Immunol Res
DOI 10.1007/s12026-013-8413-z

TREATMENT OF AUTOIMMUNITY

Immunostimulation in the treatment for chronic fatigue syndrome/myalgic encephalomyelitis

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Abstract Chronic fatigue syndrome (CFS)/myalgic encephalomyelitis (ME) has long been associated with the presence of infectious agents, but no single pathogen has been reliably identified in all patients with the disease. Recent studies using metagenomic techniques have demonstrated the presence of thousands of microbes in the human body that were previously undetected and unknown to science. More importantly, such species interact together by sharing genes and genetic function within communities. It follows that searching for a singular pathogen may greatly underestimate the microbial complexity potentially driving a complex disease like CFS/ME. Intracellular microbes alter the expression of human genes in order to facilitate their survival. We have put forth a model describing how multiple species—bacterial, viral, and fungal—can cumulatively dysregulate expression by the VDR nuclear receptor in order to survive and thus drive a disease process. Based on this model, we have developed an immunostimulatory therapy that is showing promise inducing both subjective and objective improvement in patients suffering from CFS/ME.

Keywords Chronic fatigue syndrome · Microbiome · Immunostimulation · Immunopathology · Infection

Introduction

CFS/ME is characterized by unexplained severe fatigue, excessive postexercise exhaustion and malaise, sleep disturbances, cognitive impairment, sensory hypersensitivity, muscle and joint pain, headache, bowel symptoms, flu-like episodes, and severe impairment of daily functioning [1, 2]. The United States Food and Drug Administration has described it as a "serious and life-threatening condition [3]." Severely ill individuals are often bedridden and unable to perform basic tasks of work or daily living. CFS/ME is considered to have no established etiology, and no single clinical marker has been detected that is useful for diagnosis. But because CFS/ME shares characteristics with many diseases considered autoimmune or immune-mediated (e.g., symptom improvement after immunosuppressive therapy, followed by relapse) [4], it has been increasingly studied in context with these other diseases. Some autoantibodies associated with the disease have been identified [5]. At the same time, novel genome-driven microbial detection methods are revolutionizing our understanding of how microbes may drive autoimmune processes.

Over the past decades, CFS/ME has been repeatedly linked to the presence of various infectious agents, yet no single infectious agent has ever been consistently detected in patients with the disease. Furthermore, no other objective markers have been reliably detected in patients with

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Published online: 11 April 2013

Full citation: Proal AD, Albert PJ, Marshall TG, Blaney GP, Lindseth IA. "Immunostimulation in the treatment for chronic fatigue syndrome/myalgic encephalomyelitis." *Immunol Res.* 2013 Apr 11.

Immunostimulation in the treatment of chronic fatigue syndrome/myalgic encephalomyelitis

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Abstract

Chronic fatigue syndrome/Myalgic Encephalomyelitis has long been associated with the presence of infectious agents, but no single pathogen has been reliably identified in all patients with the disease. Recent studies using metagenomic techniques have demonstrated the presence of thousands of microbes in the human body that were previously undetected and unknown to science. More importantly, such species interact together by sharing genes and genetic function within communities. It follows that searching for a singular pathogen may greatly underestimate the microbial complexity potentially driving a complex disease like CFS/ME. Intracellular microbes alter the expression of human genes in order to facilitate their survival. We have put forth a model describing how multiple species – bacterial, viral and fungal – can cumulatively dysregulate expression by the VDR nuclear receptor in order to survive and thus drive a disease process. Based on this model, we have developed an immunostimulatory therapy that is showing promise inducing both subjective and objective improvement in patients suffering from CFS/ME.

Table 1. Key Points

- Several well-characterized pathogens have been associated with CFS/ME, however, because incidence varies by study, causality is uncertain.
- Molecular tools have recently identified thousands more microbes in *Homo sapiens* with the potential to contribute to disease.
- The microbial genomes that persist in *H. sapiens* interact with the human genome; humans are superorganisms.
- In lieu of a single pathogen, many microbes seem to interact collectively to cause CFS/ME.
- Those pathogens that persist inside nucleated cells interfere with transcription, translation, and cellular repair processes.
- A key mechanism of persistence is suppression of innate immunity via the VDR nuclear receptor.
- Immunostimulative therapies that reverse the VDR dysfunction in patients with CFS/ME represent a new therapeutic approach.

Introduction

CFS/ME is characterized by unexplained severe fatigue, excessive post-exercise exhaustion and malaise, sleep disturbances, cognitive impairment, sensory hypersensitivity, muscle and joint pain, headache, bowel symptoms, flu-like episodes and severe impairment of daily functioning [1, 2]. The United States Food and Drug Administration has described it as a "serious and life threatening condition."[3] Severely ill individuals are often bedridden and unable to perform basic tasks of work or daily living. CFS/ME is considered to have no established etiology and no single clinical marker has been detected that is useful for diagnosis. But because CFS/ME shares characteristics with many diseases considered autoimmune or immune-mediated (e.g., symptom improvement after immunosuppressive therapy, followed by relapse) [4], it has been increasingly studied in context with these other diseases. Some autoantibodies associated with the disease have been identified.[5] At the same time, novel genome-driven microbial detection methods are revolutionizing our understanding of how microbes may drive autoimmune processes.

Over the past decades, CFS/ME has been repeatedly linked to the presence of various infectious agents, yet no single infectious agent has ever been consistently detected in patients with the disease. Furthermore, no other objective markers have been reliably detected in patients with the syndrome. This has left researchers with pieces of a puzzle that appear to have few clear connections. Often, the response has been to assert that CFS/ME is psychosomatic, which has severely limited the patient's ability to deal with the disease. Instead, when pathogens cannot be reliably isolated from patients suffering from a disease that has an

otherwise infectious presentation, it is important to first re-examine the methods being used for microbial detection. Metagenomic-based tools have already been employed to detect pathogens in tissue and blood [6, 7]. It is important that we begin to re-examine CFS/ME in the context of these previously occult pathogens.

Infection and CFS/ME

CFS/ME was first described as a physiological disease by the U.S. CDC in 1994 [2]. For decades however, it was already suspected to have a viral or bacterial cause [1]. The syndrome has been so frequently associated with signs and symptoms of infection that it has variously been referred to as "Chronic Epstein-Barr Virus", "Postviral Fatigue Syndrome", and "Chronic Mono-nucleosis." The disease has a relapsing and remitting nature and is very often reported to have a flu-like onset. Chronic symptoms, including myalgia, weakness, arthralgia, low-grade fever, sore throat, headache, and swollen tender lymph nodes are also flu-like in nature. Furthermore, CFS/ME has often been documented following exposure to an acute pathogen [1]. For instance, long-term fatigue, CFS/ME, or both, have been reported after infectious mono-nucleosis[8], viral hepatitis[9], viral meningitis[10], giardia[11] and Q fever[10].

In addition, higher titers of several well-known pathogens have been detected in patients with CFS/ME. One of the most well studied microbes in this syndrome is Epstein Barr virus since patients often have higher titers of IgM to the EBV viral capsid antigen [12]. Also, antibodies against cytomegalovirus and human herpes virus-6, and Parvovirus B19 have been detected more often in some CFS/ME patients [13, 14]. Likewise, a higher prevalence of *Mycoplasma* has been reported in CFS/ME patients compared to healthy subjects [15].

Yet the species and prevalence of any one microbe identified in a particular cohort vary by study, limiting their utility for inferring causation. Most studies of infectious agents in CFS/ME were performed in an era dominated by culture-based microbial detection methodologies. Those using PCR do not routinely screen their primers against today's metagenomic databases and thus may have missed species diversity.

The metagenome

Rather than focusing on the study of single microbes and their genomes, the new field of metagenomics has described how entire microbial communities, through sharing of genetic traits, can be responsible for widespread metabolic dysfunction and disease. During the past five years, novel culture-independent tools, such as pyrosequencing and single cell sampling, are becoming small, inexpensive, and suitable for a laboratory setting. These have allowed researchers to identify thousands of new microbes whose metabolic pathways contribute to health and disease processes. Two recent initiatives – the NIH Human Microbiome Project (HMP) and its European-based partner, MetaHIT – have initiated many efforts to better characterize and identify the body's microbial inhabitants and their genomes (the human microbiome).

These initiatives have unexpectedly detected and characterized so many novel microbes in *Homo sapiens* that our bodies are now understood to harbor at least ten microbial cells for every human cell. The millions of genes possessed by these microbes dwarf the 20,500 genes that comprise our human genomes[16]. The microbial communities frequently and rapidly share genes via horizontal gene transfer and homologous recombination, drastically increasing the number of specific variants in the body at any given point in time. These insights have forever changed the manner in which human biology is perceived. It is clear that the human body is a superorganism whose metabolism represents an intermingling of human and microbial attributes.

Diverse viruses and bacteriophages (constituting the virome) are key components of the human microbiome. While research has focused largely on looking for only a handful of viruses in CFS/ME patients, we now know that a tremendous number of viruses are

present. Gordon *et al.* analyzed the fecal virome of monozygotic twins and their mothers [17]. Eighty one percent of the reads generated from this virome did not match those of any known viruses. Researchers at University of California San Diego recently reported that in addition to bacteria, many previously uncharacterized bacteriophages dominate the oral cavity and contribute to disease by serving as reservoirs for pathogenic gene function[18].

One of the most fascinating aspects of the HMP, MetaHIT and related initiatives is the sheer number of new bacterial genomes identified that have the potential to contribute to disease processes. As of October 2012, the Genomes Online database lists 1,965 separate bacterial genomes fully completed, with 14,743 in progress (Figure 1). Even so, a substantial fraction of the metagenome is still not well represented by these reference genomes [19]. For example, analysis of the human oral cavity by Nasidze *et al.* identified 101 bacterial genera in the mouth as well as an additional 64 genera previously unknown to science [20].

While pathogens persist on the mucosal surfaces, they are also present in tissue and blood. There is growing evidence that signature/unique blood microbiomes may be associated with one or more chronic inflammatory diseases [21]. Polybacterial and chronic infections have been detected in atherosclerotic plaque

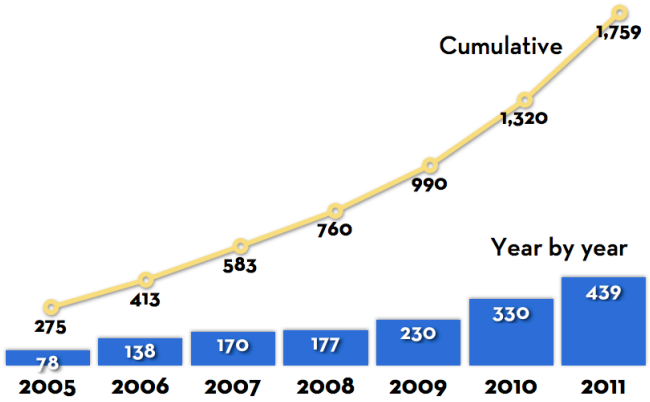


Figure 1. *Bacterial genomes in Genomes OnLine Database (GOLD) since 2005.*

[22, 23]. Amar *et al.*'s 2011 study measured blood serum concentration of 16S rDNA, a widely used bacterial marker, in 3,280 subjects without diabetes or obesity at baseline. The 16S rDNA concentration was significantly higher in those who would later develop diabetes [24]. Eighteen different bacterial taxa were recently detected in 15% of women in preterm labor with intact membranes. The positive predictive value of PCR for preterm delivery was 100% [25]. The metabolites produced by these microbes also pervade the interior of the body. The human gut microbiome alone contributes 36% of the small molecules that are found in human blood [26]. These findings are revolutionizing microbiology, and open new avenues for us to re-examine CFS/ME.

Chronic inflammatory disease appears to be polymicrobial

Most research at the intersection of infection and CFS/ME continues to be guided by Koch's postulates, which stipulate that only single species of cultivable microbes can contribute to development of a syndrome. In the era of the metagenome, efforts to understand chronic disease have forced a shift from reliance on this "one microbe, one disease" model to a focus on how entire populations of microbes can become dysregulated by their pathogenic participants. It is most likely that the structure of entire communities of microbes shift in individuals as they become ill. Indeed, one of the main goals of the HMP was to compare populations of microbes in healthy individuals with equivalent populations of microbes in their diseased counterparts. Numerous studies have revealed widespread discrepancies in the composition of microbial communities among healthy and ill individuals in chronic inflammatory conditions with overlapping symptoms to CFS/ME. These diseases include Crohn's disease[27], irritable bowel syndrome [28], rheumatoid arthritis[29], and type 1[30] and 2[31] diabetes.

Many illnesses previously believed to have psycho-somatic origins are also being tied to shifts in

microbiome composition[32]. In 1973, George Engel stated that individuals with ulcerative colitis often became ill after an emotional relationship with a maternal figure was threatened either in fact or in fantasy [33]. Now it is understood that specific bacteria of the *Enterobacteriaceae* family may act together with a disordered microbiome to increase the risk of ulcerative colitis. Lepage *et al.* found that patients with ulcerative colitis had significantly less bacterial diversity, including a higher ratio of *Actinobacteria* and *Proteobacteria* when compared to their healthy twins[34]. In 2008, Kiliç *et al.* concluded that psoriasis patients have a distinctive temperament and character when compared with the control group.[35] Recently, Gao *et al.*'s study of psoriatic patients compared to healthy controls found that psoriatic skin had double the percent of bacteria from the Firmicutes phylum as well as 84 species never before identified in the skin [36].

In 1957, Knapp and Nemetz wrote that the development of asthma was just one of the many ways in which emotional difficulties manifest themselves [37]. Yet today it is clear that the airways of asthmatics are infected by a richer, more complex collection of bacteria. Newborns who harbor certain types of bacteria in their throats, including *Streptococcus pneumoniae* and *Haemophilus influenzae* are at greater risk for developing recurrent wheeze or asthma early in life [38]. Even in cancer, psychological factors, including "malfunctionings" of the maternal sex drive, were once thought to influence disease, but such concepts have been replaced by studies increasingly tying cancers to changes in communities of infectious agents [39-41].

In addition to disease-related factors, there are profound shifts in an individual's microbiota across months, weeks, and even days [42, 32]. Small changes, such as diet alterations, can lead to rapid and pronounced shifts in the intestinal microbiome. The composition of the vaginal microbiota varies with menses [43]. Even seasonal variation or time of day can affect microbial balance. For example, the naso-pharyngeal microbiota in young children changes depending on the time of year [44]. After using microarray analysis to sample the ileal microbiome of patients with Crohn's disease, Booijink reported that in one asymptomatic patient the fluctuations in the gut microbiome composition over the course of a single day exceeded those from samples taken at the same time over several days [45]. Thus, our focus should not only be on detecting and naming species but understanding what their genomes do to drive disease dysfunction.

VDR dysregulation allows pathogens to persist

We have previously described how pathogens may interact in order to drive the dysregulation of metabolic function associated with autoimmune disease.[46] We believe that these same mechanisms drive the pathogenesis of CFS/ME. Because they persist in the cytoplasm [47], intracellular microbes already implicated in CFS/ME – such as EBV, HHV-6 and CMV – can directly interfere with the expression of human genes [47]. Bacteria have similar survival mechanisms, altering transcription and translation by human cells. This results in changes in apoptosis [48], vesicular traffic [49], membrane traffic [50], cytokine production [51], and even the release of toxins that mutate human DNA [52]. In one study, infection of a single cell by *Mycobacterium tuberculosis* resulted in changed expression of 463 proteins [53].

The ability of intracellular pathogens such as EBV and *M. tuberculosis,* to dysregulate the Vitamin D Receptor (VDR) – a type 1 nuclear receptor – provides an excellent example of how pathogens alter human gene expression in order to more effectively persist in the chronic state that drives dysbiosis – or microbial imbalance – of the microbiome. The VDR has been confirmed to regulate nearly 1,000 genes, including important components of the innate immune response [54]. In addition to Toll- like-receptor 2 (TLR2), it also controls expression of several key families of antimicrobial peptides (AMPs), including cathelicidin

and the beta-defensins. These AMPs are crucial endogenous antimicrobials that allow the innate immune system to target the intracellular pathogens [55, 56].

Cathelicidin possesses antibacterial, antiviral, and antifungal activity. Human beta-defensin plays a key role in allowing the immune system to target both gram-positive and gram-negative bacteria [57]. It also mitigates viral virulence [57]. Human beta-defensins HBD-1, HDB-2, and HBD-3 have also been shown to target the opportunistic yeast species *Candida albicans* [58], which has been detected in patients with CFS/ME. In addition the AMPs play an essential role in the protection of skin and other organs [59]. They not only kill microbes but also regulate host physiologic functions such as inflammation, angiogenesis, and wound healing [59].

This means that any microbe capable of dysregulating expression of the VDR will greatly enhance its chances of intracellular survival. In one study, EBV very effectively downregulated expression of the VDR in immature lymphoblastoid cell lines by a factor of over thirty [60]. The subsequent drop in innate immune activity allows EBV and other pathogens to persist with increasing ease. This also raises the likelihood that the host will acquire other pathogens that survive in a similar fashion. *Borrelia burgdorferi,* CMV, and *Mycobacterium leprae* have recently also been shown to slow VDR activity [61]. The VDR expresses TACO, a protein critical to intraphagocytic survival of *M. tuberculosis*, Not surprisingly then, *M. tuberculosis* has also evolved to slow activity of the receptor [53]. Other microbes create ligands that can directly dock into VDR binding sites in order to decrease transcription by the receptor. *Cytophaga, Capnocytophaga, Sporocytophaga,* and *Flexibacter* generate capnine, a sulfonic acid with a high affinity for the VDR binding pocket [62]. The fungus *Aspergillus fumigatus* has been shown to secrete gliotoxin, a toxin which dose-dependently downregulates VDR mRNA and protein levels [63]. Indeed, dysregulation of the VDR is such a logical survival mechanism that these microbes likely represent only a handful of those that persist by dysregulating the receptor.

Flow-on effects of VDR dysregulation

The primary ligand activating the VDR is 1,25-dihydroxyvitamin-D (1,25-D). VDR dysregulation reduces expression of CYP24A1, an enzyme primarily responsible for the breakdown of 1,25-D. The dysregulation of this pathway allows 1,25-D levels to rise. It is standard practice for clinicians and researchers to test only levels of the precursor 25-hydroxyvitamin-D (25-D) in patients with CFS/ME. We have found that if 1,25-D is also tested, observed levels are often elevated above normal range. Indeed, elevated levels of 1,25-D can serve as a fairly reliable marker of disease progression. We have shown that in a Canadian cohort of patients with CFS/fibromyalgia, 38 of 43 subjects had levels of 1,25-D above 110 pmol/L [64].

We have previously proposed a model describing how these elevated levels of 1,25-D can displace the native ligands of other nuclear receptors including the glucocorticoid receptor, the androgen receptor and the thyroid receptor [65, 66]. This can lead to a host of endocrine problems, including those commonly found in CFS/ME such as adrenal, thyroid, and sex hormone imbalances [67]. The subsequent dysregulation also generates further immune dysfunction since, like the VDR, each of these receptors controls the expression of several important families of antimicrobials. Brahmachary *et al.* have shown that the Androgen Receptor, the Glucocorticoid Receptor, and the Vitamin D Receptor, are respectively in control of 17, 20, and 16 families of the AMPs analyzed by the team [68]. This means that direct and flow-on effects of VDR dysregulation may well disable the bulk of the body's AMPs, leading to profound suppression of the innate immune system's ability to respond to intracellular attack.

Molecular mimicry leads to metabolic dysfunction

As an increasing number of intracellular pathogens are incorporated into the microbiome, the metabolites and proteins created by these microbes interfere with the metabolism of the host. This leads to the accumulation of foreign proteins, enzymes, mRNA and waste-metabolites in the cytoplasm. As these metabolites begin to accumulate, interactions with the host metabolites disrupt the body's metabolic pathways. A 2011 report identified tens of thousands of protein-protein interactions between the human genome and the genomes of *E. coli*, *Salmonella,* and *Yersinia* [69].

The fact that microbial and human metabolites often have very similar structures aggravates the impact of this dysregulation. For example, the pathways that allow both *Escherichia coli* and *Homo sapiens* to metabolize glucose are very similar, so that both organisms generate nearly identical intermediates in the process. The "molecular mimicry" or sequence homology between the structures of many of these products, and the genes that create them, makes it difficult for the body to distinguish "foreign" from "self." Similar mimicry creates significant systemic dysfunction and contributes to both immune and endocrine imbalances, including dysregulation of the hypothalmic pituitary axis often described in patients with CFS/ME.

Successive infection

Thus, in VDR compromised individuals, each acquired pathogen can incrementally slow the immune response, creating a snowball effect as the host metabolism shifts further away from homeostasis. Dysfunction due to molecular mimicry accumulates. The genomes of intracellular pathogens alter expression of the human immune response, facilitating subsequent infection. We refer to this process as successive infection. As the rate of successive infection increases, patients may begin to present with symptoms characteristic of CFS/ME or related inflammatory conditions. A number of studies have shown that the CFS/ME proteome differs substantially from that of healthy controls. For example**,** a 2011 analysis by Schutzer *et al.* found that while healthy subjects and patients with CFS/ME harbored many of the same proteins in the cerebrospinal fluid, 738 of the 2,783 detected proteins were unique to those with CFS/ME [70]. Because of the greater diversity of genes in the microbial metagenome, these proteomic shifts are likely a direct reflection of changes in microbiome composition.

Successive infection does not necessarily result in increased species diversity. For example, the community of vaginal bacteria in patients with bacterial vaginosis is much more taxon rich and diverse than in subjects without the disease [71]. The opposite may prove true if acquired pathogens outcompete diverse populations of more innocuous microbes. This is the case in Crohn's disease, where species diversity of the colonic microbiota of patients with the illness has been shown to diminish with increased dysfunction [72].

Because so many microbes have the potential to cause disease, it is unlikely that any two people undergoing successive infection would acquire the exact same mix of pathogens. In fact, there are a semi-infinite number of possible interactions between the human and microbial metabolomes. It follows that an individual's specific symptoms are a reflection of the location, virulence, and interplay of their pathogenic load. Thus, a particular patient's set of CFS/ME symptoms may result from their infectious history, accounting for the variety and variability of symptoms in patients with the syndrome.

A CFS/ME diagnosis requires patients to present with four out of eight required symptoms. Typically, patients with CFS/ME also suffer from a multitude of symptoms not included in the diagnostic criteria. Often a patient with CFS/ME might be diagnosed with another disease, such as rheumatoid arthritis**,** but the characteristic antibodies for that definitive diagnosis are not present. Because patients with CFS/ME suffer from such a range of diverse symptoms, some researchers have argued that CFS/ME patients should be grouped into "subsets"

that could be studied independently. However, if successive infection drives the disease process, the focus of future research should shift towards better understanding the common pathogenesis shared by all subjects.

The metabolic variability resulting from successive infection helps explain why studies that search for single pathogens in patients with CFS/ME often return equivocal results. If different pathogens slow the immune response in order to persist in a similar fashion, then no one microbe needs to be present if two people are to develop similar symptoms. Furthermore, by altering nuclear receptor activity, viruses can aid the survival of bacteria, and fungi, and vice versa. If one of the first pathogens a patient acquires is EBV, the virus may slow innate immune activity to the point where the endogenous microbiota dominates, readily resulting in a CFS/ME presentation. In this case, EBV could be described as a "trigger" or instigator of the disease. In another CFS/ME patient however, other pathogens might rapidly decrease immunity, causing the individual to acquire EBV years into the progression of their disease, causing a relapse or the presence of new symptoms. Additionally, patients who eventually receive a CFS/ME diagnosis may suffer from dysbiosis caused by non-EBV components of the microbiota and may never acquire or test positive for EBV.

High levels of stress can lower immunity by inhibiting lymphocyte populations, natural killer cell activity, and antibody production. CFS/ME is often reported to develop after a traumatic event, such as a car accident, a pregnancy, or extreme stress at the workplace. Yet many people under high levels of stress do not get CFS/ME. This suggests that patients who develop CFS/ME under/after difficult conditions may harbor pathogens whose presence is already impeding the immune response. Acquisition of a new pathogen or the difficulty of enduring a traumatic event may simply push the immune system to a critical mass such that previously subclinical infections become obvious. Reports of several CFS/ME "outbreaks" over the past decades, in which dozens of people have developed the illness at relatively the same time, may well represent this phenomenon at work. For example, in 2004 many cases of chronic fatigue were reported to occur simultaneously after a water reservoir in Bergen, Norway was contaminated with *Giardia lamblia*[11]. Nonetheless, of the approximately 48,000 people who were exposed to the contaminated water, only 5% of the people went on to develop symptoms characteristic of CFS/ME [11].

Comorbidity

A number of studies have detected enterobacteria in the gut of patients with CFS/ME. Maes *et al.* found that the prevalence and median value for serum IgA against the lipopolysaccharides of enterobacteria are significantly greater in patients with CFS/ME [73]. The presence of chronic disseminated enteroviral infection, as determined by stomach or muscle biopsy, has also been identified in a subset of patients with the syndrome. Acquisition of these pathogens as part of the successive infection process may well contribute to common CFS/ME "comorbidities" such as irritable bowel syndrome or sensitivities to certain foods.

CFS/ME patients often complain of neurological symptoms including cognitive dysfunction and memory problems. Patients with the disease are also more likely to suffer from depression and suicidal ideation. While in many cases this may simply reflect the frustration of living with a debilitating "medically unexplained" illness, an increasing number of studies are also linking mental illness to the presence of microbes. Furthermore, the ability of gut microbiota to communicate with the brain and thus modulate behavior is emerging as a provocative concept in health and disease [74]. It follows that successive infection may also contribute to many of the physical and neurological "comorbidities" associated with CFS/ME, and that these seemingly disparate conditions may best be studied in concert [46].

Autoantibodies

A number of autoantibodies have been detected in subsets of patients with CFS/ME. Several research teams have found antiphospholipid antibodies in patients with the syndrome [75]. Others have found that some CFS/ME patients have autoantibodies to components of the nuclear envelope, in particular to nuclear envelope laminin B1 molecule [76]. Moreover, antibodies to neurotransmitters – such as serotonin, adrenals, adrenocorticotropin hormone, and receptors like muscarinic cholinergic receptor 1 and mu-opioid receptor 1 – have also been detected in patients with CFS/ME. The most prominent of these autoantibodies are anti-serotonin, anti-CHRM1m, MAP2 and ssDNA [75].

However, autoantibodies are notoriously polyspecific. The autoantibodies detected in CFS/ME may actually be created in response to pathogens and thus possess a high degree of molecular mimicry. When the immune system generates antibodies in an effort to target pathogens, a proportion that are polyspecific may collaterally target human proteins. For example, Lekakh *et al.* found that autoantibodies with polyspecific activity in the serum of healthy donors were able to cross-react with DNA and lipopolysaccharides (LPS) of widespread species of bacteria including *Shigella boydii, E. coli, Salmonella,* and *Pseudomonas aeruginosa* [77].

There are numerous cases in which patients harboring infectious agents test positive for common auto-antibodies. RO, La or dsDNA autoantibodies are often generated in response to Epstein-Barr Virus [78]. Autoantibodies to ssDNA have been associated with both viral and bacterial infection. Berlin *et al.* examined blood sera from 88 patients with acute infections (41 bacterial, 23 viral, 17 parasitic, and 7 rikettsial) [79]. Elevated titers of autoantibodies including ASCA, annexin-V, prothrombin, ANA, or antiphospholipid antibodies were detected in about 50% of the subjects, with 34 individuals harboring elevated titers of at least two autoantibodies. High titers of rheumatoid factor (RF) have been detected not only in patients with rheumatoid arthritis, but also in patients suffering from a number of viral, bacterial, and parasitic infections such as subacute bacterial endocarditis.

Studies that have searched for the presence of "autoantibodies" in patients with CFS/ME have generated equivocal results. For example, while Tanaka found that mean anti-CHRM1 antibody index was significantly higher in patients with CFS/ME, a positive reaction was only found in 53.3% of CFS/ME subjects [80]. These findings reflect the variability inherent to successive infection in which patients never acquire the exact same mix of pathogens. Because most of the human microbiota is not yet well characterized, numerous antibodies are likely created in response to microbes not yet detected by current tests. We may be mistaking many of these antibodies for autoantibodies as well.

Microbes are acquired everywhere

The pathogens that contribute to successive infection are acquired in a plethora of ways. Microbes can cross the placental barrier [81] and persist in both the sperm and ovum [82]. The microbiome begins to accumulate before birth, especially if a parent already suffers from one or more inflammatory diagnoses. For example, Weyermann *et al.* has shown that infected siblings, mothers, and fathers are all major sources for *Helicobacter pylori* acquisition among young children, with the infected mother likely to be the main source for childhood.[83] Pathogens such as Human papillomavirus type 16 can also pass from mother to child during breastfeeding [84, 85].

Microbes persist in nearly every environmental niche, from the midst of glaciers to hydrothermal heat vents deep in the ocean. A 2012 high throughput sequencing study of the air in domestic homes found many microbes that are absent or rare outdoors, including taxa closely related to potential human pathogens [86]. Pinto *et al*. performed 16s rRNA gene based pyrsosequencing on water at a variety of stages in a drinking water distribution system. The team found a

consistent diversity of bacteria at every stage of the multi-stage process including 16 phyla in the disinfection tank [87].

The foods we eat contain a plethora of microbes. Recently Vignaroli *et al.* showed how genes that contribute to antibiotic resistance in farm animals could be transferred to humans via produce and other foods. [88] In the past century people commonly ate locally produced foods while today they often consume food from around the world. This greatly increases the number and diversity of pathogens potentially acquired from eating. In 2010, the U.S. imported 82.015 billion dollars of agricultural products, double the amount from years before.[89] According to the Food and Agricultural Organization of the United Nations, the global import food bill surpassed a trillion dollars in 2010 [90]. The frequency of international travel has also increased greatly over the past decades, again exposing certain individuals to a much wider array of pathogens. According to a 2012 estimate, world air travel has grown 5% per year since 1980 and is expected to almost triple over the course of the next 20 years [91].

We also acquire pathogens via blood transfusions, surgery and organ donation. For example, healthy individuals have been shown to develop the lung disease sarcoidosis after receiving an organ from a patient who suffered from the disease [92]. Recently, a number of national blood banks adopted measures to discourage or prohibit individuals diagnosed with CFS/ME from donating blood, and decline donations when aware that the potential donor has been diagnosed. These new guidelines are long overdue and extremely important.

Our treatment approach - immuno-stimulation

If successive infection drives the CFS/ME disease process, then treatments that slow the immune response make it easier for such dysbiosis to occur, increasing the likelihood of relapse over time. Paradoxically, immunosuppressive therapies often result in short-term symptom improve-ment or resolution by decreasing the cytokine, antibody and endotoxin release associated with an inflammatory response towards acquired pathogens. This temporary symptom improvement should not be mistaken for actual reversal of the disease process. The monoclonal antibody rituximab, which has recently been used to deplete the B cells of patients with CFS/ME[4], may be an example of a therapy that elicits "improvement" in this fashion. It's instructive to note that the apparent progress noted among CFS/ME patients taking the medication is almost always followed by profound relapse. Furthermore, the use of rituximab has been linked to the resurgence of latent infections including hepatitis B reactivation, multifocal leukoencephalopathy, and Pneumocystis pneumonia [93].

Subsequently, over the last decade, our group has developed a novel therapy that seeks to stimulate rather than suppress the innate immune system [94]. Instead of slowing the immune response, we aim to directly target microbes by stimulating immune defenses. Central to the treatment is the use of olmesartan medoxomil. While this drug was approved as an angiotensin II receptor agonist (ARB), we showed that it also has a high affinity for the VDR nuclear receptor**,** for which it is most likely a partial agonist [95]. In a VDR-compromised individual, olmesartan medoxomil stimulates the expression of the many endogenous antimicrobials under VDR control, and helps to gradually restore the activity of other nuclear receptors, which may have been impacted by VDR dysregulation.

In humans, cathelicidin is processed to release LL-37, a 37 amino acid peptide that is differentially spliced into a plethora of different cathelicidin-derived peptides, each with a variety of targets. Even then, LL-37 represents <20% of the cathelicidin-derived peptides. These smaller cathelicidin-derived peptides are able to target an increasing number of diverse microbial forms.

VDR expression of beta-defensin is modulated in response to cytokines, chemokines, and specific pathogenic stimuli, allowing it to target a broad range of pathogens depending on the nature of the microbial threat. Consequently, when the VDR is activated by olmesartan, the innate immune system is able to adapt its response so that patients who have accumulated very different types of pathogens can still marshal their immune defenses.

For its antihypertensive indication, olmesartan medoxomil is normally dosed at 20-40 mg once or twice a day. However, because the VDR has a short half-life (2-8 hours) before it is broken apart by protease activity, our immunostimulative therapy requires that olmesartan be dosed more frequently in order to maintain VDR expression between doses. We have found 40 mg olmesartan administered every 4-8 hours to be optimally effective. Fortunately, according to the FDA, olmesartan's antihypertensive actions begin to plateau after a single 20 mg daily dose. The maximum pressure effect is only 12 mm Hg on the systolic and 8 mm Hg on the diastolic.[96] This is easily managed by most of our cohort. Thus, physicians using the therapy have generally been able to prescribe olmesartan to these guidelines without patients suffering from morbid hypotension.

Olmesartan also has a secondary effect on immune activity. By inhibiting the ability of Angiotensin II to bind to its receptor, olmesartan reduces expression of nuclear kappa factor B (NF-kb) and the inflammatory cytokine production via that pathway. This anti-inflammatory effect offers a degree of palliation, which offsets the inflammation resulting from endogenous antimicrobial activity. Therefore, the olmesartan dosage must be individualized to minimize inflammatory symptoms. Some patients have reported that this palliative effect increases with even more frequent doses of olmesartan, but the exact mechanism for this has not yet been validated.

Olmesartan has an excellent safety profile and the U.S. FDA has not set any unsafe dosing level. The FDA package insert shows that the overall frequency of adverse events was not dose-related. In placebo controlled trials, the only adverse event that occurred in more than 1% of patients treated with olmesartan medoxomil and at a higher incidence versus placebo was dizziness (3% vs. 1%).[96] Furthermore, olmesartan has multiple beneficial effects, including the ability to reduce cardiovascular and kidney disease, inhibit liver fibrosis, prevent migraines, and reduce oxidative stress.

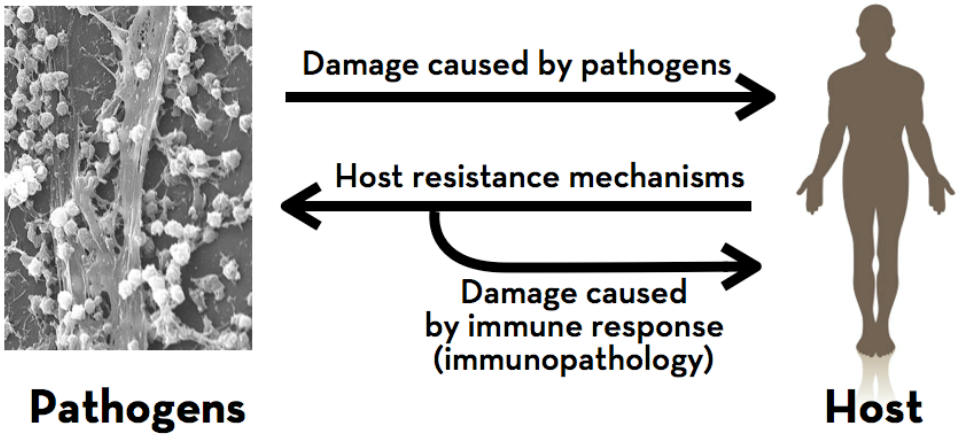
In addition to olmesartan, some patients have used low doses of pulsed bacteriostatic antibiotics including minocycline and clindamycin. After treatment with olmesartan is initiated these antibiotics appear to have greatly potentiated effects. Over the past several years we have received an increasing number of reports suggesting that olmesartan's immunostimulative properties are sufficiently potent so that many patients can achieve symptomatic improvement on the drug alone. Thus, the usefulness of prescribing the antibiotics has been de-emphasized.

Immunopathology

Despite its excellent safety profile, when CFS/ME patients are administered olmesartan according to our treatment guidelines, they generally experience a temporary surge of their CFS/ME symptoms. We do not believe these symptoms are side effects of more frequent olmesartan dosing. While such symptoms may wax and wane for months or years, if a CFS/ME patient continues our treatment for long enough, these same symptoms very frequently improve or resolve. Furthermore, healthy individuals administered these same doses of olmesartan experience no such reaction.

These symptom flares are likely due to immuno-pathology - a cascade of reactions including inflammation, cytokine release, and endotoxin release that occur as part of the immune response to microbial death (Figure 2). The death of intracellular microbes is particularly hard for the host to manage, as the body must deal with debris generated from apoptosis and phagocytosis as well as the remains of the dying

Figure 2. *Immunopathology is a cascade of reactions including inflammation, cytokine release, and endotoxin release that occur as part of the immune response against microbes (From Ruslan Medzhitov, La Jolla Immunology Conference, October 2011)*



microbes that once inhabited the cells. The adaptive immune system may also respond to the presence of this pathogenic and cellular debris, generating antibodies in the process.

Immunopathology resulting from microbicidal treatment has been documented for over a century, with symptom presentation varying depending on the nature of the pathogen targeted. First referred to as the Jarisch-Herxheimer reaction, it was originally observed during therapy of secondary syphilis using mercury. Researchers have subsequently noted this reaction in a broad spectrum of chronic diseases such as relapsing fever, *Leptospirosis*, *Brucellosis* and tuberculosis among others. Short-term immunopathology is also part of common acute infectious illness. When a patient develops the flu, symptoms are generated primarily as the immune system releases a host of cytokines and chemokines *in response* to the presence of the infectious agent [97].

More recently, an inflammatory syndrome similar to immunopathology has been documented in HIV/AIDS patients undergoing Immune Reconstitution Inflammatory Syndrome (IRIS) following treatment with Highly Active Antiretroviral Therapy (HAART) [98]. This condition occurs as HAART enables the once compromised host to target pathogens acquired during periods of severe immunosuppression. A number of prominent and easy-to-culture pathogens have been linked to IRIS: the herpes viruses, cytomegalovirus, hepatitis B and C, *Mycobacterium avium* complex, *M. tuberculosis*, and *Cryptococcus neoformans*. The presence of IRIS in culture-negative patients is common, suggesting many pathogens that cannot be detected without metagenomic tools, might also be involved.

An obvious option for managing immunopathology is the use of palliative therapies. However, because the actions of many of these medications and supplements on immune function are not well documented, we recommend limiting their use. Patients are asked to avoid exogenous vitamin D because of the seco-steroid's immunosuppressive properties. For instance, higher levels of 25-D have been positively correlated with serum antibodies to EBV, suggesting that EBV is able to better proliferate in patients who consume vitamin D [99].

A number of physicians and clinical groups have been treating CFS/ME patients with our immunostimulative therapy. We have been collaborating with them to document the therapeutic outcome.

JD is a 43 year-old operating room nurse with a long-standing history of fatigue, depression, chronic sinusitis, recurring periodontal infections, obesity and muscle pain. For several years, she was treated with Effexor and Lithium with little improvement. She suffered from recurring sore throat, sinus infections and bronchitis, which were treated with high doses of

amoxicillin, clarithromycin, cipro-floxacin and cephalexin, but never improved. In December 2005 her condition worsened to the point where she officially met the Canadian criteria for a CFS/ME diagnosis. She was forced to stop working as a nurse. One month later JD limited her vitamin D ingestion and UV exposure and was administered 40mg olmesartan four times daily. Over the course of the next three years, she was also prescribed rotating combinations of certain subinhibitory antibiotics including minocycline and clindamycin. Initially all her symptoms worsened after olmesartan admin-istration. This symptom exacerbation persisted through 2006 with several episodes of acute respiratory and sinus infections, recurring arthralgia and myalgia, mood swings, depressive episodes, insomnia, fatigue, and periodic diarrhea. By January 2007 her fatigue began to stabilize and she reported improvement in her chronic sinusitis and periodontitis. By July of the same year she reported that her muscle pain and insomnia were also markedly improved. Her depression improved somewhat, although it still fluctuated in intensity. Her weight began to stabilize and her fatigue decreased to the point where she was able to return to her nursing job part-time. By June 2008, she was able to exercise regularly and began kickboxing, and renovating her house. She no longer needed to take anti-depressant medications and her mood was improved and stable. She had no sinus or dental infections in over a year. Soon after she began working full time as a clinical case manager and staff supervisor in an extended care facility.

She experienced significant suffering over the course of treatment. Her symptoms clearly became worse before they improved. This strongly suggests that the temporary increases in symptoms were due to immunopathology. This is especially true since JD was taking the same amount of olmesartan at the start of treatment, as she was three years into therapy, when the majority of her symptoms began to resolve. We realize that the standard of care calls for physicians to reduce the suffering of their patients. Nonetheless, many treatments exist in which patients are more than willing to tolerate additional symptoms or discomfort if there is a significant possibility of long-term improvement. Chemotherapy is an obvious example.

Depending on the severity of the underlying dysregulation and the nature of the pathogenic threats, patients with CFS/ME on our treatment generally take anywhere from three to eight years to recover. This is not without precedent, as some patients experiencing IRIS or being treated for tuberculosis also often need to tolerate immunopathology for several years. Improvement on our therapy is a gradual process; so tolerating immuno-pathology generally becomes easier as the patient recovers. While this long time frame may reflect possible inadequacies associated with the therapy, it could also imply the advanced degree of dysbiosis associated with the CFS/ME disease state. While patients with other autoimmune diseases have also reported improvement on our immunostimulative therapy, our collaborating physicians report that many CFS/ME patients take longer to recover than patients with these diagnoses. CFS/ME patients often experience more severe and widespread immunopathology. This tends to underscore the relative severity of CFS/ME diagnoses.

Managing Immunopathology

While additional discomfort may be necessary in order for patients to respond, it is important that immunopathology be kept in a tolerable range. To a certain extent, the olmesartan dosing interval can be adjusted so that patients proceed at a pace that best suits their lifestyle. Those with strong support systems and ample time to rest may be able to tolerate additional immunopathology. Those still attempting to work, or with significant household responsibilities, may need to proceed more slowly. In many cases, immunopathology can be very strong and disabling. Clear and regular communication between physicians and patients is paramount.

JD's neurological comorbidities, including depression and mood swings, responded to the therapy in the

same manner as her physical symptoms. This is not surprising since, as previously described, an increasing number of neurological illnesses are being linked to the presence of microbes. We have found that a range of neurological disorders, including obsessive compulsive disorder, anxiety, dyslexia, cognitive dysfunction, and mania often respond to our treatment. Consequently, it is important that physicians actively work with their patients to ensure that neurological immunopathology remains in a tolerable range.

Subclinical Illness

It is common for some patients to develop unexpected symptoms as a result of immunopathology. The same "unmasking" of symptoms has been documented in patients experiencing IRIS.

ZW is a 51 year-old female real estate developer who developed progressive and disabling symptoms after contracting flu in January 2005. She was affected with flu symptoms for six weeks. She had an episode of loss of consciousness that resulted in a visit to the ER and subsequent internist consultation where no diagnosis was available even after extensive workup. By April 2006 she met the Canadian criteria for a CFS/ME diagnosis. Her primary symptoms were fatigue, muscle pain, anxiety, unrefreshing sleep, cognitive dysfunction (problems with short-term memory and executive functions), irritable bowel syndrome, and reduced libido. She was taking desiccated thyroid 60 mg OD. Her 1,25(OH)(2) vitamin D was at 140 pmol/L. After reducing vitamin D intake and UV exposure, she started olmesartan medoxomil 40mg four times daily in May 2006. Nearly all of her symptoms worsened or remained the same after olmesartan administration, except for her muscle pain and libido, which improved. She subsequently began taking subinhibitory doses of minocycline and azithromycin. In August and September of 2006 she rated all of her symptoms as worse, except for a continued improvement in libido. By January of 2007, her symptoms had started to improve and her fatigue had stabilized. Muscle pain and reduced libido were no longer an issue. Her 1,25-D had dropped to 90 pmol/L. Additionally she began taking subinhibitory doses of clindamycin in April 2008. This caused her symptoms to worsen again. Nevertheless, by June she once again reported improvement in her symptoms and her fatigue began to re-stabilize. She showed symptoms of hyperthyroidism requiring a reduction and then discontinuation of her thyroid hormone supplement. By July, she was regularly exercising and had hired a personal trainer. She was able to lose weight. In September she had an outbreak of herpes zoster of the right trigeminal nerve with associated headache and malaise. This resolved within a month without antiviral medications or sequelae. By July of 2009 she was asymptomatic. Nevertheless, she remained on olmesartan until May 2010 when she officially stopped the treatment. She was fully functioning in her career, had begun training in another profession, and was exercising regularly including running.

ZW had a temporary outbreak of herpes zoster virus of the right trigeminal nerve three years into treatment. This could be viewed as evidence of a new infection; we would interpret it in a different fashion. As microbes dysregulating the cell metabolism are increasingly eliminated, and infected cells replaced by more healthy counterparts, the immune system strengthens. In ZW's case, it is likely that her herpes outbreak occurred due to unmasking of a previous subclinical infection. This infection could have been acquired years before the symptoms associated with its presence became obvious.

Furthermore, some of ZW's CFS/ME-like symptoms preceded the 2005 flu-like illness. In the decade before her 2005 diagnosis, she had already reported recurring incidents of flu. In addition, she had suffered from itchy and plugged ears, constipation, mood swings, depression, and loss of libido. She had previously started thyroid hormone supplementation, after complaining of an itchy scalp and hair loss. This supports our observation that even patients who are diagnosed with "acute onset" CFS/ME still seem to accumulate the pathogens that cause their disease

gradually over time, as dysbiosis accumulates over time.

Treatment failure

We have found that nearly all CFS/ME patients who have commenced olmesartan therapy report what appears to be immunopathology. In some cases, the symptom exacerbation waxes and wanes for years before a patient notes definitive improvement. Faced with such uncertainty, some patients choose to discontinue treatment. Additionally, some patients have reported that their immunopathology is simply too strong to tolerate, and they have also stopped therapy or decided to take a break.

For example, BL is 31 year-old male who was diagnosed with CFS/ME in 2001. His main symptoms included profound fatigue, neurological hypersensitivity, sensory disturbances, cognitive deficiency, exercise intolerance, and unrefreshing sleep. He had a positive tilt table test indicating severe postural hypotension. He reduced vitamin D and ultraviolet light exposure and started olmesartan 40mg four times daily in February 2006. He also began taking subinhibitory doses of minocycline and azithromycin. Seven months later, after a period of strong immunopathology, his symptoms became overwhelming. He decided to stop therapy. Around three months later his symptoms returned to a more manageable level. Because of a lack of other viable options, he and his physician decided he should resume therapy after a three-year break. He started treatment again in November 2010. During this second attempt he used a monotherapy of olmesartan 40 mg 4-6 times daily, with no antibiotics. His immunopathology proved to be more tolerable. He continues to experience fairly strong immunopathology to this day. None of his symptoms have improved perceptibly. Some of his neurological symptoms are worse than they were pre-treatment.

BL has communicated with other patients who have improved, even after long periods of limited or no apparent response. He and his physician have chosen for him to remain on the therapy, despite discomfort, because he has exhausted other therapeutic options and there remains an expectation he may need more time before noting improvement.

Preventative and predictive medicine

The cases above describe a long and difficult path to recovery because of the immunopathology. The vast majority of CFS/ME patients tend to be quite ill upon initiating therapy. Most patients have been sick for years or decades, with many resorting to our treatment as the last in a line of previously unsuccessful therapies.

We have some reports from patients with a subset of indications for a CFS/ME diagnosis who have started our treatment quite early. Such patients tend to improve more rapidly and experience less immunopathology. This suggests that if our treatment were used as a first-line therapy, patients might encounter less immunopathology and recover more quickly. Recently, several research teams have shown that "autoantibodies" can often be detected months or years before a patient becomes symptomatic. More attention should be paid to autoantibody testing in patients with suspected CFS/ME as they could serve as a valuable new marker allowing patients to be diagnosed and treated at an earlier date. Additionally if 1,25-D is suspiciously high[64] patients could begin olmesartan therapy. Since many CFS/ME comorbidities appear to arise from a similar microbiome dysbiosis, they should be considered in the decision to withhold or commence therapy. These predictive markers, in combination, could be used to initiate therapy aimed at preventing disease progression.

Discussion

CFS/ME has been associated with signs and symptoms of chronic infection since it was first described. Still, the ability to reliably connect any single pathogen to the disease process has been stymied by culture-based studies that are often too insensitive or too selective to

detect most of the pathogens making up the human microbiome. Today, novel metagenomic technologies, including single cell sampling, are revolutionizing the field of microbiology by allowing researchers to detect microbes in cells, tissue, and blood based on their DNA sequences. Such a huge number of novel microbial species have been detected in *Homo sapiens* that the genes they express vastly outnumber our own human genes. These technologies are seldom used to search for pathogens in patients with CFS/ME. However, in patients with other autoimmune conditions, entire communities of microbes have been shown to shift over time, with pathogens interacting together in order to drive dysbiosis. Thus, Koch's postulates, which dictate that a single microbe must cause a single disease, are of little relevance in a metagenomic chronic disease**.**

We have previously described a model in which pathogen-induced nuclear receptor dysregulation via the VDR is able to drive the dysbiosis currently associated with an autoimmune disease state. We propose that CFS/ME results from this same pathogenesis. Intracellular pathogens already linked to CFS/ME, including Epstein-Barr virus and cytomegalovirus, are able to downregulate VDR activity, decreasing expression of key endogenous antimicrobials under VDR control and facilitating their survival. The host can subsequently accumulate other pathogens – bacterial, viral, and fungal – that incrementally dysregulate the VDR. Human gene expression is upregulated or downregulated by acquired components of the microbiota. Endocrine dysfunction caused by molecular mimicry increases as host cells progressively confuse microbial proteins for human proteins with similar amino acid sequences. Because there are such a huge number of discrete species in different niches**,** each patient who develops CFS/ME acquires a different mix of pathogens over time, resulting in the unique symptoms reported by each individual. "Autoantibodies", the prevalence and type of which also differ between CFS/ME patients, could be expected as the adaptive immune system responds to the presence of these microbes.

Patients with CFS/ME are responding to our novel immunostimulative therapy. Paramount to its success is the ability of patients to suffer through the immunopathology resulting from microbial death. We developed the therapy in a collaborative clinical environment where the intent was the "practice of medicine." Anticipating the spectrum of potential immunopathology may make it difficult to design a conventional controlled research environment. Nevertheless, our initial success points towards an entirely new understanding of CFS/ME and of its therapeutic options. Additionally, it appears that the disease may be prevented from progression if treatment is initiated as the first symptoms appear. Future characterization of the CFS/ME proteome and metabolome should allow earlier diagnosis and better tracking of therapeutic efficacy.

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