused by an unknown microbe/virus that we cannot detect with current methods of investigation. The observation that cognitive behavior therapy and graded exercise have produced the most effective benefit in the treatment of CFS [17, 114] strongly implicates psychosocial causative factors but may not preclude an organic or microbial role in the pathogenesis. The value of these therapies is modest and the ultimate prognosis to return to normal activity after several is still poor [11].

6.6 Future Direction

A full understanding of the pathogenesis of CFS is still needed and this may lead to more effective treatment. Larger population-based, prospective, multidisciplinary studies over several years need to be performed in both developed and developing

countries. Assessment of various factors should be repeated over time with appropriate matched controls, including healthy controls and those with stress, anxiety, or depression without chronic fatigue. Investigations with modern methods to determine the combined effects of microbes, including the gut microbiota, and interaction on the immune/inflammatory pathways with psychosocial and genetic factors, should be performed.

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Chapter 7
Can Microbes Play a Role in the Pathogenesis of Alzheimer Disease?

7.1 Alzheimer Disease Background

With the increasing age of the world's population, greater in developed than developing nations, it is predictable that there will be an epidemic of dementia. It is projected that by 2030 there will be 66 million people worldwide living with dementia, and by 2050 this will rise to 115 million [1]. Alzheimer disease is the most common form of dementia with an estimated lifetime risk of nearly one in five for women and one in ten for men. There are two forms of the disease, familial or presenile dementia which accounts for 5 % of the disease and represents the original description by Alzheimer, and sporadic disease which represents 95 % of the cases. The exact cause[s] of late onset alzheimer disease remains unknown or elusive despite extensive research for more than 50 years. Based on our current understanding of the pathogenesis of the disease, it is unlikely that a single etiology will be found to explain all cases of late onset or sporadic Alzheimer disease [AD]. There are some similarities between the development and pathogenesis of AD and atherosclerosis, and it would appear that both conditions could be the result of injury or stress to the cells [neurons or vascular endothelium] with subsequent pathological changes as a response to chronic injury. The main clinical manifestations of AD are selective impairment and dementia, usually preceded by a period of mild cognitive impairment, with gradual deterioration and death.

Sporadic AD is classically a disease of older age, especially after 80 years of age, and the incidence increases progressively after 65 years of age. AD is slightly more common in women than men, with a relative risk of 1.5 even after adjustment for greater longevity [2]. Patients with Down syndrome develop AD at a younger age, 10–20 years younger than the general population [2].

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7.1.1 Genetics of Alzheimer Disease

Onset of early AD affects members of families between the ages of 30 and 60 years of age, with at least three affected individuals in two or more generations. Most but not all families have an autosomal-dominant inheritance pattern [3]. Responsible mutations in three genes identified on chromosomes 1, 14, and 21 account for 60–70 % of early onset AD [3, 4]. These genes encode the amyloid precursor protein [APP], presenilin 1 [PSEN 1], and presenilin 2 [PSEN 2].

The genetic basis of late onset AD is more complex and until recently the only established risk factor was the apolipoprotein epsilon 4 allele gene [APOE-e4] [3]. The frequency of the APOE-e4 allele varies according to ethnicity, one e4 allele may increase the risk of late-AD 2–3 fold, whereas two copies [e4 homozygous] increase the risk 8–12 fold compared to noncarriers [3, 5]. Since 2009 genome-wide large-scale studies on the genetics of AD have revealed at least nine new risk loci [6]. These novel AD susceptibility variants' risk-increasing effect is much smaller than the APOE-e4 gene variant, and the proportion of late AD in a population that may be attributable to the nine novel variants could be as high as 35 % [6].

7.1.2 Pathogenesis of Alzheimer Disease

The neuropathology of AD was first described about 100 years ago. The hallmark of the disease consists of abundant senile plaques, composed mainly of beta-amyloid peptide Aβ-42, outside neurons, and neurofibrillary tangles inside the neurons [7]. Based on the amyloid cascade hypothesis, amyloid-β [AB], accumulation is the trigger for AD pathogenesis. The exact mechanism for neuronal injury and neurodegeneration is unknown but several postulates exist. Extracellular Aβ is in close proximity to activated astrocytes and microglial cells [tissue macrophages] which are the source of inflammatory cytokines. The endoplasma reticulum produces APP, which is important for maintaining synaptic function, but is degraded by proteolytic enzymes [a, b, and γ-secretase]which are increased with aging and AD, to form amyloid peptides. The presenilins 1 and 2 function as γ-secretase and mutations in the encoding genes result in excessive long-chain peptides [Aβ-42], which maybe cytotoxic and highly amyloidogenic [8]. However, the burden of Aβ does not correlate well with the degree of neurodegeneration and dementia. The numbers of neurofibrillary tangles correlate better with neuronal loss and dementia than the amount of amyloid plaques [9]. In animal experiments the amount of neurofibrillary tangles is a marker of neurotoxic effect and neuronal response, and the critical component appears to be a modified tau protein [7, 10]. The tau protein is a normal constituent of microtubules of the cytoplasm of neurons and promotes growth, membrane interactions, and functioning of nerve terminals [8]. Phosphorylation of tau regulates microtubule binding and assembly. In AD the tau protein is hyperphosphorylated [phospho-tau], which then accumulates in the neurons and form fibrillar tangles. Phospho-tau leads to instability of microtubules and

subsequent neurodegeneration and is considered to be the main harmful entity [11]. Tau appears to play an essential role in the pathogenesis of AD, as reducing the level in animal models results in attenuation of neuronal dysfunction [12], and in humans the extent of tau pathology correlates with cognitive decline [13].

Implication of the amyloid cascade in the pathogenesis of AD was based on the pathological findings of accumulation of the Aβ protein in senile plaques, and in familial early AD genetic mutations lead to overproduction of Aβ. Another recent finding that a rare variant of the APP gene was associated with reduced production of Aβ and this protects the elderly from AD is supportive evidence of a pathogenic role of amyloid plaques [14]. However, failure of anti-Aβ targeted drugs in recent trials in large numbers of AD patients raises question about the critical role of the amyloid cascade in the pathogenesis of this disease [15]. Thus, the exact mechanism in the pathogenesis of AD remains unclear and the relationship between amyloid and phospho-tau accumulation, neurodegeneration, and cognitive decline needs further clarification. There is pathological evidence that AD is associated with atrophy of the rhino-temporal and hippocampal cortex.

7.1.3 Risk Factors for Alzheimer Disease

There are four well-established predispositions for late onset AD and these include: increasing age, being the strongest factor, APOE-e4 allele, Down syndrome, and recurrent traumatic brain injury. However, there is evidence of multiple other risk factors and many of these are also considered to be predisposition for atherosclerosis and vascular dementia.

Family history of a first-degree relative with dementia has a 10–30 % increased risk of developing sporadic AD, but this is lower and similar to the general population if the relative developed dementia at >85 years of age [16, 17]. Clustering of multiple risk factors may also have an additive effect [18]. In a longitudinal cohort study, the risk of vascular dementia and AD progressively increases with 1–3 risk factors, diabetes, hypertension, heart disease, and smoking, with a hazard ratio of 1.8, 2.8, and 3.4, respectively [19].

Disturbances in cholesterol metabolism and transport are directly involved in atherosclerotic diseases, vascular dementia, and sporadic AD. APOE-e4 allele is a strong genetic risk factor for late onset AD and is also a major atherosclerotic susceptibility gene factor [20]. APOE is a cholesterol transport protein found in plasma and cerebrospinal fluid [CSF] and is implicated in the cholesterol homeostasis in the brain. APOE-e4 mediates neuronal protection and repair and may be involved in Aβ deposition [21]. Oxidized APOE-e4 binds Aβ and may affect the sequestration in plaques and in addition it appears to bind to tau protein and affect the function of neurofibrillary tangles [22, 23]. Besides the effect on the lipid transport system, APOE may be involved in compensation regrowth and adaptive remodeling following AD-associated brain injury [24], and differentially affect choline acetyltransferase activity in the hippocampus of AD patients [25].

Hypercholesterolemia, independently of APOE status, has been associated with increased risk of dementia and AD in some but not all studies [18]. It is postulated that high cholesterol may enhance the formation and deposition of Aβ, or increase cerebrovascular risk and development of AD by increasing local inflammation or affect tau protein metabolism [26]. Elderly patients on statins to reduce cholesterol and cardiovascular disease have been found to have substantially reduced risk of developing dementia, independent of the presence or absence of hyperlipidemia [27]. In a rabbit model fed high cholesterol diet, hypercholesterolemia increases Aβ production and accumulation in the brain, and this effect was associated with increased levels of beta-secretase [28]. Beta-secretase expression increases with age and is elevated in the brain cortex of AD patients [29].

Several large, prospective, population-based cohort studies have found an increase of vascular dementia and AD with diabetes [18]. In a systematic review of the topic, diabetes was found to be associated with 50–100 % increased risk of AD and dementia overall, and a 100–150 % increased risk for vascular dementia [30]. Insulin modulates cognition and other activity of normal brain function, and insulin resistance is characterized by reduced brain insulin levels and activity, despite chronic elevated systemic insulin levels [31]. Insulin resistance increases level of Aβ and inflammatory mediators in brain and increases the risk of the acquired immunodeficiency syndrome [AIDS]-related memory impairment and AD.

Epidemiological studies show a greater predisposition of women to develop AD compared to men of the same age, which was assumed to be secondary to decline in estrogen levels in menopause. Therefore estrogen was thought to be protective for development of AD. However, The Women's Health Initiative Memory Study [WHIMS] found no beneficial effects of replacement estrogen, and there appeared to be an increased risk of dementia in a low-risk group [18]. It has recently been postulated that increased gonadotropin concentrations [luteinizing hormone and follicle-stimulating hormone] postmenopausal and not decreased in estrogen may be the central causative factor for greater risk of AD in elderly women [32]. Other potential risk factors with increasing cumulative evidence include recurrent trauma to the head, as in sport-related concussions, and more controversial factors such as hypertension, obesity, smoking, and alcoholism.

7.2 Biomarkers in Alzheimer Disease

The molecular pathogenesis of AD may involve four major factors, in a setting of a variety of risk factors, and these include extracellular deposition of amyloid, accumulation of intracellular phospho–tau, upregulation of inflammatory cytokines, and oxidative neuronal damage [33]. This neurodegenerative process is associated with decreased level of choline acetyltransferase in the brain. Accumulated evidence indicates that lipid peroxidation occurs early in the development of AD, even in patients with mild cognitive impairment preceding development of overt AD [34]. In a longitudinal study of 113 patients with mild cognitive impairment and 28 healthy controls,

baseline CSF samples underwent proteomic analysis by surface-enhanced laser desorption/ionization times-of-flight mass spectrometry. After 4–6 years follow-up, 57 patients progressed to AD and 56 patients remained stable. Seventeen potential biomarkers could distinguish between patients with stable mild cognitive impairment and patients who progressed to AD [35]. Five of the biomarkers were identified and may be important in the molecular pathways involved in the pathogenesis of AD.

The innate immune system is believed to play a role in the development of neurodegeneration. With increased aging there is a shift within the immunity toward a proinflammatory state. Cytokines such as tumor necrosis factor-alpha [TNF-α], or interleukin-1 beta [IL-1β], combined with interferon-gamma [IFN-γ] can affect the metabolism of APP, to increase the concentration of Aβ [36]. The production and degradation of AB can trigger chronic inflammatory processes in microglial cells and astrocytes to perpetuate the disease. In both human and animal studies there were close communication with the systemic and central brain innate immune systems. In animal models systemic inflammation exacerbates the central innate immune response which may cause progression of neurodegeneration [37]. Clinical studies in patients with AD also showed increased cognitive decline in response to systemic inflammation. Increased plasma cytokines levels have been reported in AD and vascular dementia but the results were inconsistent. In a study of 60 patients with late onset AD, 80 patients with vascular dementia, 40 subjects with cerebrovascular disease without dementia, and 42 controls, plasma levels of IL-6, TNF-α, IL-1β, and IL-10 were measured. Higher levels of IL-1β and TNF-α, but not IL-6, were associated with AD compared to controls, and high IL-6 levels were associated with vascular dementia compared to controls [38]. Another recent study measured multiple inflammatory markers in the brain of 28 nonimmunized AD patients and 11 AD patients immunized against Aβ-42 [AW1792]. The findings indicate that different microglia population exists in the brain of AD and that local inflammatory status within the gray matter is linked to tau pathology [39]. The results of this study also suggested that long-term Aβ immunotherapy could reduce microglia activation and inflammatory cytokines upregulation, and therefore possible decrease in neurodegeneration [39]. Several epidemiological studies also suggested that the inflammatory pathway was important in the pathogenesis of Aβ, as patients on anti-inflammatory nonsteroidal drugs [NSAIDS] for many years for rheumatoid arthritis had decreased risk of developing AD. However, randomized, controlled trials in patients with mild-to-moderate AD with these agents failed to show any significant improvement [40].

The cumulative evidence over the years from animal and human studies, however, strongly supports a role of chronic low-grade inflammation in the pathogenesis of AD, and the failure of anti-inflammatory drugs maybe “too little too late.” The influence of chronic inflammation on the biology of dementia has been postulated to be related to aging, influence of hormone homeostasis, environmental factors, and polymorphisms of genes encoding inflammatory mediators [41]. Support for the function of inflammation is also derived from prospective cohort and pathological studies. In a long-term study over 25 years of Japanese-American men, the Honolulu-Asian Aging Study, highly sensitive C-reactive protein [CRP] was correlated with the development of dementia. Men with CRP of the upper three

quartiles compared with those in the lowest quartile [<0.3 mg/L] had a threefold significant increased risk for AD and vascular dementia [42]. Furthermore in other studies, immunohistochemical analysis of brain samples from AD patients demonstrated that amyloid plaques and fibrillary tangles are infiltrated with activated microglial cells, attempting to phagocytose and degrade amyloid components [43]. Microglia cells also activate and recruit astrocytes and together stimulate cytokines, prostaglandins, and generate free radicals and reactive oxygen species to contribute to neuronal damage [42, 43].

There is also evidence that mitochondrial dysfunction is an early event in the mechanism of development of AD and is an initial trigger for Aβ production, which may further accelerate mitochondrial malfunction and oxidative stress [44]. Theoretically this may result in increased levels of CSF lactic acid and may warrant investigation in mild cognitive impairment or predementia, AD, and controls, to determine whether or not CSF lactic acid would be a useful biomarker of AD and the progression.

7.3 Microbes and Alzheimer Disease

Microbes as a source of chronic low-grade inflammation could potentially be important in the pathobiology of AD. Chronic infection and AD share several common pathways considered necessary for the development of the disease. It has been postulated for more than a century that microbes could be involved in the etiology of AD. At about the same time, it was discovered that *Treponema pallidum* in tertiary neurosyphilis [general paresis] resulted in dementia, brain atrophy, and cerebral amyloidosis [45]. There is no substantial data, however, to indicate that AD is due to a chronic spirochetal infection although this has been suggested.

7.3.1 Potential Microbial Agents

7.3.1.1 Viruses

Interest in the herpes group of viruses as pathogenic agents in development of AD has existed for more than three decades. Herpes simplex virus-1 [HSV-1] is the most widely studied and appealing candidate as it is a neurotropic virus that remains latent in the brain in most elderly people. It is postulated that although HSV-1 normally latently infects the trigeminal ganglia, immune protection decreases with aging and herpes reactivation may occur and spread to distant cortical cells to produce low-grade inflammation. In a population-based cohort study of 512 elderly persons, initially free of dementia, and followed for 14 years the development of AD was correlated with IgG and IgM antibodies to HSV-1. Subsequently 77(15 %) patients developed AD, and after controlling for age, gender, educational level, and APOE-4 status, subjects

with IgM antibodies showed a significantly higher risk of developing AD [hazard ratio 2.55, 95 % CI 1.38–4.72], but not for IgG antibodies [46], thus suggesting that HSV-1 reactivation may contribute to the pathogenesis of AD.

Detection of HSV-1 in brain tissue of patients with AD compared to controls without dementia has been performed in at least 12 studies by 2011 [47, 48]. A total of 556 brains were examined, 210 of 344 [72.2 %] with AD had detection of HSV-1 DNA, versus 118 of 212 [55.7 %] controls [48]. Three studies reported a significantly higher prevalence of HSV-1 DNA in AD patients with APOE-e4 compared to controls [49–51], but this was not supported by another group [52]. In another study brain samples from 34 AD patients, 40 patients with Parkinson's disease, and 40 controls were examined for herpes viruses DNA by polymerase chain reaction [PCR]. Only one AD patient [2.9 %] was positive for HSV-1 DNA, 88.2 % for human herpes virus-6 [HHV-6] and 26.5 % for varicella-zoster virus [VZV] DNA; versus in controls 25 % were positive for HSV DNA, 87.5 % for HHV-6, and 27.5 % for VZV, not significantly different between the groups [53]. Others, however, have reported the detection of HHV-6 in much higher proportion of AD patients than age-matched normal brains, 70 % vs. 40 %, $p=0.003$ [54]. The presence of raised antibodies in the CSF compared to serum has also been examined. A raised antibody index in the CSF of >1.5 was defined as indicative of reactivation of the virus. Raised intrathecal HSV-Ig G was similar between 27 AD patients and 13 age-matched controls [52 and 69 %] [55]. Raised antibody index to HHV-6 was found in 22 % of AD patients and in no controls. This study does not support the hypothesis of replicating HSV-1 as playing a substantial role in the development of AD, and a minority may have replicating HHV-6.

HSV-1 DNA has also been reported in 90 % of amyloid plaques in AD brains and 80 % of plaques of normal aged brain, which contained lower burden of plaques. However, plaque-associated viral DNA in close proximity was found in 72 % of AD and 24 % of normal brains, $p<0.001$ [56].The authors posit that HSV-1 is a major cause of amyloid plaques and that in normal aged people there is less production and greater removal of Aβ. An alternative explanation could be that this association represents an epiphenomenon, and the presence of HSV simply represents an innocent bystander effect. However, there is some in vitro and animal data that provide biologically plausible role of HSV in Aβ genesis. HSV-1 infection of cells in culture causes Aβ and phospho-tau accumulation [57–59]. Also HSV-1 infection of mice exhibits Aβ formation [60], and treatment of infected cell cultures with antiviral agents can reduce Aβ and phospho-tau production [61].

7.3.1.2 Bacteria and Alzheimer Disease

It has been known for over a century that chronic bacterial infection can be associated with amyloid accumulation. Several spirochetes have been found in the brain and CSF of patients with AD more than brains of controls, and these include oral Treponema spirochetes and even *Borrelia burgdorferi* [45]. However, these have been small studies and the findings have been inconsistent [62–64].

Chronic periodontitis is a prevalent condition and has been associated with the pathogenesis of atherosclerosis and AD. It is caused by a dominance of gram-negative anaerobic species and is associated with mild increase in systemic inflammatory markers such as CRP [65]. The prevalence of periodontitis increases with age and 50 % of people older than the age of 55 have this condition. One small study reported the detection of oral spirochetes, using PCR and monoclonal antibodies, in the brain of 14/16 [90 %] AD patients and 4/18 [22.2 %] controls [66]. In a more recent study of monozygotic twins [106 twin pairs] discordant for AD, tooth loss early in life before age 35 was found to be an increased risk factor for AD, odds ratio, 5.5 [67]. Tooth loss was used as a surrogate marker for periodontitis as it is a major reason for loss of teeth. Another more recent study reported that TNF-α and antibodies to periodontal bacteria were elevated in 18 AD patients compared to 16 normal healthy controls, and were independently associated with AD [68]. In a longitudinal study of 158 subjects, cognitively intact, data was collected annually for up to 12.5 years, and baseline antibodies to periodontal bacteria were found to be significantly increased in those developing AD several years later, compared to controls who remained cognitively intact [69]. While these studies are intriguing much larger population-based, prospective studies over many years are warranted to confirm these findings. *Chlamydia pneumoniae* is a common respiratory pathogen that infects more than 70 % of the population by age 60 years and can remain dormant intracellularly [70, 71]. *C. pneumoniae*, similar to periodontitis, has been linked to the pathogenesis of atherosclerosis and coronary artery disease. *C. pneumoniae* was first linked to late onset AD in 1998, when the organism was detected in 17/19 [89 %] AD brains and only 1/19 of control brains [72].The bacteria were localized to perivascular areas, microglial and astroglial cells, which were positive for tau protein. Subsequent studies by the same investigators reported that higher loads of *C. pneumoniae* DNA were detected in patients with APOE-e4 allele [73], and that the organism in AD brains was colocalized with amyloid plaques and neurofibrillary tangles [74]. Several investigators, however, have failed to detect *C. pneumoniae* in the brain of AD patients [75–79]. Viable *C. pneumoniae* has been cultured from the brain of 2 AD patients, and amyloid plaques have been induced in the brains of the 3-month-old nontransgenic BALB/c mice by intranasal inoculation [80, 81].These studies were reported by members of the original team of investigators first describing the association of AD with C pneumoniae and have not been confirmed by others. A combination of antibiotics, doxycycline, and rifampin with antichlamydia activity was also tested in a randomized controlled trial of 101 patients with mild to moderate dementia presumably due to AD [82]. Antibiotic treatment had minor benefit on cognitive function at 6 months and no effect at 12 months. Although this is not a very large trial and it is not powered to show even moderate clinical improvement, it does not support a major role of *C. pneumoniae* in the pathogenesis of dementia or AD. Interestingly, both tetracycline and rifampin were previously shown to have antiamyloidogenic activity in vitro, independently of their antimicrobial properties [83, 84].

Helicobacter pylori, the etiology of most peptic ulcer disease, can cause chronic persistent asymptomatic gastric infection for life from childhood and predisposed to

gastric cancer in the genetically susceptible subjects [85]. Few case-controlled studies have linked chronic *H. pylori* infection with dementia and AD. In a small case-controlled study of 30 AD patients and 30 controls, serum IgG and IgA antibodies against *H. pylori* were significantly more prevalent in AD [86]. Another small study diagnosed *H. pylori* infection by gastric biopsy and found the microorganism in 88 % [$n=50$] of AD patients compared to 46.7 % [$n=30$] of age-matched controls [87]. These investigators also found that *H. pylori*-specific IgG antibody in the blood and CSF of 27 AD patients was significantly higher than 27 controls [88]. Infection with *H. pylori* diagnosed by histology was also found to be significantly higher in 63 subjects with mild cognitive impairment [predementia] compared to 35 normal controls [89]. In a recent study from France, the impact of *H. pylori* infection was significantly associated with greater cognitive impairment, higher CSF phospho–tau, and increased homocysteine levels in 53 AD patients [90]. Increase in plasma homocystine has been reported to be an independent risk factor for dementia and AD [91]. It has been postulated that chronic atrophic gastritis secondary to *H. pylori* infection could decrease absorption of vitamin B12 and folate, leading to hyperhomocysteinemia [45]. However, the prevalence of infection is much higher in Asia than Europe, and in a study from Japan 385 AD patients and 97 controls were assessed for the rate of *H. pylori* infection. There was no significant difference in the rate of infection between patients and controls without dementia, 59.7 and 62.0 % [92].

7.4 Unraveling the Link Between Microbes and Alzheimer Disease

The common link between seemingly unrelated predisposing conditions, such as aging, diabetes, APOE-4, Down syndrome, recurrent head trauma, and chronic infections, is the presence of chronic inflammation or neuroinflammation [93]. Recent studies in the mouse model have further elucidated the role of inflammatory cytokines in the pathogenesis of AD. Vom Berg et al. [94] demonstrated that inhibiting the signal and expression of the cytokines interleukin-12 [IL-12] and interleukin-23 [IL-23] results in reduction of microglial activation, concentration of soluble Aβ, and in amyloid plaque burden [94].The current understanding of the sequence of events in the inflammatory pathway begins with neuronal stresses which trigger the expression of amyloid precursor protein by neurons, resulting in the release of soluble fragments of APP extracellularly, which activates microglia to synthesize and release IL-1, resulting in further neuronal expression of APP and stimulation of IL-12 and IL-23 with proinflammatory activity. Binding of IL-12 and IL-23 to their common receptor is associated with increased concentration of soluble Aβ, elevation of plaque density, and cognitive decline [93, 94].

There is evidence of connection between AD, APOE-4, lipid metabolism, and infections [95]. APOE, in addition to its role in lipid transport and regulation of lipid metabolism, have other physiological properties, i.e., antioxidant, antiapoptotic, immunomodulatory, and atheroprotective attributes [95].Some of the receptors

involved in APOE and lipoprotein cellular binding and uptake are also involved in virus entry and neutralizing of bacterial lipopolysaccharide. APOE-e4 may predispose to greater vulnerability to Aβ-induced oxidative damage and cytotoxicity [96, 97], lysosomal leakage [98], and increased secretion of inflammatory mediators [99] and microglia activation. In animal models APOE-e4 is associated with greater risk of spread to the brain and establishing latency with HSV-1 [100].There is also some evidence that HIV-infected patients who are APOE-4 carriers have a greater tendency for developing dementia and neuropathy than noncarriers [101]. Also in vitro APOE-e4 positive neurons with HIV infection were more prone to oxidative damage from the viral neurotoxic tat-protein than APOE-e3 cells which provided a protective effect [102].

HIV infection is the commonest infectious cause of dementia and analysis of the pathobiology may shed light on the pathogenesis of AD and the relationship with microbes. In the era before highly active antiretroviral therapy [ART], clinical dementia from subacute encephalitis was recognized in 50 % or more of patients with the acquired immunodeficiency syndrome [AIDS]. Pathological studies of the brains of patients of AIDS-dementia revealed ventricular dilatation and sulcal widening, with chronic inflammatory reaction and microglial nodules, foamy macrophages, sometimes associated with giant cells and variable areas of multifocal pale myelin, with axonal swelling and gliosis [103]. Initially pathological reports did not include the presence of amyloid plaques or neurofibrillary tangles. The HIV did not directly invade the neurons and oligodendrocytes but infected the mononuclear cells, macrophages, and microglia cells. Damage to the neurons is posited as secondary to the release of cytokines and soluble molecules produced by infected microglia. The believed pathogenesis of AIDS-dementia, although not clearly delineated, has some similarities to that of AD pathophysiology with the exception of the absence Aβ plaques and fibrillary tangles. Since the advent of ART AIDS-dementia has dramatically decreased and is replaced by HIV-associated neurocognitive disorder [HAND], which is found in nearly 50 % of HIV-chronically infected patients irrespective of viral control and CD4+ cell count [104, 105]. Clinically HAND resembles the mild cognitive impairment that may precede development of dementia or AD. Also shared risk factors between the two conditions include increased age, underlying cardiovascular disease, and elevated inflammatory markers. The neuropathogenesis of HAND is associated with neuronal damage and cell death, considered to be secondary to the inflammatory response to viral particles [i.e., tat protein], with release of numerous soluble molecules by microglia or macrophages [106]. The common key feature of both AD and HAND is the presence of neuroinflammation. Many in vitro and in vivo studies demonstrate that HIV-induced neurodegeneration is associated with macrophage/microglia production of cytokines/chemokines, excitatory neuronal injury, and oxidative stress [106]. Recent pathological studies on the brains of HIV patients since the advent of ART have reported new findings that were not appreciated before. In a study of 145 HIV-infected patients, 46 treated with ART and 99 without, significant deposition of Aβ was demonstrated by immunostaining in almost 50 %, predominantly in the frontal cortex and less abundant in the hippocampus [107]. Aβ was detected in both extracellular

plaques and within neurons, and there was a clear trend with greater amyloid deposition in patients treated with ART. The main differences noted compared to the brain of patients with AD were less abundant extracellular deposition of Aβ and the absence of intracellular fibrillary tangles [107]. Amyloid plaques can be detected in the brains of patients with AD many years before development of dementia by positron imaging markers [108]. Whereas, a recent study failed to demonstrate any amyloid deposit by similar imaging of patients with HIV and cognitive impairment/HAND [109].

Two recent clinical trials have raised doubt on the critical role of Aβ in the pathogenesis of AD, as being the main driver or force leading to the development of neurodegeneration and dementia in AD. In phase 3 double-blind, randomized trials of two humanized monoclonal antibody against Aβ, no significant improvement in cognition or functional ability was found in two large trials involving 4,000 patients combined [110, 111]. The negative results maybe an indication that treatment was started too late to result in significant benefit, or that amyloid deposition is a marker or response to neuronal injury and is not a causative factor in the genesis of AD. Thus, the role of Aβ and neurofibrillary tangles in the mechanism of disease in dementia and AD needs further elucidation. Other pathways or molecules have recently been implicated in the pathogenesis of AD. Genome-wide association studies have found a significant association of triggering receptor on myeloid cells 2 [TREM2] protein and AD [112]. TREM2 is a member of the immunoglobulin family and is a bacterial phagocyte receptor that also may be critical for microglia in the clearance of apoptotic neurons and has an anti-inflammatory role [113]. Although variants of the TREM2 gene in the population are rare [114] this low frequency variability has a medium size effect modulating disease development that is similar to that of APOE-e4 [115].There is also evidence that the metabolic pathway that may influence longevity and many chronic diseases later in life maybe important in the pathogenesis of AD. The family of nicotinamide adenine dinucleotide [NAD]-dependent protein deacetylase, termed sirtuins, appears to be important as antiaging molecules [116]. SIRT1 upregulation in mice can reduce the Aβ burden in the brain by proteolytic cleavage through the activation of α-secretase [117]. SIRT1 can also deacetylate tau protein to destabilize it and reduce neurofibrillary tangles in the brain of mice [118]. Based on our current understanding of the pathogenesis of AD, a diagrammatic outline of the mechanisms leading to its development is shown in Fig. 7.1.

7.5 Conclusion

Dementia and AD are multifactorial disorders with strong genetic predisposition, with no single factor or agent that can account for the heterogeneous predisposition of these conditions. Although infections can clearly cause dementia syndromes, the present role in the etiology of AD is conflicting and it is unlikely that microbes are the primary culprit in the genesis of this prevalent disease. However, there is significant biological plausible evidence that microbes, viruses, or bacteria may play

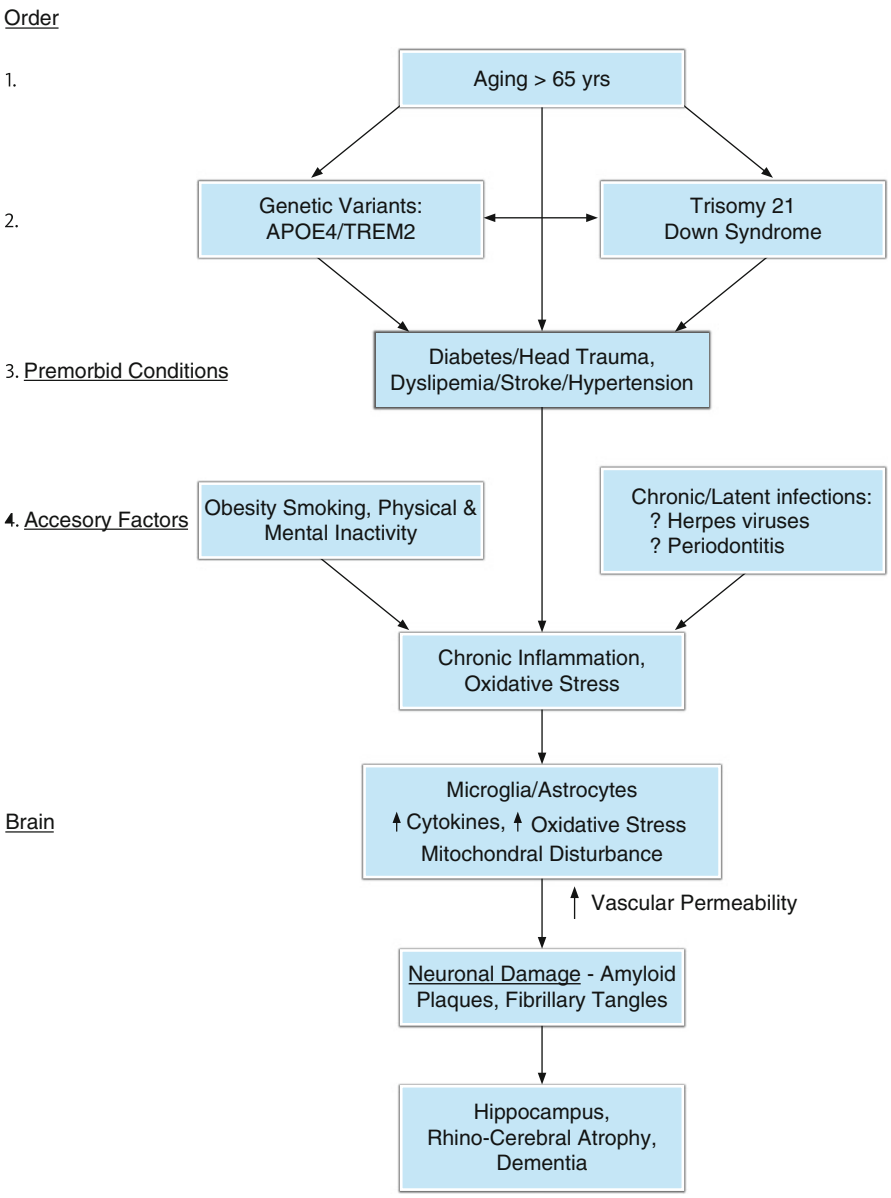

Fig. 7.1 Pathogenesis of sporadic Alzheimer disease

a secondary or adjunctive role in the pathogenesis of AD. The role of chronic infections in the presence of other factors in the genesis of AD will likely be the effect through sustained low-grade systemic inflammation that influences biologic markers in the brain to drive neuroinflammation and causing neuronal loss with eventual dementia.

Determining the reasons for recent decline in the incidence of AD reported from affluent countries would help provide clues as to the etiology of the disorder. It appears unlikely that this is due to improved lifestyle changes such as increased exercise activity and better dietary habits, as there should be a concomitant decline in rates of obesity and overweight populations. It is possible that increased liberal use of statins in the older population for prevention of cardiovascular disease could contribute to the recent decline of AD, but this will require further investigations to confirm this theory. To fully understand the mechanisms in the genesis of AD we need to explain the observation that the risk of disease is lower in subjects with higher education, who maintain an active social life and, continue physical and mental activity in older age, compared to the general population.

7.6 Future Directions

Future large population-based, longitudinal studies over 20 years are needed to clarify the possible role of chronic infections, latent or active, in the pathogenesis and development of AD. There is increasing evidence that early changes on special imaging and certain biomarkers precede the development of dementia by 15–20 years [119]. Therefore, further studies on the pathogenesis and therapeutic trials would best be performed on patients with predementia who are cognitively intact but with a high risk of AD and with imaging or biomarker changes indicative of early disease or in subjects with mild cognitive impairment. For example, a large population of people in their late 50s or early 60s could be enrolled in a longitudinal study until they reach the mid-80s and assessed on a yearly basis for periodontal disease, markers of AD, and clinical dementia. Randomized controlled trials with NSAIDS should also be tested in subjects with mild cognitive impairment or cognitively intact for an accurate assessment of their therapeutic value.

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Chapter 8
Multiple Sclerosis and Microbes

8.1 Introduction

Multiple sclerosis [MS] is a chronic demyelinating, immune-mediated disorder of the brain and spinal cord of unknown etiology. Although MS is not considered a common disease as such, it is not a rare disorder and it is estimated to be the second commonest cause of neurological disability after traumatic injuries. There is a marked geographical variation in the incidence of the disease, more prevalent in temperate regions and rare in tropical and subtropical countries. In the United States MS affects about 35,000 persons and worldwide 2.5 million people are afflicted [1]. In most temperate regions of the world [North America, Northern Europe, Southern Australia, and South New Zealand] the prevalence of MS is 0.1–0.2 % of the population, whereas in the tropics and Middle East the prevalence is 10- to 20-fold less [1]. The age of onset of MS is typically between 20 and 40 years, about 5–10 % occurs in children less than 18 years, and women are affected threefold more common than men.

Based on the geographical variation it has been proposed that sunlight exposure is protective through the production of vitamin D. Low serum vitamin D levels are common in the population of temperate zones, and prospective studies show that vitamin D deficiency is associated with a greater risk of MS and for relapses [1]. The clinical and pathological extent of MS is very variable and heterogenous, and the course is often characterized by spontaneous relapses and remissions early in the disease.

8.2 Pathobiology of Multiple Sclerosis

Initially in the early stages of MS, patchy inflammation with focal lymphocytic infiltration is the primary pathological feature in the brain, which leads to damage of the myelin and axons [2]. The inflammation is often transient with remyelination and recovery of neurological dysfunction early in the course. Over time the predominant

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pathological changes consist of widespread microglial activation with extensive chronic neurodegeneration and plaques of demyelination [2]. Normally myelin is produced by mature oligodendrocytes which are adjacent to axons of the white matter tracts in the central nervous system [CNS].

It is believed that MS is initiated by some environmental factor that stimulates autoreactive lymphocytes in the CNS. Although MS is considered an autoimmune disease, transfer of antibodies directed against self-antigens [identified in MS] failed to cause MS-like disease in animals [3]. Despite that, T-cells reactive to myelin components, especially myelin basic protein [MBP], are activated in MS patients but not in controls [4]. There is some evidence that uncontrolled autoreactive lymphocytes may induce inflammation [predominantly by perivascular CD8+ cells] to cause neuronal damage due to dysfunction of regulatory lymphocytes and regulatory mechanisms in the CNS of MS patients [5]. Failure of regulatory lymphocytes to suppress autoreactive T-cells appears to be related to overexpression of β-arrestin1, which is a promoter of naïve and activated CD4+ T-cell survival [6]. Previous animal models of experimental allergic encephalomyelitis had supported a critical role of Th1-type γ secreting cells [3], but recent studies indicate that the inflammation in MS is driven by T-lymphocyte subtype that secretes interleukin [IL]-17 under IL-23 control [7].

Myelin proteins may not be the only target of autoreactive lymphocytes, and there is evidence that antibodies against neurofascin may mediate axonal injury in MS [8]; and autoimmune response against αB-crystalline prevents counter-regulatory suppression of inflammation [9]. Cortical biopsies of brain lesions in early MS have revealed perivascular inflammation with CD3+ and CD8+ T cells in the majority of cases [which were highly inflammatory], and 27 % of cortical plaques also contained B-cells [10]. In this study [10] cortical demyelination was present in 40 % of patients and 66 % of the lesions contained foamy macrophages, and all had activated microglia, indicating ongoing demyelination.

8.2.1 Pathogenesis of Multiple Sclerosis

The pathogenic mechanisms of MS are complex and involve multiple genetic and environmental factors. Epidemiological studies have implicated increased risk of MS by gender, sex hormones, ethnic origin, geographical location/latitude/distance from the equator, smoking, viral exposure, and vitamin D status [2, 11–13]. In family studies, first-degree relatives showed a 20- to 40-fold increased risk for MS, and identical twins display 300-fold increased risk over the general population [14]. Genetic studies have reported that genes in the major histocompatibility complex [HLA] region are associated with MS [15]. The primary association was with DRB1 gene in African Americans and individuals of European descent [16, 17]. Further genome-wide association studies have reported about 50 genes associated with MS [18]. However, MS concordance rate in monozygotic twins is only approximately 30 %

and this suggests that environmental factors have a major influence on genetic trait. This would be consistent with epistatic interaction, where two or more independent factors promote disease only when combined [19]. In a large genome-wide association study, DRB1 risk alleles had the strongest association with MS, and HLA-A gene variation had a protective effect [18]. A multitude of genes encoding cytokine pathways and immune related mechanisms were overrepresented, particularly those implicated in T-helper cell differentiation, and acting on cell surface receptors. This study also implicated genes encoding pathways for vitamin D function and targets for therapies for MS, such as VCAM1 [natalizumab] and IL2RA [daclizumab] [18]. Several of the genes are also associated with other autoimmune diseases [IL2RA and IL7RA], and these pathways are involved in regulation of autoimmunity in animal models [20, 21]. It has also been suggested that several genetic variants [IL7RA, IL2RA, MGAT1, and CTLA-4] lead to dysregulation of N-glycosylation that cause pathogenesis in MS [22]. Faulty N-glycosylation of cytotoxic T lymphocyte antigen 4 [CTLA-4] and T-cell receptor [TCR] generates T-cell hyperactivity and promotes autoimmunity in mice, which induces a spontaneous MS-like disease [23, 24].

It has also been proposed that environmental factors may regulate disease manifestation by modulating the epigenome in MS to promote changes in the immune system and brain [25]. These epigenetic mechanisms include DNA methylation, regulation of noncoding miRNAs, and post-translational modification of histone that can be affected by smoking, diet, exercise, and possible previous infection.

8.2.2 Hypovitaminosis D in MS

Geographical regions of the world with limited sunshine for 4–6 months of the year, beyond the 40th parallels North or South, are those with the highest prevalence of MS [26]. Several epidemiological studies have found a vitamin D insufficiency in the great majority of MS patients, including in the early stages [27]. Vitamin D receptors are present in numerous tissues and cells, including circulating immunity cells [lymphocytes, macrophages, and monocytes], brain [microglia], intestine, bone, kidney, gonads, breast, pancreas, and cardiovascular tissues. Besides its classic role in calcium homeostasis and related metabolic functions, vitamin D and its active metabolite 1,25-dihydroxy vitamin D have other important functions: anti-inflammatory, anti-infective, immunomodulatory, antiproliferative, and neurotransmitter, which may be involved to prevent many autoimmune diseases, including MS [27]. The major action of vitamin D that may be important in the pathogenesis of MS is through immunomodulation. Hypovitaminosis D may affect cell proliferation of CD4+ T cells, the proportion and function of regulatory T lymphocytes [28–30]. Thus, in the final global analysis, the current data is supportive of hypovitaminosis D as a risk factor for MS, but acting in combination with other environmental factors in the genetically predisposed individuals.

8.3 Role of Microbes in Multiple Sclerosis

Viruses have been suggested to play a role in the development of MS since the early 1990s, and this debate still continues today [31]. There are several lines of evidence that link viral infections with MS and these include: (1) epidemiological evidence which consistently demonstrate increased risk of MS with some past infections; (2) the CD8+ lymphocyte infiltration in MS lesions is consistent with a viral-immune response; (3) and the CSF oligoclonal IgG bands typically present in MS are also present in CNS viral infections. Moreover, many naturally occurring demyelinating CNS diseases of animals and humans are of known viral origin. This topic of virus-induced demyelination was previously reviewed in 2003 [32]. There are two primary mechanisms by which viruses produce demyelinating CNS disease. The first of these is by an autoimmune process, as exemplified by postinfectious [also postvaccination] encephalomyelitis, which is preceded by a viral infection [i.e., an exanthema] with no evidence of direct invasion of the CNS [33]. For some viral demyelinating diseases there is direct CNS invasion and replication of the agent, with neuronal and axonal pathology. A very rare but well described example is subacute sclerosing panencephalitis due to chronic measles infection of the brain at an early age, <2 years old [33]. In this condition the measles virus can be recovered from the brain and histology demonstrates cytoplasmic and nuclear inclusion bodies, with signs of astrocytes and microglia activation and neuronal loss. Measles is also one of several viruses that can present more acutely with postinfectious encephalomyelitis.

In postinfectious encephalomyelitis the hypersensitivity reaction may occur both against viral and host antigen. In a study of measles postinfectious encephalitis, immune response was demonstrated to MBP with early destruction of myelin in about 50 % [34]. Other viral infections associated with demyelination include JC-virus in progressive multifocal leucoencephalopathy, seen mainly in immunosuppression, human immunodeficiency virus [HIV] itself in subacute HIV encephalopathy, and the human T-cell lymphotropic virus type 1 [HTLV-1]-associated myelopathy affecting the spinal cord [32]. There are also several naturally occurring animal viruses that produce demyelinating CNS disease, some of which are used as animal models to study the biology of MS. These include Theiler's virus, neurotropic strain of mouse hepatitis virus, and Semliki Forest virus [32].

Exposure to microbes in early childhood has also been proposed to influence the development of autoimmune disorders such as MS, the hygiene hypothesis. This could explain the geographical differences in incidence of MS, greater in developed nations of the temperate zones with advanced hygienic communities compared to poorer countries in tropical and subtropical regions with substandard hygiene, but lower rates of MS. There are also marked differences in the incidence of MS in persons migrating from one country to another in which the rates are different. In Israel, MS is common in immigrants from Europe and rare among immigrants from Africa or Asia, whereas in native born Israelis of African or Asian descent have similar rates of MS as the European migrants [35]. These differences cannot be

explained by sunlight exposure or genetic factors. Similar trends have been reported in US migrant studies with a large number of MS cases [>5,000] [36]. It is postulated that multiple infectious exposures [even carriage of parasites in the gut] could reduce the risk of MS by modulating the immunity toward helper T cells [Th]2 and regulatory T cells, with attenuation of the proinflammatory Th-1 cellular immunity [37, 38]. Decreased antigenic stimulation from low childhood infections and reduced microbial exposure burden may result in decreased levels of regulatory cytokines, Il-10, and transforming growth factor B[TGF-B], which are produced by CD 25+ T cells and other regulatory T-cells, to downregulate both Th-1 and Th-2 mediated immune responses [39]. Intestinal helminth, which induces predominantly Th-2 response, has been reported to produce a beneficial effect in patients with MS [40, 41].

8.3.1 Specific Microbes

The role of microbes in autoimmune disorders is complex, some infections can trigger autoimmune responses and others may prevent these reactions [37]. This could be related to the microorganism itself, host genetic trait, age of onset, and epistatic effect with other environmental factors.

8.3.2 Epstein–Barr Virus

Epstein–Barr virus [EBV] is strongly implicated as playing an important role in the pathogenesis of MS. EBV is a human herpesvirus that infects B-cells in nearly 95 % of the population and persists latently in the memory B-cell pool for life. It was proposed more than 50 years ago that MS may be caused by infection that is harmless to the host and confers protective immunity when acquired in early childhood, but become pathogenic later in life [42]. This could apply to EBV infection and the pattern of infection between resource endowed and resource deprived countries of the world could explain the discrepancy in geographic distribution of MS. Almost all children are infected with EBV at an early age in developing countries of tropical and subtropical regions of the world, with a seropositivity of >90 % by 4 years of age, whereas in Europe and the United States only 30–40 % are infected at the same age [42]. The prevalence of age-related EBV infection is also increased with lower socioeconomic status and overcrowding. Infection in later years is more commonly manifested by symptomatic disease and pathology, such as clinical infectious mononucleosis, peak incidence 15–25 years of age in developed countries, whereas infection at a very young age is largely asymptomatic [43]. The epidemiology of MS in developed countries is strikingly similar to that of infectious mononucleosis with respect to age of onset [44]. MS risk in EBV-negative adults is extremely low but is increased in those with previous infectious mononucleosis. There is a 20-fold increased risk of MS in adolescents

and young adults with a history of infectious mononucleosis compared to those who are EBV negative, even for similar childhood hygienic environment [45]. In a meta-analysis of 13 case–control studies comparing MS patients and matched controls, 99.5 % of MS patients were EBV seropositive compared to 94 % of controls, but the risk of MS in EBV-seronegative subjects was extremely low, odds ratio 0.6, highly significant [46]. The mean interval between primary EBV infection and onset of MS [during the vulnerable age of 15–40 years] is estimated to be 5.6 years [47]. A clinical history of infectious mononucleosis increases the risk of MS more than twofold with a relative risk of 2.3 [48].

A few prospective studies have reported that elevated IgG antibodies to EBV nuclear antigen-1 [EBNA1] were found to increase the risk of MS [49–51]. In a study involving US military personnel before onset of MS, high serum titers to EBNA1 increased the risk 36-fold for developing the disease later [51]. Seroepidemiological studies of EBV in children with MS and matched controls have shown a similar pattern as in adults but somewhat less robust. EBV-seropositivity rate in MS children varies from 86 to 94 %, compared to 64–72 % in age-matched controls [52–54]. It can be argued that EBV infection is not essential for development of MS, as 14 % of children with this disorder were EBV seronegative [53]. However, in a recent meta-analysis of 22 adult and 3 pediatric studies on the risk of development of MS in EBV seronegative individuals, it was concluded that EBV appears to be present in almost 100 % of MS patients [55].

The increased synthesis of antibodies in the CSF as reflected by the corrected Antibody Index [56], also support a role of EBV in the pathogenesis of MS [57–59]. CSF oligoclonal IgG bands in MS patients predominantly consist of antibodies against EBV proteins, EBNA1 and BRRF2 [58]. However, increase in CSF antibodies to other viruses [measles and rubella] has also been reported [56, 57]. It is proposed that EBV infection of the CNS could stimulate dominant anti-EBV antibody response and promote synthesis of other viral antibodies by infecting B-cells [60].

A key issue in determining the pathogenic role of viruses in MS is the presence of the microorganism in the CNS and demyelinating plaques. Various studies have examined brain tissue samples for EBV by different methods, such as in situ hybridization, immunohistochemistry, and PCR, in subjects with MS with conflicting results. Serafini et al. [61] previously reported that B-cells and plasma cells of 21 of 22 MS brain sections had detectable EBV by in situ hybridization and immunohistochemical stains. However, Willis et al. [62] detected EBV in only 2 of 24 MS brains by real-time [RT] PCR and none by in situ hybridization nor immunohistochemistry. A subsequent focused workshop was held in Vienna in 2011 to review the data of EBV in MS brain [63]. Overall most studies using PCR for EBV detection from brain sections or CSF failed to detect the virus, except on rare occasions. The data on detection of EBV by in situ hybridization and immunohistochemistry were more mixed with varying results [63]. Hence, unequivocal proof that EBV exist in the brain lesions of MS patients, compared to EBV-related tumors, is still lacking.

The detection of EBV DNA in the blood of MS patients and matched controls by PCR methods also had been reported. In one study from Spain, EBV was detected in the blood of 70/75 [93.3 %] MS patients versus 123/186 [66.1 %] of controls,

$p<0.001$ [64]. Moreover, dual infection with types 1 and 2 EBV was detected in 63 [90 %] of MS patients and only 37 [30 %] of controls, $p<0.001$. In contrast, in a nested study from Australia with 215 MS cases and 216 controls, detectable EBV in blood was similar [55.8 and 50.5 %], and there was no difference between the two groups in EBV DNA load [65]. However, similar to other reports, past history of infectious mononucleosis, high anti-EBV titers, and HLA-DR B1 status had additive risk for MS.

8.3.3 Human Herpesvirus-6 in Multiple Sclerosis

Human herpesvirus-6 [HHV-6], which causes roseola in early childhood, infects >90 % of the population and remain latent probably in lymphocytes. The virus has been associated with meningoencephalitis in immunocompromised hosts on occasion [66] and also has been associated with MS. There is evidence that there is cross reactivity with MBP and HHV-6 in MS patients, which could activate autoimmune reactivity through molecular mimicry [67]. Phosphorylation of HHV-6 protein U24 may confound signaling and other pathways normally utilized by phosphorylated MBP that could precipitate the pathological process in MS [68]. Some studies have reported evidence of HHV-6 reactivation with MS activity but others have not. In one study measuring viral mRNA in peripheral blood mononuclear cells [PBMC] by RT-PCR, and plasma IgG and IgM antibodies, the prevalence of HHV-6 active infection was significantly higher in MS patients than other neurological diseases and in blood donors [69]. Moreover, there was correlation with reactivation of HHV-6 and with relapsing and progressive MS. In another study with 1 year of follow-up, serum samples were analyzed by quantitative PCR to assess HHV-6 prevalence and viral load. Among 63 patients with relapsing–remitting MS only 19.1 % of samples in relapse had active infection compared to 7.9 % of samples in remission [70]. HHV-6 DNA was found in 16 of 64 [25.4 %] MS patients at least once but in none of 63 healthy blood donors, $p=0.04$ [71]. In another report from the same group of investigators, only 16 % of 105 MS patients had active HHV-6 infection versus 0 of 49 healthy controls, but the viral load was higher during attacks than during remission, $p=0.04$ [71]. A subsequent study of 57 MS patients and 57 controls followed for a year also found that reactivation or new infection with HHV-6 variant A was related to relapse of symptoms, with a prevalence of 80.7 % in MS cases and 29.8 % in controls [72]. These investigators also assessed the effect of beta-interferon [IFN-β] treatment on HHV-6 viral load in MS subjects. Treatment with IFN-β was given to 105 patients and 84 were untreated; the viral load of HHV-6 was twice as high in untreated than treated cases in relapse [73]. IFN-β treatment reduced HHV-6 viral burden in patients in relapse but not in remission.

Other investigators have reported discrepant results of the presence of HHV-6 in MS. In the Finnish twin study of 17 MS twin pairs, serum and CSF were analyzed for HHV-6 DNA by PCR and for IgG and IgM antibodies [74]. The prevalence of antibodies was similar between twins with MS and healthy twin siblings, 88 and 86 %;

and there were no detectable antibodies in any CSF sample and no HHV-6 DNA was found in serum or CSF. Other negative studies for HHV-6 DNA in serum or CSF in MS patients have been reported by several groups [75–77].

Several studies have been performed to detect the presence of HHV-6 genome or antigen in brain lesions of MS patients with varying results. HHV-6 genome was reported in acute brain lesions of all five MS cases [78] and in 58 % of established plaques [79]. However, HHV-6 viral mRNA can be detected in both MS plaques and normal appearing white matter, although at higher levels than normal control samples [80]. In another report HHV-6 DNA was detected at similar rates, 41 and 44 %, and quantity in MS and control samples [81].

A previous systematic review of the association of HHV-6 and MS was reported in 2010. Overall, 25 of 61 [41 %] studies showed a significant positive correlation but only 15 of the studies were considered of high [A] quality [82]. Thus, the role of HHV-6 in the pathogenesis of MS remains unclear and correlation with disease activity is not robust.

8.3.4 Human Retrovirus in Multiple Sclerosis

Human endogenous retrovirus [HERV] genetic elements comprise about 1–8 % of the human genome and are believed to be remnants of ancestral infections of exogenous retroviruses during our evolution [83, 84]. HERVs are divided into specific families and may occur in up to 1,000 copies distributed throughout the human genome and inherited by a Mendelian pattern [85]. Although not replication competent, HERV genes may be intact and encode functional proteins [86]. HERV have been implicated in carcinogenesis and autoimmune diseases in both animals and humans [84]. In the late 1980s a novel retrovirus element was isolated from cells derived from CSF of a MS patient and was named MS-associated retrovirus [MSRV], and later was incorporated in the HERV-W family [87]. Although HERV elements are considered normal constituents of the human genome which are rarely expressed in cells, activation in cell culture to develop viral bodies [and possibly in humans] may be precipitated by environmental and endogenous stress. Activation could be an epiphenomenon after flares of inflammatory cytokines, but there is some evidence that specific HERVs may act as auto-, super-, or neoantigen that could enhance inflammation or induce autoimmune reactions [88]. In a humanized SCID mouse model, MSRV retroviral particles injection caused acute neuropathological changes with multifocal brain hemorrhages, mediated by the expression of inflammatory cytokines through T-cell stimulation [89].

Viral RNA from HERVs has been detected by reverse-transcriptase [RT]-PCR in blood and brain of MS patients, but not exclusively [88]. It has been postulated that herpesviruses may activate [transactivation] HERV-W particles and enhance immunopathological reactions in MS [90, 91]. In a recent in vitro study, EBV activated the potentially neuropathogenic HERV-W/MSRV/syncytin-1 in cells derived from blood and brain [92]. The authors proposed a model that include EBV as initial

trigger of future MS, and years later interaction of HERV-W/MSRV/syncytin-1 contributes to MS pathogenicity, paralleling the observed relationship of EBV infection in MS patients. Several studies have found elements of HERV-W family in blood, CSF, and brain lesions of MS subjects and significantly less in controls [93–95]. HERV particles in CSF of early MS cases, followed for 10 years, were associated with greater risk of disability and progression of disease in a small number of subjects [96]. Also B-cells and monocytes from patients with active MS exhibit increased surface expression and high antibody reactivities in sera to HERV epitopes, more than stable MS cases and controls [97].

However, other studies have failed to confirm the association of MS and HERV elements. In a study of 92 CSF samples, 48 from MS patients and 23 from other inflammatory neurological diseases, and 21 from patients with non-inflammatory CNS diseases no HERV sequences were found in any sample [98]. Analysis of humoral and cellular immune responses against MSRV/HERV-W antigens in 50 MS cases and 59 controls, in another study failed to detect any appreciable immune responses [99]. The majority of HERVs are present in 100 % of healthy humans and the paucity of functional genes argues against a causative role in disease. However, recently a new class of polymorphic HERVs has been described with widespread differences in geographic and racial distribution that could explain the geographic variation in MS distribution, if implicated in disease pathogenesis. A subtype, HERV-K113 is present in 0–28 % of humans and could be a disease causing HERV [100]. In a large family-based study, genomic DNA samples from 951 MS patients and 1,902 unaffected parents were tested for the presence of HERV-K113 allele by PCR [101]. HERV-K113 provirus was detected in only 70 of 951 [7.3 %] MS patients and 6.5 % in the parents, which did not support a role in MS. To cloud the issue further, another member of the HERV-K family has been reported to be associated with MS in a large study population. HERV-K18 is considered an EBV-associated superantigen and is a plausible candidate to influence the genetic susceptibility to MS. In a nested case–control study of 207 MS cases and 403 matched controls, with analysis replicated in 909 MS patients and 339 controls, risk of MS was threefold higher in individuals with HERV-K18 env alleles [102].

8.4 Conclusion

Microbes, especially viruses that remain latent in the host for life, are biological plausible triggers or key factors in the development and pathogenesis of MS. This maybe through molecular mimicry, alteration of the immune response to other antigens, and by genetic influence [through HERV elements], acting in a manner similar to gene variation such as with single nucleotide polymorphism. However, it is difficult to explain the discrepancies noted with different viruses in various studies and their findings on the relationship with MS. The most likely explanation is the difference in methods of detection used, which are not standardized or commercialized but locally developed without independent validation.

The present data indicate that the timing of primary EBV infection at a certain age or period in life [teenage to young adulthood] in those genetically susceptible plays an important role in the development of MS. The exact mechanism of this relationship remains elusive. The hypothesis that genetic influence of ancestral endogenous retrovirus interaction with latent EBV is an attractive paradigm but remain unproven.

8.5 Future Direction

Further studies on the role of microbes in MS need to concentrate on the most attractive theory with the best data available, in order to expend valuable resources on a large, prospective cohort with observation over several years. This would preferably be implemented by an international collection of interested investigators, with different interest and expertise, using standardized or validated methods of investigation, repeated at intervals over the years from blood, CSF, and brain samples where feasible. Such a study would be best performed in subjects with strong family history or evidence of genetic predisposition to MS.

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Chapter 9
The Role of Infections and Microbes in Atherosclerosis

9.1 Introduction

Atherosclerosis and its complications, strokes or heart attacks and gangrene of limbs, are the leading cause of death worldwide. It is a multifactorial disease with no single cause and the process of atherosclerosis starts in all humans from childhood, but without symptoms until later adulthood. The development of atherosclerosis of arteries is a complex, active process and is not just deposit of cholesterol. The concept that atherosclerosis involves chronic low-grade inflammation as a result of response to injury exists for well over a century [1]. Soon after the germ theory of disease was established by Robert Koch, in the latter part of the 19th century, it was proposed in Europe that atherosclerosis was the result of a microbial infection. Although this is an unproved hypothesis it is still a contentious issue. Interest in infections and relationships with atherosclerosis and coronary artery disease [CAD] was reignited in the late 1990s after several studies showed association with CAD and *Chlamydia pneumoniae* infection and periodontitis [2]. Although in vitro and ex vivo biological studies and animal models supported the concepts of microbes playing a major role in the pathogenesis of atherosclerosis [3], this was not confirmed by several therapeutic clinical trials. Thus, after a series of negative antibiotic trials in adults with CAD, interest on this topic dramatically declined in the past decade. However, since the advent of highly active antiretroviral therapy [ART] for human immunodeficiency virus [HIV], associated with longer survival of HIV infected patients and higher prevalence of symptomatic CAD in this population, there is again renewed interest in the association of atherosclerosis and microbes.

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9.2 Biology of Atherosclerosis

The pathobiology of atherosclerosis has been extensively studied for more than 60 years, and the mechanisms of developing the pathological changes of the blood vessel walls are well established. The progression of the pathological changes is over many years and starts in young children as early lesions that are small lipid enriched deposits that do not affect blood flow but precede advanced lesions. Advanced atheromas cause disorganization of the structure of the intima and alter the contour of the arterial segment, and with progression leads to narrowing of the vessel lumen [4]. The earliest changes in the development of atherosclerosis are endothelial dysfunction and denudation of the endothelium, associated with adhesions of leukocytes and platelets and increase in vascular permeability. There are many potential causes of endothelial dysfunction that could initiate the process, including genetic alteration, traditional risk factors of CAD, and infectious microorganisms [1]. Interaction of the leukocytes and endothelium in response to injury results in cascade of events with release of cytokines, growth factors, and chemokines. This results in migration across the endothelium of monocytes, T-lymphocytes, and accumulation of foamy macrophages laden with lipids or oxidized low density lipoprotein [LDL] to form early lesions or fatty streaks. Activated macrophages and activated T-cells result in upregulation of the inflammatory reaction, resulting in further accumulation of leukocytes, foamy macrophages, and smooth muscle cells. Production of growth factors and proteolytic enzymes, with fibroblast production leads to damage and repair of the vessel wall. Cell-mediated immune responses are also likely involved in atherogenesis [5]. Potential antigens that activate inflammatory response include oxidized LDL, heat shock protein [HSP] through an autoimmune response, and infectious agents [6]. Eventually mature atheromas develop around the second to third decade of life or before, consisting of a necrotic core of degenerating foamy macrophages, extracellular lipids, cell debris and dead cells, smooth muscle cells, with an overlying fibrous cap, and encroaches on the arterial lumen [4, 6]. Further progression of mature plaques involves increased deposition of smooth muscle cells and increased synthesis of collagen to form fibroatheromas which can become calcified. Most acute symptoms of ischemia [i.e., acute heart attack] do not occur from progressive narrowing of the lumen of arteries, but from complicated atheromatous plaques that fissure, rupture, or erode and produce an acute thrombus from activation of platelets and release of procoagulant material from the core. This results in acute occlusion of a partially obstructed blood vessel.

9.3 Risk Factors and Pathogenesis

There are well-established traditional risk factors for atherosclerotic diseases [i.e., CAD] and risk factors which are less well established. The traditional causative risk factors include hypercholesterolemia, hypertension, smoking, obesity

[now considered globally to be the leading factor], diabetes mellitus, dyslipidemia, and genetic predisposition [7]. The emerging risk factors include estrogen deficiency, markers of inflammatory reaction [increased fibrinogen, C-reactive protein, cytokines and adhesion molecules, phospholipase A, and amyloid A], increased clotting factors [factor VII, endogenous tissue plasminogen activator, and plasminogen activator inhibitor type 1], chronic renal failure, homocystinemia, and certain infections [7].

Chronic low-grade inflammation plays a key role in the pathogenesis of atherosclerosis. The markers of inflammation such as C-reactive protein [CRP] are predictors of future atherosclerotic ischemic events, best established for cardiovascular events such as acute myocardial infarction [AMI]. Immune-mediated inflammatory cells and effector molecules are involved in the initiation, progression, and precipitation of acute ischemic events [8]. Although the inflammatory markers are predictors of atherosclerotic vascular disease, they are likely not causal factors but may reflect the burden and extent of atherosclerosis in the body. However, it is unclear at present whether or not endogenous extravascular inflammation plays a major role on the progression or precipitation of atherosclerotic events. There is also a close association and interaction between the inflammatory and coagulation pathways. Platelets and leukocytes interaction occurs at the initial onset of atherogenesis, and tissue factor expression is increased in the developing stages of atherosclerosis, and these elements are key factors for the acute thrombotic stage of an ischemic event [9, 10]. Moreover, inflammation can activate the coagulation pathway leading to thrombosis and thrombosis itself can amplify the inflammatory pathway.

9.4 Possible Mechanisms of Infection and Microbes

There are multiple possible mechanisms by which microbes and chronic infections could influence the development and pathogenesis of atherosclerosis. Indirectly the gut microbiota may play a role in the development of traditional risk factors such as diabetes and obesity [see previous chapters on these subjects]. Chronic inflammation and infections can affect cholesterol and low-density lipoprotein [LDL] metabolism by several mechanisms to produce an environment conducive to progression of atherosclerosis [11]. Oxidative modifications of lipoproteins play a major role in atherogenesis, and inflammatory mediators induced by infection can induce LDL oxidation in vivo [12]. The main effect of infection and inflammation on the metabolism and lipoprotein is hypertriglyceridemia. This is mainly due to increase in the very low density lipoprotein [VLDL]. In chronic infections with HIV there is a change in the large buoyant LDL component to a small dense LDL [subclass B] which is more proatherogenic [13].

Another major role in atherogenesis of chronic infection/inflammation is a consequential reduction in serum levels of high density lipoprotein [HDL]. Low HDL has been shown to increase the risk of cardiovascular disease. The antiatherogenic properties of HDL may include removal of cholesterol from cells [i.e., foamy macrophages of atheromatous plaque] for elimination by the liver [reverse cholesterol transport]

and protection against LDL oxidation. Infection/inflammation result in reduction of HDL concentrations, and there is evidence of impaired ability to prevent oxidation of LDL by several mechanisms, including decrease in transferrin concentration which is an antioxidant that improves HDL function [12].

It is presently unclear whether the general increase in first phase reactants [CRP and fibrinogen] seen with acute or chronic infection plays role by themselves in the progression and exacerbation of atherosclerosis. However, there is significant clinical evidence that acute infections, including influenza and pneumonia, can precipitate acute ischemic events such as AMI. Many observational studies have found an increased incidence of acute vascular thrombosis [AMI and strokes] in previously stable patients after influenza or influenza-like illnesses [14–16]. Several case–control studies have reported that influenza vaccination before the winter season decreased the cardiovascular and cerebrovascular outcomes in high risk but stable patients [17–20]. In a large international study of 31,546 participants from 40 countries observed over 4 influenza seasons, influenza vaccination was associated with significantly lower risk of major vascular outcomes [OR = 0.62], during the years that circulating influenza matched the vaccine antigen, but not during the years that there was an incomplete match between circulating influenza and vaccine antigen [21]. In a recent meta-analysis and review of high quality studies, influenza vaccination was found to be associated with a lower risk of composite cardiovascular events, 2.9 % versus 4.7 %, $p = 0.003$ [22].

The possible explanations for these observations include a nonspecific effect of the stress of an acute infection in subjects with critical narrowing of coronary or cerebral arteries, with increased demand for oxygen and excessive secondary catecholamine production resulting in acute ischemia. In this situation it would be expected that the vascular events would occur primarily in patients with unstable CAD or advanced cerebrovascular disease. An alternative explanation is that this systemic infection causes local exaggerated inflammation in atheromas, resulting in vulnerable plaques that erode or rupture to produce acute thrombosis and ischemia [23]. These effects could be mediated by the systemic production of cytokines, chemokines, and tissue factors to facilitate acute thrombosis on narrowed abnormal arteries.

It has been postulated that microbes could initiate the activation of inflammatory cells to produce adhesion and migration through the vascular endothelium, resulting in endothelial dysfunction and the initiation of atherogenesis. This process could involve direct infection of endothelial cells, smooth muscle cells, or macrophages, or indirectly an autoimmune response via heat shock protein-60; or indirectly via bacterial lipopolysaccharide [LPS] from periodontal pathogens interacting with toll-like receptors [TLRs], scavenger receptors, and upregulation of cytokines [24, 25]. Moreover, microbes have been found both by cell culture methods and animal models to induce early lesions of atherosclerosis [fatty streaks] and caused acceleration of the existing lesions produced by lipid rich diet [24]. In addition, theoretically infections could lead to acute plaque rupture or erosion through increased upregulation of matrix metalloproteinases [MMP], increased apoptosis and necrosis, and stimulate thrombosis of plaques by increased tissue factor and activation of factor X [3].

9.5 Specific Microbes in Atherosclerosis

9.5.1 HIV Infection

Since the advent of ART and prevention of opportunistic infections, chronic conditions typical of the aging population such as cardiovascular disease, diabetes, bone disease, and non-AIDS related malignancies have become the major concerns of surviving HIV population. The rising rate of atherosclerotic vascular complications especially cardiovascular disease has become the biggest threat. The World Health Organization [WHO] projects that by 2030, ischemic heart disease will be the leading cause of death in both low and high income countries [26].

Multiple studies comparing HIV-infected patients with control subjects have consistently shown an increased risk of CAD in HIV subjects over time in several countries. Data from the California Medicaid claims of >3 million patients had shown significantly increased incidence of CAD in men age ≤34 years and in women age ≤44 [27]. In Boston data from the Partners Healthcare System also revealed an increased incidence of AMI in HIV-infected patients versus HIV-uninfected subjects, after adjusting for common CAD risk factors, relative risk [RR] for AMI 1.75 [95 % CI, 1.5–2.2] [28]. A population-based cohort study in Denmark reported a significantly increased risk of first hospitalization for CAD in HIV-infected patients compared to controls, adjusted RR, 2.2, 95 % CI, 1.62–2.76 [29]. Similar increased incidence of AMI in HIV-infected patients more than matched controls has been reported in France and Canada (Quebec) and as well in women [30–32]. The effect of HIV infection and the treatment [ART] on the incidence of cardiovascular disease appears to be greater for women than men [33]. In a recent systematic review and meta-analysis of studies on this topic, it was concluded that CAD is increased approximately twofold in HIV infection [34].

There are a few studies reported in HIV-infected patients on the detection of subclinical atherosclerosis with imaging techniques. Computed tomographic [CT] angiography can be used to assess arterial wall, size, and characteristics of atherosclerotic plaques with a high degree of reliability [35]. An increased prevalence of subclinical coronary atherosclerosis has been reported in HIV-infected men using this technique [36]. Moreover, noncalcified coronary plaques were associated with elevated soluble CD163, a marker of activated macrophages [37]. Similar findings were also reported in young asymptomatic HIV-infected women compared to HIV-uninfected controls, with similar demographic and cardiovascular risk factors [38]. The HIV-infected women had good immune control with CD 4+ T-cell count of 597 ± 297 cells/μL and were infected for 15 years and had been receiving ART for a mean of 8 years. The HIV-infected women demonstrated increased noncalcified coronary plaques and increased monocyte/macrophage activation [38].

A more recent larger multicenter, cross-sectional, cohort study has been reported in men who have sex with men, age 40–70 years, with noncontrast CT performed on 1,001 participants and coronary CT angiography on 759 of these subjects. HIV infection was associated with greater prevalence of any coronary plaques or noncalcified

plaques compared to non-HIV controls, even after adjustment for traditional risk factors, $p<0.005$ [39]. There was also greater prevalence of coronary artery stenosis ≥50 % in HIV-infected men versus uninfected men, $p=0.02$ [39]. The longer duration of ART and lower nadir CD4+ T-cell count were also associated with greater coronary artery stenosis ≥50 %, $p=0.005$. There is also evidence that HIV infection significantly impacts on pathogenic mechanisms in the early stages of atherogenesis, such as endothelial dysfunction [40, 41] and this is also seen with ART [42]. Furthermore, HIV infection or the treatment plays a role in progressive changes in the development of atherosclerosis, such as increased carotid intima-media thickness which is a validated marker of preclinical atherosclerosis [43–46].

9.5.1.1 Mechanisms in HIV-Associated Atherosclerosis

Development of atherosclerosis in HIV infection is likely a complex interaction of multiple factors, including traditional risk factors of vascular disease and factors associated with HIV infection itself, as well as the drugs used for treatment [ART]. Some traditional risk factors such as smoking, diabetes, hypertension, and dyslipidemias have been found to be increased in HIV-infected subjects over the general population [47–51], and their contribution to the development of vascular complications are probably similar to the general population. It has been found that genetic risk factors for cardiovascular disease in HIV-infected subjects had a similar effect as in normal uninfected persons [51].

The chronic inflammatory response to HIV infection itself may be causally related to the pathogenesis of atherosclerosis. There is indirect evidence that systemic inflammation from chronic noninfectious inflammatory diseases [i.e., rheumatoid arthritis] increases the risk of AMI and vascular complications from atherosclerosis [52]. Increased markers of inflammation and enhanced atherosclerosis are present in both patients with uncontrolled HIV infection and in elite controllers, who demonstrate good immune parameters and suppressed virus without any treatment [53]. Intima-media thickness of carotid arteries and highly sensitive CRP are greater in HIV-elite controllers compared to uninfected matched controls [53, 54]. It has been postulated that even in well-controlled HIV-infected patients that chronic immune stimulation and persistent inflammation occurs from bacterial translocation from the gut [55]. Bacterial LPS bind to CD14+ monocytes and macrophages to form the TLR-4/CD14 complex and induce systemic immune activation, and produce soluble CD14 [SCD14] [56]. In a study of 55 HIV-infected patients with good immunity and fully suppressed virus, serum markers of microbial translocation predicted subclinical atherosclerosis progression over 3 years but not in uninfected controls [57]. Protease inhibitor therapy in this study was not associated with changes in carotid intima-media thickness but the sample size was likely inadequate to detect a significant clinical difference.

The ART medications themselves have been implicated in the vascular complications of atherosclerosis. A large proportion of HIV-infected subjects on long-term ART have developed lipodystrophy, visceral lipohypertrophy, and metabolic disturbances conducive to accelerated atherosclerosis. HIV-infected people have higher prevalence

of dyslipidemia, insulin resistant diabetes, and consequential cardiovascular complications than the general population [58]. Fat redistribution occurs in about 50 % of HIV-infected subjects on ART for several years and general obesity is also common [59, 60]. Recent clinical and laboratory studies indicate that adipose tissue plays an important role in the systemic inflammatory state and adaptive immune response in normal host and HIV infection [61]. Both viral proteins and ART can alter adipocyte biology to enhance inflammation in untreated or treated HIV-infected persons. Excessive adipose tissue with high mitochondrial [mt] DNA load has greater capacity for excessive immune activation and sustained inflammation, whereas adipose depletion results in lower immune cells and reduced response to antigens [61].

A new biomarker of inflammation, lipoprotein-associated phospholipase A2 [Lp-PLA2], is a promising marker of cardiovascular events in the general population. Lp-PLA2, also known as platelet-activating factor acetylhydrolase, plays a key role in atherosclerosis development by oxidizing phospholipids on LDL in the arterial intima to produce oxidized free fatty acids and lysophatidylcholine, molecules that are considered the main culprits in causing arterial damage [62]. Elevated Lp-PLA2 has been shown by multiple studies to be an independent predictor of future CAD events and other vascular complications [63]. In a study of 341 HIV-infected women, high Lp-PLA2 activity was correlated with abnormal carotid intima-media thickness and coronary artery calcium [64]. ART and protease inhibitor-based treatment were significantly correlated with high Lp-PLA2 activity even after adjustment for multiple risk factors.

Protease inhibitors [PI] have been implicated in visceral fat accumulation, insulin resistance, and dyslipidemia, associated with reduced adipokine expression and insulin signaling [61]. Some PIs increase very low density lipoprotein [VLDL] particles in animal models and in HIV-infected patients [65]. It has been estimated, however, that only about half the PI effect on cardiovascular risk appears to be associated with changes in cholesterol and triglyceride levels [66]. Ritonavir, a commonly used PI to boost the blood levels of other PIs in combination, has been found in the atherosclerosis-prone mouse to enhance lipid accumulation in macrophages [foam cells] by a direct effect without plasma lipid alterations [67]. Although PIs have been associated with insulin resistance, clinical studies indicate that this is a temporary effect which correct over time by a compensatory increase in adiponectin [65].

There is evidence that insulin resistance from ART may be more likely secondary to the nucleoside reverse transcriptase inhibitors [NRTIs] [68]. It has been proposed that NRTIs, especially didanosine, stavudine, and zidovudine, cause insulin resistance and lipoatrophy by mitochondrial dysfunction [69]. Four weeks of stavudine caused impaired insulin sensitivity and a decrease in mtDNA in skeletal muscle in HIV-infected volunteers [70]. Systemic inflammation via cytokine upregulation in HIV infection itself may predispose to insulin resistance and diabetes [71]. This is associated with a baseline prevalence of diabetes in 14 % of HIV-infected men before starting ART compared with 5 % in matched controls and a fourfold increase risk of developing diabetes on ART [72]. Hence, diabetes which is a major established risk factor for atherosclerosis is increased from HIV infection itself and the medical treatment, and the incidence increases with accumulative exposure to ART [73].

9.5.2 Chlamydia pneumoniae *and Atherosclerosis*

A detailed review of the association of *C. pneumonia* with atherosclerosis including epidemiological data, biological in vitro and ex vivo studies, pathological and microbiological evidences, animal experimentations, and clinical trials was previously published by this author [24]. Since the publication of several negative therapeutic trials, no major advances have been made to settle the debate of the importance of *C. pneumoniae* in the development of atherosclerotic vascular complications. However, further in vitro and ex vivo studies have strengthened the possible biological role of *C. pneumoniae* in the pathogenesis of atherosclerosis [3].

There were over a dozen clinical therapeutic trials using antimicrobials with antichlamydial activity that failed to show any clinical benefit in patients with established CAD [74]. This was based on the premise that a high proportion of patients with CAD, based primarily on serology, had viable latent intracellular *C. pneumoniae* in coronary arteries that could cause or accelerate progressive atherosclerosis, or precipitate acute coronary events. Although the clinical trials may have failed to show any benefit with antibiotic treatment because of an incorrect premise, there are several other explanations even if the hypothesis were correct. Serology has been found to be inaccurate in predicting the presence of *C. pneumoniae* in atheromas [3, 24], and studies using this for patients' selection may have been underpowered. Furthermore, no single antimicrobial agent was able to eradicate *C. pneumoniae* persistent latent form in either continuous cell culture methods or experimental pneumonitis animal models [75, 76]. Therefore, in the previous clinical trials the antibiotics used would not be effective in eradicating *C. pneumoniae* from coronary atheromas or even within monocytes [77]. In addition, most of the patients entered in these trials were maintained on standard statin medications, which have been shown in cell culture to suppress the inflammatory or cytokine response to *C. pneumoniae* [78]. This effect appears to be due to the reduction of *C. pneumoniae-mediated* histone modification and gene expression to downregulate cytokine production [79]. There is also evidence that statins can prevent early endothelial dysfunction of coronary arteries after acute *C. pneumoniae* infection in a swine model [80].

It is almost a decade since the results of the negative therapeutic trials of antibiotic treatment for CAD were published [81–83]. Since then there has been no major study to disprove conclusively that *C. pneumoniae* can play a role in the pathogenesis of atherosclerosis nor establish a role in this process. Several studies continue to provide support for biological plausible role of *C. pneumoniae* in atherosclerosis. These include studies that demonstrate that this microorganism can promote vascular smooth muscle cell migration [84] and can initiate HSP 60-dependent inflammation in the early stages of atherosclerosis. Previous animal studies have shown that *C. pneumoniae* can induce early changes of atherosclerosis de novo or accelerate cholesterol-induced atherosclerosis [24]. More recently *C. pneumoniae* was shown to enhance atherosclerosis produced by secondhand cigarette smoke in the mouse model [85].

Although there is no good animal model of a vulnerable atheromatous plaque to test the hypothesis of infection ability to precipitate an acute vascular event, there is some indirect evidence to suggest that this is biologically possible. It is believed that matrix metalloproteinases [MMP] play an important role in plaque erosion or rupture of vulnerable plaques, and a study of human coronary plaque specimens found a significant association of MMP-9 and the intravascular presence of *C. pneumoniae* [86]. There is also evidence experimentally that apoptosis of vascular smooth muscle cells [VSMC] is important in the thinning of the fibrous cap of atheromas that leads to plaque instability [87]. *C. pneumoniae* has been demonstrated to induce apoptosis and necrosis of human coronary artery epithelial cells [88] and can infect human VSMC [24]. In a minipig model infections with *C. pneumoniae* and influenza virus could increase coronary endothelial cell death and exacerbate cholesterol-induced intimal thickening and foam cell accumulation [89].

9.5.3 Periodontal Pathogens in Atherosclerosis

Interests in the concept that periodontitis and oral pathogens maybe important in atherogenesis started in the late 1990s and diminished after 2005. Over the past decade there have been no pivotal studies to confirm or disprove this hypothesis. Comparable to the data on *C. pneumoniae*, there are epidemiological, in vitro biological, and human histopathological and some experimental animal models to support a role of periodontitis in atherosclerosis [25, 90, 91]. See Table 9.1 for a summary of the biological mechanisms by which some microbes can influence the development of atherosclerosis.

In a cross-sectional study of 3,585 persons >40 years old, in the National Health and Nutritional Examination Survey [NHANES], a significant association was found between periodontitis and peripheral vascular disease [OR = 2.25, $p < 0.05$], which remained significant after adjustment for other factors [92]. There is also evidence of direct relationship between periodontal bacterial burden and subclinical atherosclerosis in 657 subjects assessed by carotid artery intima-media thickness [93]. In apolipoprotein E deficient [APOE–null] mice, polymicrobial infection-induced periodontal disease was associated with accelerated atherosclerosis, along with increased serum amyloid A, and increased cholesterol and triglycerides [94]. Although genetic elements of a variety of oral and gut microbes can be found in atherosclerotic plaques by molecular methods their significance remains unknown, as this could represent an innocent bystander effect from migrating phagocytic leukocytes [95].

Overall, the accumulating evidence is supportive of an indirect role of periodontal pathogens on progression of atherosclerosis through low-grade chronic systemic inflammation [90]. Oral pathogens via TLR-2-induced inflammatory responses can maintain a chronic state of low-grade inflammation systemically, at sites distant from the oral infection [96, 97].

Table 9.1 Potential mechanisms of microbes on atherosclerosis

Atheroma Stages	*C. pneumonia*	Periodontal pathogens	HIV/ART	Microbial burden/gut microbiota
Initiation	Endothelial dysfunction, invasion, HSP-related.	Endothelial dysfunction via LPS and cytokines	Endothelial dysfunction, immune activation.	Endothelial dysfunction, altered metagenome
Early disease	Induce foam cells, oxidize LDL, induce fatty steaks in rabbits.	Induce foam cells, increase CRP and amyloid A.	Increase VLDL and triglycerides, decrease HDL leads to foam cells.	Increase in IL-6, CRP, and fibrinogen enhances foam cells/fatty streaks.
Acceleration of atheromas	Enhance foam cells, SMCs, lymphocytes, cytokines, activate NF-kβ	Enhance lipid-induced atheroma in mice; upregulation of NF-kβ.	Dyslipidemia, increase Lp-PLA2 and oxidized LDL.	Altered gut metagenome decreases β carotene, lycophene, anti-inflammatory, and antioxidant activity.
Precipitation of acute events.	Increase MMP and gelatinase, apoptosis and necrosis of atheroma; increase tissue factor and PAI-1.	Increase inflammation and procoagulation via proteinase activator receptor; activate factor X.	Inflammation and ART induce insulin resistance, increase in lipid accumulation in macrophages leads to vulnerable plaque.	Increase inflammation, fibrinogen, and procoagulant state.

CRP C-reactive protein, *HDL* high density lipoprotein, *IL-6* interluekin-6, *LDL* low density lipoprotein, *LPS* lipopolysaccharide, *Lp-PLA2* lipoprotein-associated phospholipase A2, *MMP* matrix metalloproteinase, *NF-kβ* nuclear factor kappa β, *PAI-1* plasminogen activator inhibitor-1, *VLDL* very low density lipoprotein

9.5.4 *Burden of Microbes and Gut Microbiota on Atherosclerosis*

A variety of other microbes including the herpesviruses, especially cytomegalovirus [CMV] have been implicated in the pathogenesis of atherosclerosis in the past [98, 99]. However, the cumulative evidence only supports a role of CMV in patients undergoing cardiac transplantation with the development of transplant accelerated atherosclerosis [98]. Hence further details of these infections will not be addressed in this chapter.

It has been opined by some experts that no specific microbes likely play a significant function in the mechanism of atherosclerosis, but likely the burden of chronic infectious agents is important [8]. Since my previous review of this topic [99], there have been several studies to support this concept. In a prospective cohort of 1,625 participants followed for a median of 8 years, quantitative weighted index of infectious burden, including, *Helicobacter pylori*, CMV, and herpes simplex 1 and 2, was associated with the risk of first stroke [100]. Furthermore, in the stroke free participants [$n = 861$] infectious burden was associated with carotid plaque thickness in this multiethnic cohort [101]. In another multiethnic study of atherosclerosis, high antibody response to multiple pathogens showed graded and significant associations with inflammatory markers [IL-6, CRP, and fibrinogen] whereas seropositivity to a single pathogen did not [102]. It has been proposed that the high burden of latent microbes from early childhood, more common among the lower social economic strata, results in chronically higher levels of inflammatory markers [102]. These mediators then act on the vasculature to promote and enhance atherosclerosis through the accumulation of leukocytes and lipids in plaques.

The gut microbiota which has been implicated as a contributor to metabolic diseases, such as obesity and diabetes, through modulation of host metabolism and inflammation, may also contribute to the development and progression of atherosclerosis. Shotgun sequencing of gut metagenome demonstrates that the genus *Collinsella* was enriched in 12 patients with symptomatic atherosclerosis [stenotic carotid artery plaques], whereas *Roseburia* and *Eubacterium* were enriched in 13 healthy controls [103]. These differences in metagenome between patients and controls were not related to smoking, diabetes, or body mass index. The metagenome of patients were enriched with genes associated with peptidoglycan biosynthesis, which may act to prime the innate immune system and enhance neutrophil function and inflammation. While in healthy controls, the metagenomes were associated with increased prevalence of phytoene dehydrogenase and β-carotene plasma levels, and with increased synthesis of anti-inflammatory molecules [i.e. butyrate] and antioxidants to retard atherosclerosis [103].

There is also a link between the intestinal microbiota, high red meat consumption, and cardiovascular risk. Dietary L-carnitine, a trimethylamine [TMA] abundant in red meat, is metabolized by the gut microbiota to a proatherogenic species, trimethylamine-*N*-oxide [TMAO] [104]. Specific bacterial taxa in human gut were associated with dietary status and plasma TMAO concentration. Increased cardiovascular disease and major vascular events [AMI, stroke, and death] were associated with

high L-carnitine and TMAO levels in a large cohort of 2,595 persons [104]. In mice chronic dietary L-carnitine altered cecal microbiota with markedly enhanced synthesis of TMA and TMAO and increased atherosclerosis. This could be prevented with changes in the gut microbiota. Previously it was shown that the metabolism of phosphatidyl-choline [lethicin] by the gut flora contributes to the pathogenesis of atherosclerosis in animal models [105]. Plasma levels of TMAO have also been found to increase with dietary choline challenge [in 2 eggs] in healthy volunteers, suppressed with antibiotics and reappeared after withdrawal of antibiotics [106]. Furthermore, in the study of 4,007 patients, followed for 3 years, increased plasma levels of TMAO were associated with major cardiovascular adverse events, after adjustment for traditional risk factors, $p<0.001$ [106]. TMAO appears to promote macrophage foam cell formation in association with increased expression of 2 scavenger receptors associated with atherosclerosis, CD36 and SR–A1 [105]. It has been speculated that TMAO might oxidize LDL as it promotes thiol-dependent oxidant stress [107]. However, one study in hamsters found TMAO plasma levels were negatively correlated with aortic cholesteryl esters, which are considered precursors of foam cell formation [108].

9.6 Conclusion

There appears to be a complex interaction of microbes and atherosclerosis and vascular complications, and this may depend on genetic influence, diet, stage of development, and host response to the microbes. There is substantial evidence that acute infections [influenza] can precipitate acute cardiovascular events in the older population and this can be partially prevented with seasonal influenza vaccination. Although the exact mechanism is unclear, an experimental animal model demonstrated a local effect in atherosclerotic arteries with increased macrophage density in plaques [109].

HIV infection can promote atherosclerosis through sustained low-grade inflammation even when well controlled, and through the effects of medical treatment. Some ART may enhance atherosclerosis through dyslipidemia and insulin resistance, often acting in concert with traditional risk factors. It is very likely that chronic or latent infections in combination can promote atherogenesis through increase persistent low-grade inflammation. However, there is some experimental evidence that chronic immune reactivity against persistent microbial antigen in the vasculature can occur [110], but this needs to be confirmed by other studies. Another mechanism by which systemic chronic infection may enhance progression of atherosclerosis is through the modulation of paraoxonases. There is increasing evidence that paraoxonase [PON] proteins, by protecting cells from oxidative stress, anti-inflammatory properties, and enhancement of HDL-mediated cholesterol efflux, can protect or retard atherosclerosis [111]. However, chronic or persistent latent infection can reduce the antiatherogenic properties of PONs [111].

The most exciting research and findings in the last several years, however, is a possible role of the gut microbiota in the development of atherosclerosis. These new findings could tie-in the relations of age-related diseases and the biological role of the gut microbiota in such diseases as diabetes, obesity, and atherosclerosis and colon cancer [112].

9.7 Future Directions

There is no need for further antibiotic clinical trials in atherosclerotic vascular diseases, and it is unlikely that adding additional anti-inflammatory agents will improve the outcome in patients with cardiovascular disease, who are already on standard medications such as statins and aspirin. This is recently exemplified by the failure of darapladib [an inhibitor of lipoprotein-associated phospholipase A2] to improve cardiovascular outcome over standard treatment in a randomized control trials of 15,828 patients, with stable CAD over 3.7 years [113].

Future studies of the gut microbiota and microbiome need to be expanded in larger population-based prospective studies over several years to assess the relationship of diet, host intestinal microflora, plasma biomarkers of atherosclerosis, and development of subclinical atherosclerosis. Furthermore, the impact of probiotics on the biomarkers and subclinical vascular disease should be undertaken. It is of interest that a recent study in APOE [null]-mice has found that the administration of a probiotic *[Lactobacillus acidophilus]* could attenuate the development of atherosclerosis [114].

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