

# Chapter 12

## Autoimmune Disease and the Human Metagenome

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### Background

In 1922, Ernst Almquist – a colleague of Louis Pasteur – commented, “Nobody can pretend to know the complete life cycle and all the varieties of even a single bacterial species. It would be an assumption to think so” (Mattman, 2000). While Almquist’s work on idiopathic bacteria in chronic disease never received the plaudits accorded to Pasteur’s work, Almquist foresaw the complexity that would later be inherent to the field of metagenomics – a field that today forces us to examine how countless microbial genomes interact with the human genome across disease states.

Yet in the decades before novel genomic technology made a metagenomic understanding of disease possible, bacteria could only be cultured *in vitro* on a limited range of growth media. As most major diseases of the time – tuberculosis, pneumonia, leprosy, and others – were linked to the presence of a handful of acute pathogens able to grow under these constraints, a “game over” attitude toward infectious agents dominated the thinking of much of the medical community. Little consideration was given to the possible role of these pathogens in autoimmune and inflammatory disease states. Instead, for most of the twentieth century, the predominant feeling about the treatment, control, and prevention of diseases with a possible infectious etiology was optimism (Cohen, 2000).

Between 1940 and 1960, the development and successes of antibiotics and immunizations added to this optimism and, in 1969, Surgeon General William H. Stewart told the US Congress that it was time to “close the book on infectious diseases” (Avila et al., 2008). With “victory” declared, increasing emphasis was directed at the “noninfectious” diseases such as cancer and heart disease. In many cases, research on infectious disease or activities on their prevention and control were de-emphasized and resources were reduced or eliminated. As recently as the 1980s, pharmaceutical companies, believing that there were already enough antibiotics, began reducing the development of new drugs or redirecting it away from antibiotics.

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Despite this rosy narrative, some microbiologists were never convinced that drugs like penicillin had ended the war between man and microbe. In 1932, Razumov noted a large discrepancy between the viable plate count and total direct microscopic count of bacteria taken from aquatic habitats (Razumov, 1932). He found higher numbers (by several orders of magnitude) by direct microscopic counting than by the plating procedure. In 1949, Winogradsky confirmed Razumov's assessment and noted that many microbes are not satisfied with laboratory cultivation conditions. He remarked that readily cultivated bacteria in natural microbial communities "draw importance to themselves, whereas the other forms, being less docile, or even resistant, escape attention" (Relman, 1998). In 1985, Staley and Konopka pointed to Razumov's discrepancy and called it the "Great Plate Count Anomaly" (Grice et al., 2008). Their review describes work in which they compared the efficacy of a fluorescent dye versus standard plating procedures in detecting bacterial species in samples of water collected from Lake Washington. They found that only approximately 0.1–1.0% of the total bacteria present in any given sample could be enumerated by the plating procedure – causing them to conclude that, unless new methods for detecting bacteria were employed, "No breakthrough in determining species diversity seems likely in the near future."

Meanwhile, some microbiologists continued their best efforts to alter the pH and growth medium of their samples in an effort to look for previously undetected bacteria in chronic disease states. Over the course of a career spanning almost 50 years, Lida Mattman of Wayne State University cultured wall-less forms of bacteria from the blood samples of patients with over 20 inflammatory diagnoses including multiple sclerosis and sarcoidosis (Almenoff et al., 1996). She authored an entire textbook on novel approaches for *in vitro* cultivation of bacteria (Mattman, 2000). Over his 39-year career at Tulane University, Gerald Domingue published dozens of papers and book chapters devoted to the role of chronic forms of bacteria in inflammatory disease. "It is unwise to dismiss the pathogenic capacities of any microbe in a patient with a mysterious disease," he wrote. "Clearly, any patient with a history of recurrent infection and persistent disability is sending the signal that the phenomenon [infection with chronic bacteria or viruses] could be occurring. The so-called autoimmune diseases in which no organism can be identified by routine testing techniques are particularly suspect" (Domingue and Woody, 1997).

Yet, scientists like Mattman and Domingue faced serious challenges in trying to convince the medical community that their work was valid. Other research teams using less rigorous techniques often failed to duplicate their findings. Many of their observations were dismissed on the premise that their samples could have been contaminated. However, the greatest impediment toward the acceptance of this work was a set of rules set in motion by the nineteenth-century German physician Robert Koch. These rules, known as "Koch's postulates," stipulate that in order for a microbe to be deemed a causative agent of a disease, certain criteria must be met. The same microbe must be identified in every person with a given disease; the specific microbe must be able to be grown on pure culture medium in the lab; and, when reintroduced into a healthy animal or person, must produce the disease again.

While Koch's postulates may have offered certain clarity during the formative stages of the field of microbiology, the rules distracted scientists from considering the possibility that multiple species could be responsible for the onset of a single disease state. Even today, Koch's notions about disease are regularly invoked (Monaco et al., 2005) despite the emergence of a number of counter-examples. Neither *Mycobacterium leprae*, which is implicated in leprosy, nor *Treponema pallidum*, which causes syphilis, fulfill Koch's postulates, because these microbes cannot be grown in conventional culture media. Viruses further invalidate Koch's postulates because most require another living cell in order to replicate (Walker et al., 2006).

In the absence of clear connections between a single microbe and a single disease, most microbiologists necessarily assumed that the body was a sterile compartment and that inflammation, which might well suggest the presence of microbes, was attributed to an idiopathic causation. Unable to grow all but a fraction of bacteria found in the human body in the confines of a Petri dish, and constrained by a lack of technology with which to detect new microbes, the theory of autoimmune disease, in which the immune system loses tolerance and generates antibodies that target self gained momentum in the 1960s.

Yet over the past decade, the role of infectious agents in autoimmune disease has once again gained momentum. The 2004 International Congress on Autoimmunity in Budapest was themed "Autoimmunity and Infection" with many subsequent conferences and papers in the same vein. However, nearly all speakers discussed the role of viruses in autoimmune disease, whereas only a few contemplated bacteria. Autoimmune conditions were repeatedly attributed to easily cultured viruses such as Epstein-Barr and Herpes 6. Where bacteria were discussed, most reports centered on select pathogens such as *Chlamydia pneumoniae*. Yet because none of these pathogens has ever been detected in any one autoimmune disease state 100% of the time, such researchers continue to paint autoimmune diseases as a mosaic – in which the hallmarks of infection are continually present in bits and pieces but cannot be drawn into a fully cohesive picture. Yet the emerging science of metagenomics is beginning to unmask entirely new populations of microbes whose genomes allow for a means by which to bridge these gaps. The following chapter examines how this metagenomic microbiota can cause the dysfunction seen in a wide range of autoimmune conditions.

## Culture-Independent Methods for Identifying Microbes

In 2007, a study orchestrated by NASA announced that the surfaces of the supposedly sterile "clean rooms," in which technicians assemble spacecraft, host an abundance of hardy bacteria (Moissl et al., 2007). Samples taken from clean rooms at the Jet Propulsion Laboratory in California, the Kennedy Space Flight Center in Florida, and the Johnson Space Center in Houston revealed the presence of almost 100 types of bacteria representing all the major bacterial phyla; 45% of the species identified were previously unknown to science. The findings came as a shock to

NASA officials, who were left to wonder exactly how many unknown microbes might have been taken to the moon and Mars.

These clean room bacteria had not been previously detected because they could not be characterized by standard cultivation techniques. To find them, the research team had used a genomic approach – RNA gene sequence analysis – to characterize the genetic material of the bacterial species in the rooms previously touted as sterile.

Similar culture-independent tools are beginning to revolutionize our understanding of autoimmune disease by allowing for a vastly more comprehensive understanding of the microbes that persist in *Homo sapiens*, microbes that may cause the generation of autoantibodies. Genomic sequencing techniques, including 16S RNA sequencing, PCR and, more recently, pyrosequencing, have made it clear that only a fraction of those microbes that persist in the human body will grow on the limited medium of a Petri dish. With the advent of these technologies, the field of metagenomics was born. Rather than focusing on the study of single microbes and their genomes, metagenomics provides a means of analyzing aspects of microbial communities through their underpinning genetics. The amount of novel microbial genetic information that is generated on a daily basis by metagenomic analysis is so great that multidisciplinary approaches that integrate statistics, bioinformatics, and mathematical methods are required to assess it effectively.

Today, the National Institutes of Health (NIH) estimates that a mere 10% of the cells that comprise *Homo sapiens* are human cells. The remaining 90% are bacterial in origin. The number of *Escherichia coli* in a single human is comparable to the entire human global population – approximately six billion people (Staley, 1997). Such knowledge has forever changed the manner in which the human organism is perceived. We may best describe the human being as a super-organism in which communities of different organisms flourish in symbiosis with the host. Yet, even with the availability of technology to explore the microbial world in depth, to date, only a fraction of the human bacterial microbiota has been genetically identified and characterized. As of late 2009, approximately 1,100 published complete bacterial genomes had been identified with 6,000 more under review (Liolios et al., 2008). Nevertheless, there are still huge gaps in our understanding of how the microbiota contributes to human health and disease.

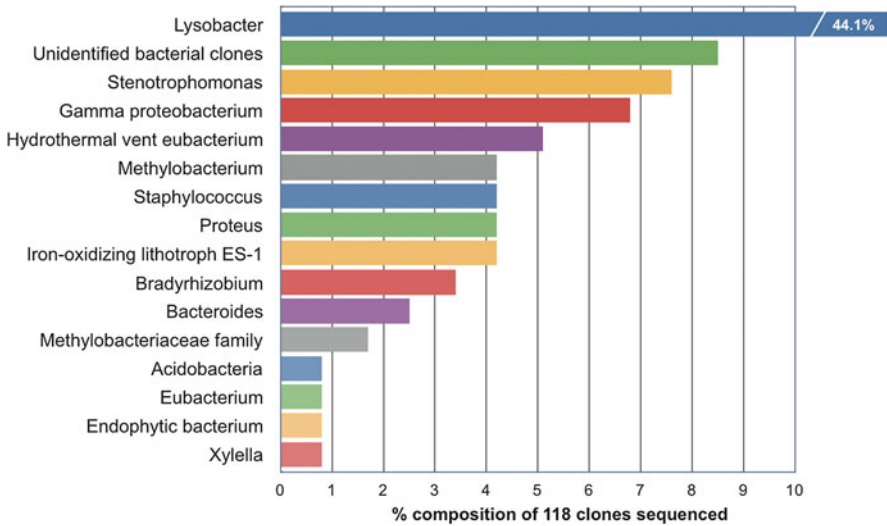
Viruses (the virome) and phages are also key components of the microbiota. Like bacteria, many of these microbes have yet to be fully characterized by high-throughput genome sequencing. However, molecular analysis has revealed that nearly all humans acquire multiple viruses, usually within the first years of life, viruses that generally remain with them throughout life. Polyomaviruses infect between 72 and 98% of humans, surviving in the kidney, lung, and skin (Virgin et al., 2009). Similarly, human herpes viruses are extremely persistent. Aneliaviruses, as well as adeno-associated virus, are now recognized to infect most humans by the end of childhood. The role of these viruses is unknown, but a significant number of people who harbor them become symptomatic later in life, suggesting that they may be capable of virulence under conditions of immune dysfunction. According

to Herbert Virgin of Washington University, “We carry, for good or for ill, many lifelong [viral] passengers” (Virgin et al., 2009).

In the next 5 years, researchers associated with the NIH Human Microbiome Project (HMP) plan to use molecular genetic sequencing in an effort to catalog the bacterial component of the human microbiome. This initiative promises to increase our knowledge of bacterial diversity. The NIH has funded many more HMP projects, with the goal that the diagnosis, treatment, and prevention of many inflammatory diagnoses can be improved by examining how the microbiota differs between those people with a disease and their healthy counterparts. Thus far, targeted conditions include Crohn’s disease (CD), inflammatory bowel disease, vaginosis, psoriasis, and other conditions now considered to be autoimmune. Early work has already demonstrated fundamental discrepancies in microbial composition between health and disease. Swidsinski et al. found that patients with irritable bowel syndrome have more bacteria from diverse genera attached to their epithelial gut surfaces than do healthy controls. Some of these microbes, such as *Bacteriodes*, were found to penetrate the epithelial layer, at times intracellularly (Swidsinski et al., 2002). Enck et al. found that irritable bowel syndrome manifests with a relative decrease in populations of bifidobacteria and significant differences in a variety of other microbes, including those that cause the production of gas (Enck et al., 2009).

Medicine has become comfortable acknowledging that bacterial populations exist in the areas of the body in contact with the external environment, such as the airways, gastrointestinal tract, mouth, skin, and vagina/penis. 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 (Nasidze et al., 2009). Yet, microbes have also been shown to persist in many other body tissues, including joints and blood vessels. Some of the same bacteria identified in the salivary microbiome, such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* – both of which cause tooth decay (Lamell et al., 2000) – have also been identified in atherosclerotic plaque (Kozarov et al., 2005). Bacterial DNA has been detected in the blood (Nikkari et al., 2001). Recently, 18 different bacterial taxa were detected in the amniotic fluid, which was previously believed to be completely sterile (DiGiulio et al., 2008). Analysis using 16S rRNA sequencing detected 28 distinct phylotypes on biofilm removed from prosthetic hip joints during revision arthroplasties – joints also removed from a body compartment also thought to be sterile. The prevalence of hydrothermal vent eubacteria, which were previously thought to persist only in the depths of the ocean since they were found at temperatures well above 176°F (80°C), was higher than the prevalence of *Staphylococcus aureus*, a common biofilm species (Fig. 12.1).

It is now more prudent to assume that tissues that become inflamed in disease most probably do so because of the actions of microscopic pathogens, rather than idiopathic causation. Different microbial populations have been identified in many nongastrointestinal autoimmune conditions including sarcoidosis (el-Zaatar et al., 1996), ankylosing spondylitis (Liu et al., 2001), chronic fatigue syndrome (Lombardi et al., 2009), rheumatoid arthritis, multiple sclerosis, Hashimoto’s



**Fig. 12.1** Bacterial species identified by 16S rRNA gene sequencing of clones from 10 prosthetic hip joints (Dempsey et al., 2007)

thyroiditis, and others (Pordeus et al., 2008). These diseases share features of microbial infection including widespread inflammation and periods of relapse. Sarcoidosis and CD' are characterized by granuloma. In more than a dozen infectious diseases, granuloma is widely acknowledged to be a host-protective structure and to occur when acute inflammatory processes cannot destroy invading agents (Zumla and James, 1996).

## The Human Metagenome

At only approximately 23,000 genes, the human genome is dwarfed by the thousands of genomes of the bacteria, viruses, and phages that persist in and on humans. Given the sheer number of microbial genes, it is no longer possible to study the human genome in isolation. Rather, the human genome is only one of myriad genomes that influence the *Homo sapiens* experience. Humans are controlled by a metagenome – a tremendous number of different genomes working in tandem. Because they are so small, thousands of microbial cells can persist inside a single infected human cell (Wirostko et al., 1989). The combined genetic contributions of these microbes invariably provide a vast number of gene products not encoded by our own relatively small genomes.

There is considerable similarity between the functions of the bacterial organisms and the human organisms. For example, humans and *E. coli* metabolize glucose-6-phosphate in a similar fashion, producing almost identical metabolites (Kuroki et al., 1993). Thus, the transgenomic interaction between an *E. coli* genome and the

human genome, as they exchange nutrients and toxins, increases the complexity of transcription and translation for both species. The dihydrofolate reductase (DHFR) antagonist trimethoprim is such an effective antibiotic because, like humans, bacterial species possess a folate metabolism. Bacteria in the distal intestinal tract of mice have been shown to significantly alter the composition of human blood metabolites, including amino acids, indole-3-propionic acid (IPA), and organic acids containing phenol groups, providing another example of the significant interplay between bacterial and human metabolism. A broad, drug-like phase II metabolic response of the host to metabolites generated by the gut microbiota was observed (Wikoff et al., 2009), suggesting that the gut microbiome has a direct impact on the host's capacity for drug metabolism.

In the pre-genomic era, diseases were classified largely on the basis of symptom presentation; while in recent decades, researchers have attempted to categorize them based on common genes. Yet metagenomics dictates that we must also consider how the many microbial metabolites affect expression of these genes. Some genes and their related pathways have already been shown to influence the pathogenesis of autoimmune disease. For example, Goh et al. has shown that PTPN22 is related to rheumatoid arthritis, lupus, and diabetes mellitus (Goh et al., 2007). Yet expression of PTPN22 is also modified by the bacterial metagenome – it has been shown to be upregulated as part of the innate immune response to mycobacteria (Lykouras et al., 2008). The importance of understanding how microbes affect PTPN22 across multiple disease states has special impetus, given the increased rate of latent tuberculosis in the global population as well as studies showing high rates of infection by *Mycobacterium avium* among autoimmune patients (Bentley et al., 2008).

Many of the most well-studied persistent pathogenic bacteria have evolved mechanisms to evade the immune response and survive inside macrophages and other phagocytic cells. These include *Francisella tularensis* (Hazlett et al., 2008), *Mycobacterium tuberculosis* (Domingue and Woody, 1997), *Rickettsia massiliae* (Monaco et al., 2005), *Brucella* spp. (Baldwin and Goenka, 2006), *Listeria monocytogenes* (Birmingham et al., 2007), *Salmonella typhimurium* (Kuijl et al., 2007), among others. This suggests that other disease-causing components of the microbiota may also persist in the cytoplasm of nucleated cells, where they have access to both human DNA transcription and protein translation machinery (Hall et al., 2008). When *Shigella* persists within a macrophage it modulates numerous host signaling pathways, including those that inactivate mitogen-activated protein kinases (Lutjen-Drecoll, 1992). *Brucella* spp. downregulates genes involved in cell growth and metabolism, but upregulates those associated with the inflammatory response and the complement system upon infecting a macrophage.

Additionally, there appears to be an entire intra-cytoplasmic microbiota within phagocytic cells. Wirostko's team at Columbia University in the 1980s and 1990s used electron microscopy to identify entities within the cytoplasm of phagocytes from patients with juvenile rheumatoid arthritis, sarcoidosis (Wirostko et al., 1989), Crohn's, and other inflammatory diseases (Wirostko et al., 1987). The wide variety of elongated and globular formations, together with both the existence and absence



of exoskeletons around the microbiota, would imply that the observed communities are metagenomic, rather than due to any one single obligate phagocytic pathogen.

## Microbial Complexity

The HIV genome consists of a single strand of RNA comprising nine genes, from which are transcribed 19 proteins. Transcription is noncontiguous, and variations abound. For example, “Tat” is transcribed in multiple pieces that are subsequently joined. Yet 1,443 direct interactions (3,300 total interactions) have been identified between just these 19 proteins and the human metabolome (Fu et al., 2009). Consider that the average bacterial genome codes for hundreds or sometimes thousands of proteins. According to one recent estimate, the average human gut microbiota codes for 9 million unique genes (Yang et al., 2009). Factor in the proteins created by viruses and phages, and efforts to understand how these proteins affect the metabolome leave an observer with little more than stochastic noise, particularly since biological systems are replete with components showing nonlinear dynamic behavior.

Subsequently, interaction between the metagenome and the human genome introduces a new level of complexity to the study of autoimmune disease – complexity that renders it nearly impossible to fully comprehend the vast number of the interactions between the human genome and those microbial genomes capable of influencing the pathogenesis of autoimmune disease. According to Bunge, the size of a gene pool for a given environmental sample can be estimated by mathematical modeling, but the size of the gene pool for a microbial biosphere, such as the human body, may be beyond any current credible model (Bunge, 2009). While this complexity poses a significant challenge to systems biology and to Koch’s simplistic one gene–one disease model, it does not impede the emergence of a better understanding of the human super-organism and the processes that cause disease.

Lifelong symbiosis between the human genome and persistent components of the metagenome has shifted the focus of microbiology away from the search for a single pathogen in a disease state. Many research teams are now striving to understand how components of the microbiota may cause disease. For example, researchers with the European MetaHIT Initiative are studying how bacteria in the gut may contribute to obesity and inflammatory bowel disease. The goal of the project is simply to examine associations between bacterial genes and human phenotypes. “We don’t care if the name of the bacteria is *Enterobacter* or *Salmonella*. We want to know if there is an enzyme producing carbohydrates, an enzyme producing gas or an enzyme degrading proteins,” explains Francisco Guarner of the project.

Studies focused on enzymes, proteins, and carbohydrates are studies of the metabolome. Metabolomic approaches can be used to characterize entire components of the microbiome that cannot easily be seen or studied directly. Because the downstream results of gene expression manifest in the human metabolome, the metabolome can be analyzed for the presence of those unique metabolites



created under the influence of the microbiota. Dumas et al. used mass spectroscopy to identify the nonhuman metabolites present in the urine of subjects living in three distinct populations – the United States, China, and Japan (Dumas et al., 2006). He found that subjects in each population produced very different nonhuman metabolites. Thus, genetic makeup, healthcare, nutrition, and external toxins, factors associated with the acquisition of a particular microbiota, caused the three populations to become significantly different. Moreover, when five of the Japanese subjects moved to the United States, their metabolomes changed to resemble those of the American population. This suggests that the metagenome is indeed the product of its environment, and that the composition of the microbiota is far more important than regional variations in the human genome itself.

In another study, the INTERMAP epidemiological study used an  $^1\text{H}$  NMR-based metabolomics approach to examine differences in the urine metabolite profiles for each of 4,630 participants from 17 populations in the USA, UK, Japan, and China (Stamler et al., 2003). Elevated blood pressure was associated with high levels of the bacterial co-metabolite formate. Interestingly, low levels of hippurate and alanine, which reflected gut microbial activities, were also found in subjects with high blood pressure (Holmes et al., 2008). This suggests that certain microbial metabolites may serve as useful biomarkers for a disease state.

The fact that components of the microbiota are seldom found as single entities further complicates the complexity of transgenomic control in *Homo sapiens*. While just a few decades ago, most of the bacteria in *Homo sapiens* were assumed to persist on their own in a planktonic form, it is now understood that large components of the microbiota persist in communities commonly called biofilms; they are sheltered by a self-created polymeric matrix that better protects them from the immune response. Hundreds of different microbes can persist in a single biofilm community, and individual bacteria often form a niche inside the biofilm that allows them to promote their own survival and the chronic nature of the infection. For example, more virulent bacteria may protect the biofilm from outside intrusion whereas other less innocuous species inside the biofilm may focus on obtaining nutrients for the community. As the biofilm forms and then develops, the collective genetic expression of microbes in the biofilm is altered dramatically. For example, the expression of 800 genes has been shown to be altered when a single bacterial species joins a biofilm (Sauer et al., 2002). Biofilms are increasingly being detected in autoimmune diseases where they were not known to previously exist. For example, Wolcott recently used pyrosequencing to demonstrate that the infectious agents that drive the development of diabetic leg, foot, and pressure ulcers are almost all in a biofilm state (Dowd et al., 2008).

Bacteria in biofilm, their planktonic counterparts, viruses, and other microbes rapidly and frequently share their DNA with other species, even distantly related species, through horizontal gene transfer. Genomic coherence is further muddled by homologous recombination. This further diversifies the variability present in the human microbiome. Horizontal gene transfer is now believed by many to occur so frequently that it has been proposed as a means by which species can acquire new genetic traits. Some argue that the number of microbes created through homologous

recombination is so high that the concept of distinct bacterial species may become obsolete (Doolittle and Papke, 2006).

Thus, the concept that a single pathogen could cause the human metabolism to fail in the myriad of ways necessary to result in an advanced, systemic autoimmune disease is increasingly recognized as an outdated nineteenth-century concept. The postulates of Koch are no longer relevant in the era of the metagenome. Brock contends in his profile of Koch that attempts to rigidly apply Koch's postulates to the diagnosis of viral diseases may have significantly impeded the early development of the field of virology (Brock, 1988). The same can be said for the field of bacteriology, where the postulates have long impeded researchers from considering that the genomes of many different bacteria and other pathogens interact together to cause the range of symptoms we associate with autoimmune diagnoses.

## Toward a More Nuanced View of the Human Microbiota

In *New science of metagenomics: revealing the secrets of our microbial planet*, the National Research Council writes, "The billions of benign microbes that live in the human gut help us to digest food, break down toxins, and fight off disease-causing microbes" (Committee on Metagenomics and National Research Council, 2007). While certain components of the microbiota clearly aid humans in these and other ways, strictly classifying microbes as either commensal or pathogenic may suggest too categorical a distinction. Emerging research suggests that bacteria are no more "good" or "bad" than their human counterparts, particularly when a commensal microbe can easily acquire a plasmid or virulence factor from another microbe. According to Fredricks and Relman, "The mobile nature of virulence-associated gene islands, transported between bacteria via plasmids or phages, creates the potential for acquired virulence in previously innocuous microbes" (Fredricks and Relman, 1998).

In September 2009, Malcolm Casadaban, an infectious disease researcher at the University of Chicago, died suddenly. An autopsy showed no obvious cause of death except *Yersinia* in his bloodstream. Dr. Casadaban, an associate professor at the university, was studying the bacteria to create a better vaccine for plague. Yet Casadaban was working with a strain of *Yersinia* that was supposed to be less virulent than those strains considered lethal. Researchers postulated that there must have been something unusual about the bacterium that caused it to be dangerous, such as a mutation. The so-called "innocuous" strain of *Yersinia* may have acquired a plasmid or gene that endowed it with newfound virulence.

Acquired virulence via horizontal gene transfer has been studied in anthrax. Although *Bacillus anthracis*, which causes fatal poisoning, and *B. cereus*, which causes nonlethal opportunistic infections, are generally classified as separate bacterial species, Hoffmaster discovered a *B. cereus* mutant that also causes a deadly form of pneumonia. Analysis revealed that the *B. cereus* mutant (*B. cereus* G9241) had acquired a plasmid with 99.6% sequence homology to pX01, *B. anthracis*' most virulent, toxin-encoding plasmid. Indeed, *B. cereus* G9241 killed mice more quickly

than *B. anthracis*. *Bacillus cereus* G9241 was deemed the product of horizontal gene transfer, causing Hoffmaster to note that, depending on the extent of horizontal gene transfer, nature could produce an unlimited number of variations and combinations of any given pathogen.

The distinction between commensalism and pathogenicity is further blurred by host-specific factors. For example, if a species of bacteria aids in the metabolism of carbohydrates from the human intestinal tract, the presence of the microbe in the intestines of famine victims could save lives. However, in many Western countries, where rates of obesity are rising at an alarming pace (Wang and Beydoun, 2007), the same microbe might contribute to excess weight gain.

Returning to the gene/disease network discussed above, the ACE gene is related to myocardial infarction, renal tubular dysgenesis, Alzheimer's, the progression of SARS, diabetes mellitus, and sarcoidosis. However, *Lactobacillus* and *Bifidobacteria*, species of bacteria considered to be innocuous or "friendly," are capable of creating a number of peptides that downregulate expression of ACE (Ramchandran and Shah, 2008). These species of bacteria are added to many of our dairy products and are clearly present in the human body. Yet by altering the expression of ACE, these "friendly bacteria" may well affect the progression of several autoimmune and chronic inflammatory diseases, albeit in ways not yet fully understood.

## Pathogens Alter the Expression of Human Genes and Receptors

Intracellular components create myriad metabolites that can interfere and alter the correct transcription of human proteins. Some of these metabolites can also disrupt cellular repair mechanisms, resulting in the accumulation of "junk" (e.g., proteins, enzymes, and mRNA) in the cytosol. For example, Machado et al. reported that *Helicobacter pylori* impairs central DNA repair mechanisms, inducing a transient mutator phenotype, rendering gastric epithelial cells vulnerable to the accumulation of genetic instability (Machado et al., 2009). If the accumulation of errors can exceed the capacity for cellular repair, such dysregulation not only has the potential to drive autoimmune processes, but can also result in early senescence (Muller, 2006), apoptosis (Knodler and Finlay, 2001; Yilmaz et al., 2008), or cancer.

One of the ways in which pathogens survive is by dysregulating the activity of several of the body's key nuclear receptors. The ability of a number of pathogens to dysregulate the vitamin D receptor (VDR), a type 1 nuclear receptor, provides an excellent example of how microbes alter human gene expression so as to gain a survival advantage. Many of the nuclear receptors play a critical role in regulating immune activity and hormonal expression.

The VDR expresses at least 913 genes, many connected to autoimmune conditions and cancers. The receptor also regulates expression of several families of key antimicrobial peptides (AMPs), including cathelicidin and the beta-defensins.

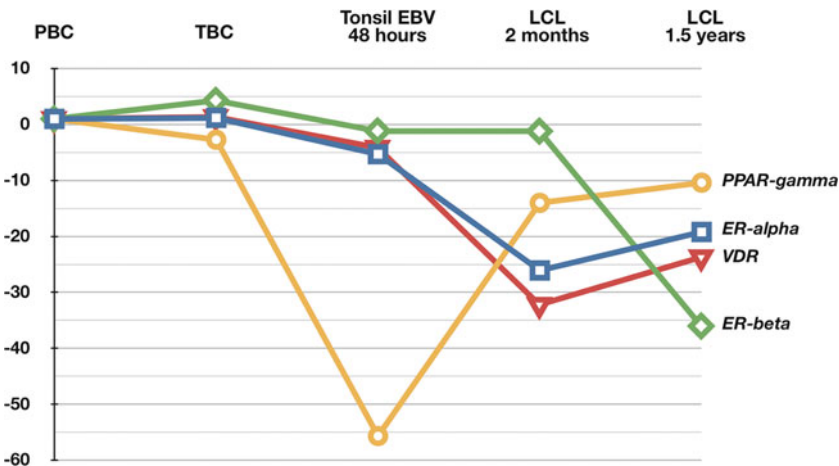
These play a vital role in allowing the innate immune system to target intracellular pathogens. For example, vitamin D-mediated human antimicrobial activity against *Mycobacterium tuberculosis* is dependent on the induction of cathelicidin (Liu et al., 2007). The VDR also transcribes Toll-like-receptor 2 (TLR2), which recognizes bacterial polysaccharides.

The TACO gene, when expressed, inhibits mycobacterial entry as well as survival. *Mycobacterium tuberculosis* (Mtb) downregulates the VDR, and thus expression of TACO in order to survive. Xu et al. showed that the VDR was downregulated 3.3 times in monocytic cell lines infected with Mtb (Xu et al., 2003). *Borrelia*, as assessed by BeadChip microarray, has been shown capable of downregulating VDR activity by a factor of 50-fold, with lysed *Borrelia* downregulating the receptor by a factor of 8 (Salazar et al., 2009). We have previously shown that at least one bacterial metabolite produced by gliding biofilm bacteria is also a strong VDR antagonist (Marshall, 2008). The HIV “tat” protein binds to the VDR in order to use this receptor to recognize its long terminal repeat (LTR) promoter region (Nevado et al., 2007). Thus, tat takes over the human VDR in order to transcribe HIV’s own genome, so the HIV LTR can be recognized and express new HIV RNA. Tat also recruits histone acetyltransferase activity, including the CREB binding protein (CBP)/p300 complex, to acetylate the HIV LTR promoter region (Romani et al., 2009).

Slowing the ability of the VDR to express elements of the innate immune function is such a logical survival mechanism that it is almost certain that the other less studied components of the microbiota would have also evolved ways to dysregulate the VDR, and the other nuclear receptors orchestrating the innate immune response. Eukaryotic cells respond to the presence of the microbiota by activating signaling cascades such as the NF-kappaB pathway. Induction of such pathways leads to the upregulation of gene expression mediating pro-inflammatory and anti-apoptotic effector proteins. Thus, in order for pathogens (and potentially, symbionts) to continue their life cycle, it is necessary to evade or repress these cellular responses. This is especially true because acquisition of resistance to AMPs by a sensitive microbial strain is surprisingly improbable. Furthermore, the extension of human life during the past century now offers additional opportunity for microbes to evolve their specialization in order to survive in man.

Indeed, Yenamandra et al. recently showed that Epstein–Barr virus (EBV) also slows VDR activity (Yenamandra et al., 2009). Infection of human B cells with EBV induces metabolic activation, morphological transformation, cell proliferation, and eventual immortalization by altering the expression of a number of key nuclear receptors. The team found that the expression of 12 nuclear receptors was downregulated in the longer-lasting, younger lymphoblastoid cells. Among them were the VDR and the estrogen receptor beta (ERB), both downregulated by a factor of about 15 times (Fig. 12.2).

EBV is found in many common chronic disease states. Indeed, EBV has been detected in a subset of patients with nearly every autoimmune diagnosis, although it has rarely been detected in 100% of patients with any given condition. In some cases, infection with the virus is described as a “precipitating factor” for



**Fig. 12.2** Nuclear receptors mRNA expression is downregulated upon infection of B cells with EBV (Yenamandra et al., 2009)

autoimmune disease. That EBV downregulates VDR and ERB expression may explain this phenomenon. If a patient acquires EBV, the virus slows innate immune activity to the point where the endogenous microbiota can become dominant.

This is particularly true because, in addition to reducing expression of cathelicidin and beta-defensin, VDR dysregulation opens a number of other pathways that also influence immune activity and hormonal regulation. Blockage of the VDR prevents transcription of CYP24A1, an enzyme that normally breaks down excess levels of the active vitamin D metabolite 1,25-dihydroxyvitamin-D (1,25-D). Activation of protein kinase A (PKA) by bacterial cytokines also causes increased production of the enzyme CYP27B1, resulting in increased conversion of 25-hydroxyvitamin-D (25-D) into 1,25-D. Both processes result in a rise in 1,25-D.

High levels of 1,25-D in autoimmune disease have been confirmed in a clinical setting. Mawer et al. found that 1,25-D levels were particularly elevated in the synovial fluid surrounding the joints of patients with rheumatoid arthritis (Mawer et al., 1991). Abreu et al. found that in a cohort of 88 CD<sup>+</sup> patients, 35 patients or 40% had elevated levels of 1,25-D, which the authors defined as above 60 pg/ml (Abreu et al., 2004). Bell noted that patients with tuberculosis, pneumonia, AIDS, disseminated candidiasis, leprosy, rheumatoid arthritis, silicone-induced granulomas, Wegener’s granulomatosis, Hodgkin’s disease, lymphoma, histocytic lymphoma, T-cell leukemia, plasma cell granuloma, leiomyoblastoma, seminoma, and subcutaneous fat necrosis all tend to manifest with higher than normal levels of 1,25-D (Bell, 1998). Blaney et al. found that of 100 patients with various autoimmune diagnoses, 85% had 1,25-D above the normal range (Fig. 12.3) (Blaney et al., 2009). Yoshizawa et al. reported that in VDR knockout mice, a circumstance that closely mimics extreme VDR dysregulation, 1,25-D levels increase by a factor of 10 (Yoshizawa et al., 1997). However, understanding 1,25-D’s role in various inflammatory disease states is complicated by the fact that most researchers determining

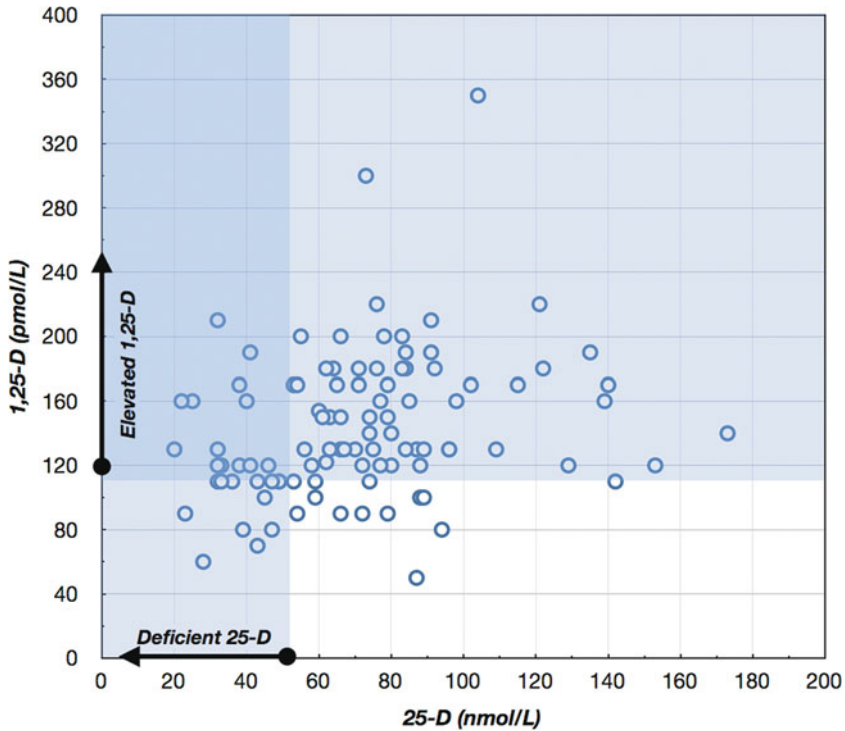


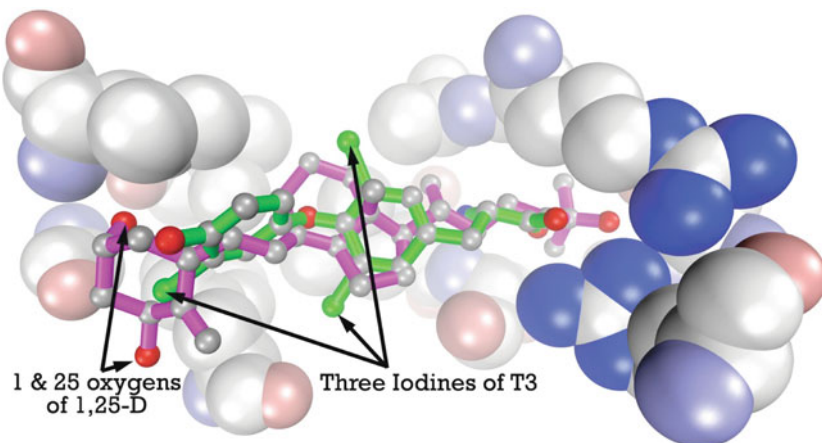
Fig. 12.3 25-D vs. 1,25-D in a cohort of 100 autoimmune patients (Blaney et al., 2009)

vitamin D status test subjects only for levels of the inactive vitamin D metabolite, 25-D.

In silico research indicates that 1,25-D has a high affinity for not just the VDR, but many of the body's other nuclear receptors (Proal et al., 2009). This suggests that at high concentrations it will displace their exogenous ligands. Those receptors affected by elevated 1,25-D include alpha thyroid, beta thyroid, the glucocorticoid (adrenal) receptor, and the progesterone receptor (Fig. 12.4). For example, 1,25-D has a very high affinity for the thyroid beta, suggesting that it can displace T3 and T4 from the binding pocket (Table 12.1) (Proal et al., 2009).

If 1,25-D prevents T3 from activating thyroid beta, then genes with thyroid beta promoters will be less energetically transcribed. This would result in thyroid disease and explain why increasing levels of thyroid hormone are necessary to maintain thyroid homeostasis as chronic disease progresses. Furthermore, since the functions of type 1 nuclear receptors are largely interdependent, if transcription by thyroid beta is dysregulated, system-wide transcription is also affected.

This leads to disruption of system-wide AMP production. Just as the VDR expresses cathelicidin and beta-defensin, other nuclear receptors also express AMPs. Brahmachary et al. have shown that the glucocorticoid receptor, the androgen receptor, and the VDR are, respectively, in control of 20, 17, and 16 families



**Fig. 12.4** The Thyroid- $\alpha$  nuclear receptor and T3, its native ligand [PDB:2H77], with the bound conformation of 1,25-D superimposed. Since the XSCORE Kd for 1,25-D is 8.4, and for T3 is 7.2, it is apparent that 1,25-D is capable of displacing T3 from binding to key receptor residues (shown here are Arg228, Asn179, Gly290, Leu292, Leu276, Ser277, Thr275, Ala263, Leu287, Ala180, Phe218, and Arg162) (Proal et al., 2009)

**Table 12.1** Affinities of native ligands and 1,25-D for various nuclear receptors (Proal et al., 2009)

Nuclear receptor	Native ligand	Native ligand (Kd)	1,25-D (Kd)
Thyroid alpha	T3	7.20	8.41
Thyroid beta	T3	7.18	8.44
Glucocorticoid	Cortisol	7.36	8.12
Androgen	Testosterone	7.38	8.05
Progesterone	Progesterone	7.53	8.09

out of the 22 analyzed (Brahmachary et al., 2006). Thus, dysregulating VDR activity yields flow-on effects that potentially disable the bulk of the body's AMPs. A patient affected in this manner would become increasingly immunocompromised, allowing disease-causing components of the microbiota to proliferate with even greater ease.

This supports a disease model in which key components of the microbiota responsible for autoimmune conditions gradually shut down the innate immune response over a person's lifetime as bacteria, and other pathogens, incrementally accumulate into the microbiota. CD' is already characterized by diminishing functional antimicrobial activity, particularly when it comes to expression of cathelicidin and the beta-defensins (Nuding et al., 2007). Eventually, genes from the accumulating microbial metagenome may instigate a clinical disease symptomology, such as one of the autoimmune diagnoses, or simply drive the inflammation associated with the aches and pains of aging. Indeed, the lifelong accumulation of an increasingly diverse microbiota directly correlates with an age-related increase in diseases and symptoms associated with inflammation. The term "inflammaging" has been coined to explain "the now widely accepted phenomenon that aging is accompanied by a



low-grade chronic, systemic up-regulation of the inflammatory response, and that the underlying inflammatory changes are common to most age-associated diseases” (Giunta, 2006).

Because 1,25-D is expressed in the human cycling endometrium and rises by 40% during early pregnancy, women may be disproportionately affected by the potential drop in AMP expression associated with VDR dysregulation (Viganò et al., 2006). This implies that females may more easily accumulate a more diverse microbiota than their male counterparts, which could help explain why women suffer from a higher risk of most autoimmune diagnoses.

## Successive Infection and Variability in Disease Onset and Presentation

The makeup of a person’s microbiota is unique: humans may share as little as 1% of the same species (Eckburg et al., 2005). Given that the human microbiome may play the principal causative role in autoimmune disease, it may not be by accident that the uniqueness with which patients’ autoimmune disease symptoms develop parallels the incredible variability of the human microbiome. Traditionally, diseases have been understood to be discrete and have their own respective and distinct pathologies, hence the emphasis on diagnosis. However, if the spectrum of autoimmune disease were driven by a common factor – namely a person’s microbial inhabitants – variability in disease could be explained by accounting for how the human microbiota accumulates and develops in any one person. Enck et al. recently analyzed fecal flora of stool samples from 35,292 adults whose ages ranged from 18 to 96 years of age in order to gauge the relative abundance and composition of various bacterial species over time (Enck et al., 2009). He found that while the number of bacteria in the fecal microbiota remained stable with age, the composition of the microbiota diversified as subjects became older, with the oldest subjects measured (over 60 years of age) representing the most profound changes. Older subjects were much more likely to have higher prevalence of microbes associated with chronic disease such as *Enterococcus* and *E. coli*.

A number of microbes that slow immune activity have already been identified indicating that bacteria/viral-driven suppression of innate immune activity may occur on a much larger scale than previously imagined. Each pathogen that decreases immune activity makes it easier for the host to pick up other pathogens, which themselves may further slow immune activity, creating a snowball effect. This process is known as successive infection and offers us a framework for understanding how not only diseases of the gastrointestinal tract develop, but also any number of other autoimmune and inflammatory diseases. As human genes are upregulated or downregulated by components of the microbiota, the body shifts farther and farther away from its natural state of homeostasis. Infected cells increasingly struggle to correctly produce human metabolites in the presence of numerous proteins and enzymes being created by the pathogenic genomes.

The ease with which a person acquires a pathogen from the environment, or from another person, depends largely on the state of their immune system. Those people who harbor low pathogenic loads and still have an active innate immune system could be expected to kill the acute and chronic pathogens they encounter. Conversely, those people with a compromised immune system will accumulate pathogens over time. We have previously discussed how VDR dysfunction, along with adrenal and androgen dysfunction, predispose to a weakened innate immune system, but there are many other factors in play. For example, Bukholm and team found that when the measles virus infects cell cultures, those cells are more susceptible to a secondary bacterial invasion (Bukholm et al., 1986).

Stress has also been shown to impede immune function, by inhibiting natural killer cell activity, lymphocyte populations, lymphocyte proliferation, antibody production, and reactivation of latent viral infections (Webster Marketon and Glaser, 2008). Already identified consequences on health include delayed wound healing, impaired responses to vaccination, and development and progression of cancer (Boscarino, 2004). Depending on the variety of stressful events that occur over a lifetime, people may be more susceptible to picking up microbes at different times. The immune response could be expected to be particularly weak after traumatic events such as surgery, a car accident, or even a pregnancy (McLean et al., 2005).

People accumulate microbiota-altering pathogens in myriad different ways, the most obvious being social contact. People in close proximity, particularly spouses and children, inevitably pick up components of each other's microbiomes (Wilhoite et al., 1993). Healthcare workers have higher rates of certain autoimmune and inflammatory conditions including breast cancer and malignant melanoma (Lie et al., 2007). Merely shaking hands causes the transfer of numerous microbes (Fierer et al., 2008). Genomic analysis of the bacteria on the hands of students leaving an exam room contained 332,000 genetically distinct bacteria belonging to 4,742 different species. Forty-five percent of the species detected were considered rare. This marked a 100-fold increase in the number of bacterial species detected over previous studies that had relied on purely culture-based methods to characterize the human hand microbiota.

Obesity is not currently accepted as an autoimmune condition, but Christakis and Fowler recently used quantitative analysis of a densely interconnected social network to conclude that obesity is transmitted among people (Christakis and Fowler, 2007). A person's risk of becoming obese increases by 57% if they have a friend who becomes obese, and by 37% if their spouse becomes obese. Although, as the team concludes, people may mimic the behavior of friends or family in ways that could cause them to gain or lose weight, it is also possible that the close proximity among many of the subjects in the study would have allowed them to directly exchange microbes. Since the composition of bacteria in the gut has, in several instances, been linked to the development of obesity (Kinross et al., 2008) – perhaps, in some cases, obesity is literally contagious. It seems likely the same could be said for any autoimmune condition with an infectious etiology.

In some cases, pathogens may be acquired in the womb, particularly if the mother already suffers from one or more autoimmune or inflammatory diagnoses.

Similarly, bacterial species including *Staphylococcus epidermidis*, *Streptococcus viridans*, *E. coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Proteus*, and others have been detected in sperm (Merino et al., 1995). *Mycobacterium tuberculosis* and influenza H5N1 have been shown to cross the placental barrier. Already implicated in implantation failure, spontaneous abortion, and preterm birth, infection with *Shigella* is now proposed to cause endometriosis (Kodati et al., 2008). DiGiulio studied ribosomal DNA (rDNA) of bacteria, fungi, and archaea from amniotic fluid of 166 women in preterm labor with intact membranes. Fifteen percent of subjects harbored microbes that together belonged to 18 different taxa – including *Sneathia sanguinegens*, *Leptotrichia amnionii*, and an unassigned, uncultivated, and previously uncharacterized bacterium. A positive PCR was associated with histologic chorioamnionitis and funisitis. The correlation between positive PCR and preterm delivery was 100%.

Pathogens can also pass from mother to child during breast-feeding. For example, Human papillomavirus type 16 (also called high-risk HPV-16), which has been linked to cervical cancer, has been detected in human breast milk collected during the early period after a woman delivers her baby (Sarkola et al., 2008). Pathogens can also be transmitted from person to person through bodily fluids released during coughing, sneezing, and other intimate contact and are found nearly everywhere in our environment. For example, nontuberculosis *Mycobacteria* and other opportunistic human pathogens are enriched to high levels in many showerhead biofilms, >100-fold above background water contents. Catheters used to treat urinary tract infections and other conditions have, in some cases, been shown to harbor copious amounts of biofilm.

## Early Infections Predispose a Person to Later Chronic Disease

Most of the bacteria implicated in autoimmune disease are slow-growing pathogens whose effects will take decades to manifest (Davenport et al., 2009). In this sense, bacteria acquired earlier in life can alter the ultimate microbiota in ways that may not be recognized for decades. According to Merkler et al., “In genetically susceptible individuals, early childhood infections seem to predispose them to [such disease as] multiple sclerosis or Type 1 diabetes years or even decades before clinical onset” (Merkler et al., 2006). A 2006 report by the Centers for Disease Control (CDC) echoes this sentiment: “A person’s age at the time of infection – from intrauterine or perinatal (the time period surrounding birth), through childhood and adolescence, to adulthood and the elder years – may further influence the risk for chronic outcome. For example, perinatal herpes virus infection dramatically increases the risk of developing adult or pediatric chronic liver disease. Recurrent infections or perhaps serial infections with certain agents might also determine a person’s risk for chronic outcome” (O’Connor et al., 2006).

Thus, while medicine generally assumes that once a person has recovered from an acute illness, they return to a state of complete health – the so-called “sterilizing immunity” – in truth, the long-term consequences of acute infection are

somewhat poorly understood. Newborns who harbor certain types of bacteria in their throats, including *Streptococcus pneumoniae* and *Haemophilus influenzae*, are at increased risk for developing recurrent wheeze or asthma early in life (Bisgaard et al., 2007). Approximately two-thirds of patients with Guillain–Barré syndrome, a suspected autoimmune condition, have a history of an antecedent respiratory tract or gastrointestinal infection (Kuroki et al., 1993). Prenatal infections such as rubella, influenza, and toxoplasmosis are all associated with higher incidence of schizophrenia – with the children of those mothers exposed to influenza in the first trimester of gestation showing a seven-fold increased risk of schizophrenia (Brown, 2006). Reactive arthritis (Reiter’s syndrome) is classically seen following infection with enteric pathogens such as *Yersinia*, *Salmonella*, *Campylobacter*, and *Shigella* (Hill Gaston and Lillicrap, 2003). Acute gastroenteritis, resulting from infection with the same pathogens, causes approximately 6–17% of patients to develop chronic irritable bowel syndrome.

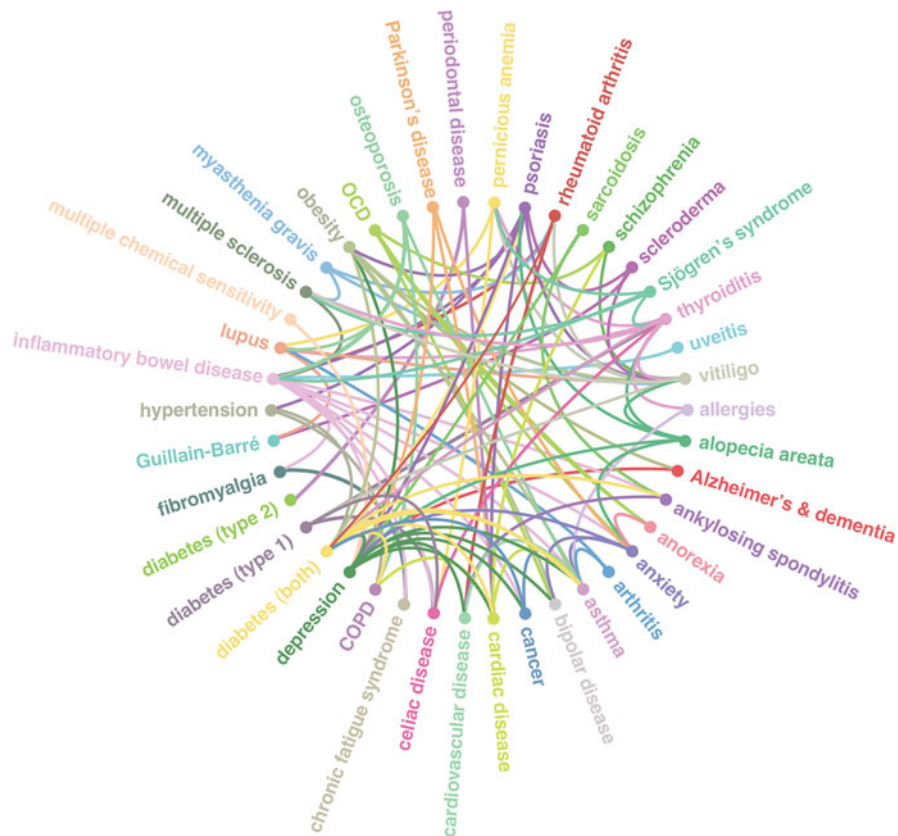
In an especially provocative experiment, a team including Doron Merkler and Nobel Laureate Rolf Zinkernagel injected cytomegalovirus (CMV) into the brains of mice that were only a few days old (Merkler et al., 2006). The innate immune systems of the mice were able to eliminate CMV from most of the tissues except for those of the central nervous system. As a result, the virus persisted in the brains of the mice. Later in life, when the same mice were challenged by infection with a similar virus, they developed a condition resembling a type of autoimmune disease and died. The team referred to this concept as “viral *déjà vu*.”

Incidents of food poisoning also point to unresolved features of acute infections. Siegler et al. noted that 10% of people who suffered from *E. coli* food poisoning later developed a relatively infrequent life-threatening complication called hemolytic uremic syndrome (HUS) where their kidneys and other organs fail (Siegler et al., 1994). According to the study, 10–20 years after patients recover, between 30 and 50% of *E. coli* survivors will have some kidney-related problem, conditions that include high blood pressure caused by scarred kidneys, slowly failing kidneys, or even end-stage kidney failure requiring dialysis.

Microbes can also be transmitted by donation of blood, bone marrow transplants, or organ donation, which, if pathogenic, can greatly disrupt the composition of the microbiota over time. The term “donor-acquired sarcoidosis” refers to the development of sarcoidosis in presumably naïve (nonsarcoidosis) transplant recipients who have received tissues or organs from donors who were not known or suspected to have active sarcoidosis (Padilla et al., 2002). Murphy studied over 8,500 people in the United Kingdom who underwent heart surgery between 1996 and 2003 (Murphy et al., 2007). Patients who had received red blood cell transfusions were about three times more likely to suffer a heart attack or stroke and were at a higher risk for infection, readmission to hospital, and death compared with heart patients who did not receive blood. The risks associated with blood transfusions were not influenced by a patient’s age, hemoglobin levels, or the extent of their disability at the time of transfusion. Writing in the journal *Circulation*, Murphy et al. concluded: “Red blood cell transfusion appears to be harmful for almost all cardiac surgery patients and wastes a scarce commodity and other health service resources” (Murphy et al., 2007).

## Comorbidity

Thus the catastrophic failure of the human metabolism we see in autoimmune disease – which at first glance appears so diverse and so different among different diagnoses – appears to be due to a single underlying mechanism: a ubiquitous microbiota, much of which has evolved to persist in the cytoplasm of nucleated cells. What differ among individuals as they gradually acquire a unique microbiota over time are the virulence, location, and combination of those pathogenic species. The high rate of comorbidity among inflammatory diagnoses (Anderson and Horvath, 2004) lends support for this explanation. Such comorbidity between seemingly unrelated diseases cannot be explained by laws of average – the risk of autoimmune disease is not evenly distributed. Figure 12.5 demonstrates the degree of comorbidity seen among various inflammatory diagnoses. Each “spoke” represents a study



**Fig. 12.5** Comorbidities among common inflammatory diseases. Each “spoke” of this wheel represents a published study appearing in MEDLINE, which shows a significant statistical relationship between one disease and another

from PubMed, which has demonstrated a significant statistical relationship between patients suffering from one inflammatory disease and the next.

In the case of multiple sclerosis, Barcellos et al. identified coexisting autoimmune phenotypes in patients with multiple sclerosis from families with several members with the disease and in their first-degree relatives (Barcellos et al., 2006). A total of 176 families (386 individuals and 1,107 first-degree relatives) were examined for a history of other autoimmune disorders. Forty-six (26%) index cases reported at least one coexisting autoimmune disorder. The most common were Hashimoto's thyroiditis (10%), psoriasis (6%), inflammatory bowel disease (3%), and rheumatoid arthritis (2%). One hundred and twelve (64%) families with a history of multiple sclerosis reported autoimmune disorders (excluding multiple sclerosis) in one or more first-degree relatives. Hashimoto's thyroiditis, psoriasis, and inflammatory bowel disease were also the most common diagnoses occurring in these family members. Such high rates of comorbidity support a model for autoimmune conditions in which no two people with the same diagnosis ever develop the exact same disease presentation; the interactions between an individual's genome and their unique metagenome are so varied that they are rarely identical.

Note that Fig. 12.1 suggests that patients with autoimmune diagnoses are also much more likely to suffer from mental conditions such as depression and anxiety. Increasing clinical evidence, including that from our own study (Perez, 2008), confirms the involvement of microbiota in neurological disease states. This suggests that both autoimmune and neurological diagnoses, which are currently balkanized into separate medical specialties, most probably result from the same underlying dysregulation of microbial populations.

Autoimmune and inflammatory conditions also suffer from specialty delineation. For example, VDR dysregulation does not just impact the autoimmune disease state. Researchers have reported epigenetic repression of VDR gene expression and activity in choriocarcinoma cell lines (Pospechova et al., 2009). Furthermore, the VDR expresses genes involved in both autoimmune and inflammatory processes. It transcribes insulin-like growth factor (IGFBP-3) (Wang et al., 2005), which influences the development of diabetes, yet also expresses metastasis suppressor protein 1 (MTSS1), which plays a vital role in repressing the cell cycle and promoting apoptosis in cancerous cells (Wang et al., 2005). Drawing a line between autoimmune and inflammatory disease makes these and other common mechanisms harder to recognize and study.

## Causation vs. Association

If most autoimmune and inflammatory conditions do indeed arise from the same underlying disease process, then we must re-examine some of the cause and effect relationships postulated to exist among inflammatory conditions. For example, it is commonly believed that obesity is a causative factor in the development of diabetes (Hibbert-Jones et al., 2004). In fact, patients with Type 2 diabetes are so likely to



become morbidly obese that the two conditions are sometimes collectively referred to as “diabesity” (Bailey, 2009). Obesity has been tied to microbial composition in the gut (Turnbaugh et al., 2006), the result of a microbial process. Roesch et al. found that the onset of Type 1 diabetes was tied to the presence of specific bacteria in the murine gut (Roesch et al., 2009). Additionally, at least one microbial species, *Streptomyces achromogenes*, secretes a substance, streptozocin, which can directly induce Type 1 diabetes (Bolzan and Bianchi, 2002). The diabetes disease process would also make it substantially harder for the immune system to regulate microbial gut composition. In particular, species that are extremely effective at extracting calories from food may thrive while their innocuous counterparts may find themselves out-competed. The expression of hormones that regulate appetite, such as leptin or ghrelin, could also become dysregulated by the bacterial microbiota (Fetissov et al., 2008). For example, *H. pylori* infection leads to a decrease in circulating ghrelin through a reduction in ghrelin-producing cells in the gastric mucosa (Weigt and Malfertheiner, 2009). In some cases, this could cause weight gain even in the absence of excess calorie consumption (English et al., 2002). In light of the above, obesity and diabetes might better be described as developing simultaneously. Treatments aimed at addressing those underlying factors contributing to both disease states might well prove the most effective.

The same dichotomy is found in other sets of parallel conditions such as tooth decay and dementia, rheumatoid arthritis and uveitis, high cholesterol and heart disease, and others. It is far more likely that both conditions arise from a common metagenomic microbiota than that one condition is causal for the other.

## Microbial Interaction and Disease

One of the more striking characteristics of nonobese diabetic (NOD) mice is that exposure to *Mycobacteria* can prevent the onset of diabetes while precipitating lupus in the same animal (Harada et al., 1990; Hawke et al., 2003). While this phenomenon is difficult to interpret by studying the murine genome alone, it may help to consider the murine metagenome. If, as in humans, the murine metagenome causes disease as it accumulates over time, then the interactions between various microbial species may be telling. Even within the context of the ultimate example of symbiotic behavior, the biofilm, bacteria have been shown to compete with one another, sometimes even “cheating” to do so (Dunny et al., 2008). We would not have many antibiotics if it were not for competition among bacterial species. For example, the early tetracycline antibiotics were derived from species of *Streptomyces*, and are toxic to a number of its competitors.

With the NOD mice, introduction of a new species of bacteria into the microbiota, *Mycobacteria*, alters the microbiota in such a way as to wipe out, or at least diminish, the diabetes disease state. At the same time, the microbiota allows lupus to proliferate or dominate. Similar competition between microbes may also explain why lupus has been shown to inhibit the development of malaria (*Plasmodium falciparum*) (Zanini et al., 2009).



Autism, an inflammatory condition that has been associated with several unique microbial populations (Nicolson et al., 2007) may have a comparable dynamic at work. In children diagnosed with autism spectrum disorder, fever associated with intercurrent bacterial or viral infections – such as upper respiratory infections – has been shown to temporarily decrease aberrant behavior such as irritability and inappropriate speech (Curran et al., 2007).

Gastric surgery invariably alters the composition of the gastrointestinal microbiota. DePaula et al. found that after 39 diabetic type 2 patients in Brazil underwent bariatric surgery all subjects no longer required insulin therapy (DePaula et al., 2008). All subjects also experienced normalization of their cholesterol levels, 95.8% had their hypertension controlled, and 71% achieved targeted triglyceride levels. This correlates with data showing that the intestinal bacterial populations of normal weight individuals, morbidly obese individuals, and people who have undergone gastric bypass surgery are distinctly different. For example, *Firmicutes* were dominant in normal-weight and obese individuals but significantly decreased in post-gastric-bypass individuals, who had a proportional increase of gammaproteobacteria (Zhang et al., 2009).

Other microbial interactions can alter the pathogenicity of one or more species involved. The pathogenic potential of *Helicobacter hepaticus* in a mammalian colitis model is altered by the presence of different strains of *Bacteroides fragilis*. When the bacterial polysaccharide PSA is expressed on the microbial cell surface of *B. fragilis*, it suppresses pro-inflammatory interleukin-17 production to *H. hepaticus* (Mazmanian et al., 2008). Hoffman et al. found that when the bacterial species *Pseudomonas aeruginosa* and *S. aureus* were incubated together, *P. aeruginosa* created a protein, HQNO, which protected *S. aureus* from eradication by commonly used aminoglycoside antibiotics such as tobramycin (Hoffman et al., 2006). Besides, in cases of *P. aeruginosa* and *S. aureus* co-infection in the presence of HQNO, small-colony variants of *S. aureus* are selected for, making *S. aureus* more difficult for the immune system to target. Although we are far from understanding the full nature of these microbial interactions, it is clear that a microbiota constantly evolves so that the symptoms of any given disease are seldom static.

## Familial Aggregation

The common disease–common variant hypothesis suggests that chronic diseases are the product of anywhere from one to thousands of disease-causing alleles. The HapMap single nucleotide polymorphisms (SNPs) cataloging project has identified over 3.1 million SNPs, with many more expected to be found as the project continues. However, only a fraction of these SNPs confers any more than a minimal statistically increased risk for disease (Chung et al., 2010). For example, in cancer, for nearly all regions conclusively identified by genome-wide association studies (GWAS), the per allele effect sizes estimated are less than 1.3. While over 85 regions have been conclusively associated in over a dozen different cancers, no more than five regions have been associated with more than one distinct cancer type (Chung

et al., 2010). According to Stephen Chanock of NIH, “Nearly every candidate SNP [associated with cancer] has failed in the long run – maybe five or six are real by rigorous standards” (personal communication).

There appear to be factors at work other than just Mendelian inheritance. The increased risk of chronic disease among nonrelations in close proximity – the so-called “case clusters” – strongly implies an infectious dynamic at work. The evidence that the autoimmune disease sarcoidosis is communicable is particularly strong. A study of 215 sarcoidosis patients found that five husband-and-wife couples both had the disease – a rate 1,000 times greater than could be expected by chance (Rossman and Kreider, 2007). The NIH ACCESS research team also noted that the risk for sarcoidosis increased nearly five-fold in parents and siblings of people with the disease. A case-controlled study of residents of the Isle of Man found that 40% of people with sarcoidosis had been in contact with a person known to have the disease, compared with 1–2% of the control subjects (Gribbin et al., 2006). Another study reported three cases of sarcoidosis among 10 firefighters who apprenticed together (Kern et al., 1993).

The literature contains many examples of unexpected familial associations among seemingly distinct disease pathologies. For example, a 2008 study of parents of children with autism found they were more likely to have been hospitalized for a mental disorder than parents of control subjects, with schizophrenia being more common among case mothers and fathers compared with respective control parents (Daniels et al., 2008). In the case of schizophrenia and autism, both have been associated with prenatal viral infection (Fatemi et al., 2008). While a fetus can acquire these and many other pathogens directly, successive infection dictates that as children age they will manifest with inflammatory symptoms that may differ from those of their parents. Major factors that would influence the development of a discrete inflammatory diagnosis include the mix of species acquired, the sequence in which the pathogens are acquired, the subsequent changes in gene expression caused by the pathogens, and the profound effect on the body’s proteins, enzymes, and metabolites caused by these changes. Because the adaptive immune response in infants takes several weeks to develop, infants are particularly prone to picking up pathogens during the first weeks of life (Bisgaard et al., 2007). Such pathogens could be acquired from any family or friends in contact with the child, especially the grandparents, who probably harbor some of the highest pathogenic loads. Palmer et al. found that infants pick up many of the species that make up their gut flora from family members within just a few weeks of birth, suggesting that nongut bacteria may easily be acquired during this time as well (Palmer et al., 2007).

## **Is Autoimmune Disease Predisposition Mendelian?**

Two decades ago, the attention of the research community shifted toward a new source in an attempt to explain the etiology of autoimmune disease: the human genome. Begun formally in 1990, the US Human Genome Project was a 13-year

effort coordinated by the US Department of Energy and the NIH. Its primary goal was to determine the sequence of chemical base pairs that make up DNA and to identify the genes of the human genome from both a physical and a functional standpoint. A working draft of the genome was released in 2000 and a complete version in 2003, with further analyses yet to be completed and published (Collins et al., 2003). Meanwhile, the private company Celera Genomics conducted a parallel project (Venter et al., 2001).

Early in the aftermath of the sequencing of the human genome, many geneticists advocated the common disease–common variant hypothesis, expressing certainty that the field would quickly determine genetic haplotypes that would correlate with and explain the bulk of chronic diseases. Dr. Francis Collins’ 2001 statement was typical: “It should be possible to identify disease gene associations for many common illnesses in the next – 7 years” (Collins and McKusick, 2001). Researchers hoped that by dissecting the human genome, patients could be informed that they had “the gene” for breast cancer, sarcoidosis, rheumatoid arthritis, or any of the other autoimmune diagnoses. Targeted gene therapies could then be developed to effectively eradicate these conditions.

It may be too early to call human genomic research an unqualified failure (Buchanan et al., 2006), but it is difficult to ignore a lack of utility in identification of disease. Recently, the limited progress in the genetic analysis of common diseases has begun to be acknowledged (Davey Smith et al., 2005; Risch, 2000). Certainly there have been no widely successful gene therapies to date, and genome-driven personalized medicine has yet to live up to its early promise. To identify what some researchers refer to as the “missing heritability,” geneticists have proposed GWA studies with historically unprecedented sample sizes. In the past year, researchers have publicly contemplated “daunting” sample sizes exceeding 500,000 subjects in concert with studies that would be conducted over periods as long as 45 years (Burton et al., 2009).

Ewald et al. argue that evolutionary forces that would cause a serious disease to be weeded from the population would also cause those people whose immune systems are prone to self-attack to be eliminated from the population (Cochran et al., 2000). An exception would occur if the disease offers a survival advantage. For example, the genetic disorder cystic fibrosis may confer resistance to tuberculosis (Poolman and Galvani, 2007). The Mendelian disorder sickle cell anemia is common in tropical countries because it confers resistance to malaria. With malaria, researchers can quantify the rate by generation at which the gene for sickle cell anemia is dropped from the population in the absence of an evolutionary advantage – as is the case when people migrate away from malaria-infested areas. However, no autoimmune diagnosis has been shown to confer any sort of beneficial survival trait. Under these circumstances, one would expect any faulty gene or network of genes associated with an autoimmune condition to be selected against, especially since many autoimmune conditions strike during the reproductive years. Chronic diseases have existed for thousands of years with manifestations of both arteriosclerosis (Azer, 1999) and cardiac disease observed in mummies of ancient Egypt (Miller et al., 2000). Ötzi the Neolithic Iceman who lived around 3300 BC had

arthritis, allowing ample time for any alleles associated with autoimmune disease to be eliminated via natural selection (Dickson et al., 2003). Instead, the prevalence of autoimmune conditions seems to have remained essentially constant until quite recently.

## SNPs and Autoimmune Disease

After noting that among his cohort of 31 patients with abdominal aortic aneurysm, SNPs in the gene *BAK1* were different in aortic tissue than in blood samples from the same patients (Gottlieb et al., 2009) Gottlieb remarked, “Genome-wide association studies were introduced with enormous hype several years ago, and people expected tremendous breakthroughs. Unfortunately, the reality of these studies has been very disappointing, and our [own] discovery certainly could explain at least one of the reasons why.” The conundrum that Gottlieb’s study has exposed is that the human genome appears to vary between the tissue and plasma compartments. Medicine has always assumed that human DNA is homogeneous throughout the human body. We now need to explore the mechanisms whereby these different genetic sequences could arise through selective pressure in different tissues such as would exist if the tissue harbored a microbiota.

One of the mechanisms proposed for genetic predisposition states that genetic haplotypes predispose for disease processes. Because it is a highly polymorphic genomic region, MHC has served as the preferred axis for studying susceptibility to immune diseases. Major changes have been detected within the HLA class I and class II genes related to various populations across the globe. For example, in Type 1 diabetes, the most common haplotype in the Western world is AH8.1 (HLA-A1-B8 DR3-SC01). However, this haplotype is almost nonexistent in the Indian population and has been supplanted by the variant AH8.1v, which differs from the Caucasian AH8.1 at several gene loci (Mehra et al., 2007). Moreover, there are additional HLA-DR3 haplotypes HLA-A26-B8-DR3, HLA-A24-B8 DR3 (AH8.3), A2-B8-DR3 (AH8.4), and A31-B8-DR3 (AH8.5) that occur largely in the Indian population alone.

Similarly, the FCRL3-169T-C polymorphism, which is significantly associated with rheumatoid arthritis (RA) in East Asian populations is not associated with RA in Caucasians of European descent (Begovich et al., 2007). Interestingly, the frequency of the rs7528684 minor allele associated with FCRL3- varies as much within each of the two ethnic groups as it does between them. Furthermore, a recent large case-controlled study found that FCRL3-169T-C was not significantly associated with RA in Korean patients (Begovich et al., 2007).

Thus, no diagnostic certainty can be obtained by measuring genes on the HLA axis. None of the HLA haplotypes causes disease 100% of the time and none causes any one immune disease consistently. Patterns of haplotype variation are more suggestive of a regional infectious model rather than a model in which an illness is caused by widespread inherited variation of HLA haplotypes.

## Potential Systematic Errors in the Interpretation of the Metagenome

Primers selected for most epidemiological studies are chosen without consideration for whether they might amplify DNA from the genomes of any intracellular microbes. As artist Pablo Picasso once remarked, “Computers are useless. They can only give you answers.” If a software program fails to make provision for the possibility that a metagenome might also be present, the chances of a false-positive increase significantly during the process of genomic analysis. Similarities between bacterial and human genes will likely cause the analysis software to not assemble the genomic data properly. The likelihood of error is not minuscule as there is growing evidence of molecular mimicry, homology between bacterial and human proteins. For example, significant sequence homology exists between human carbonic anhydrase II and alpha-carbonic anhydrase of *H. pylori* (Guarneri et al., 2005). Moreover, the homologous segments contain the binding motif of the HLA molecule DRB1\*0405. The group A streptococcal carbohydrate antigen *N*-acetyl-glucosamine is able to cross react with cardiac myosin (Cunningham, 2003). Microbes including *E. coli*, *H. pylori*, *P. aeruginosa*, Cytomegalovirus, and *H. influenzae* share sequence homology with human pyruvate dehydrogenase complex-E2, which has been tied to the development of primary biliary cirrhosis (Bogdanos et al., 2004). The core oligosaccharides of low-M(r) LPSs of *C. jejuni* serotypes that are associated with the development of Guillain–Barré syndrome are homologous to neural gangliosides.

Before we can be certain that all measured SNPs and HLA haplotypes are a product of only the human genome and not the metagenome, researchers must begin to actively choose PCR primer pairs that are unlikely to amplify microbial DNA. Primers need to be certified not only to amplify a unique sequence in the human genome, but also as not likely to amplify genes from any of the thousands of bacterial and viral genomes in the metagenomic databases. Although PCR amplification usually involves more than one stage of genomic selectivity, the increasing use of arrays of RNA probes increases the likelihood that a fragment of metagenomic RNA will unexpectedly match a probe, and increases the possibility of a false-positive being signaled for the particular SNP being sought.

## Antibodies in Response to Microbial DNA

Autoimmune diseases are characterized largely by the presence of autoantibodies. Although autoantibodies were reported over a century ago, many scientists at the time were unwilling to accept the possibility that the immune system attacks its own cells. Ehrlich argued that autoimmunity was not possible and proposed the theory of *horror autotoxicus* to describe the body’s innate aversion to immunological self-destruction by the production of autoantibodies. Now that humans are understood to be the product of multiple genomes, increasing evidence supports Ehrlich’s view. When an innate immune system is forced to respond to a chronic microbiota,

the resulting cascade of chemokines and cytokines will also stimulate an adaptive response. Antibodies are notoriously polyspecific, and the likelihood that antibodies generated to target metagenomic fragments will also target human proteins (target “self”) is finite.

A litany of research implies a re-evaluation of the “autoantibody.” Recently researchers have shown that certain autoantibodies are created in response to several well-studied pathogens. “Lupus-specific autoantibodies” such as RO, La, or dsDNA are often generated in response to EBV (Barzilai et al., 2007). Similarly, anti-EBNA-1 antibodies are able to bind lupus-specific autoantigens such as Sm or Ro (Harley and James, 2006). Casali and Slaughter found that, in humans, EBV is a polyclonal B cell activator, and in vitro transformation with EBV results in production of rheumatoid factor (RF) (Casali et al., 1987; Slaughter et al., 1978). Posnett et al. argues that high titers of RF are not only associated with severe rheumatoid arthritis but also appear in a number of other diseases including viral, bacterial, and parasitic infections (Posnett and Edinger, 1997). Maturation of RF can be initiated by chronic infections (Djavad et al., 1996). For example, patients with subacute bacterial endocarditis, which is frequently tied to the presence of *Streptococcus*, also often present with high levels of RF (Russell et al., 1992). Williams et al. showed that once the offending infectious agent is removed with antibiotic therapy, the RF disappears (Williams and Kunkel, 1962). Similarly, the autoimmune disease thrombocytopenic purpura (ITP) is mediated by what are considered to be anti-platelet autoantibodies. However, Asahi et al. found that eradication of *H. pylori* is effective in increasing platelet count in nearly half of ITP patients infected with the bacterium (Asahi et al., 2006). Barzilai and team also found that Hepatitis B shares amino acid sequences with different autoantigens, further suggesting that the so-called autoantibodies may actually be created in response to pathogens (Barzilai et al., 2007). Autoantibodies have been detected in patients without autoimmune disease during periods of infection. Berlin et al. collected sera from 88 patients with acute infections (41 bacterial, 23 viral, 17 parasitic, and 7 rikettsial (Berlin et al., 2007)). Elevated titers of autoantibodies including annexin-V, prothrombin, ASCA, ANA, or antiphospholipid antibodies were detected in approximately half of the subjects, with 34 individuals harboring elevated titers of at least two “autoantibodies.”

EBV, *E. coli*, *Salmonella*, and other pathogens discussed above are easily detected by culture-based methods that may explain why their presence has already been tied to “autoantibody” production. Yet the vast majority of the human microbiota is understudied. This means that what we now consider to be autoantibodies in many autoimmune diagnoses may also indicate the presence of pathogens, but pathogens that have yet to be fully characterized and named. Thus, in addition to looking for antibodies to well-characterized pathogens, it is also important that we look for antibodies indicating the presence of the underlying chronic microbiota, some of which we may also be mistaking for autoantibodies. Like the pathogens that may create them, many of these antibodies may not yet be detected by standard testing. If this is the case, hundreds of pathogen-induced antibodies may exist and impact the autoimmune disease state, but the possible detection and correlation of

such antibodies with specific components of the microbiota remains difficult until a much larger portion of the microbiota has been characterized.

Because many antibodies demonstrate a high degree of polyspecificity, it is possible that in some cases, antibodies initially directed against pathogens could also attack human tissue (Christen et al., 2010). According to Bozic, oxidative alterations, affecting either the hypervariable region or the receptor site of IgGs, may influence their functions (Bozic et al., 2007). Similarly, McIntyre reported the appearance and disappearance of antiphospholipid antibodies subsequent to oxidation reactions in human blood (McIntyre, 2004). Dimitrov et al. have shown that a fraction of antibodies present in all healthy individuals begin to recognize large number of self-antigens only after a transient exposure to certain protein-destabilizing conditions, including low or high pH, high salt concentration, chaotropic factors, and redox-active agents (Dimitrov et al., 2008). This points to at least one mechanism whereby the oxidative stress that accumulates in inflamed tissue could be at least partly responsible for the apparent polyspecificity of antibodies and autoantibodies.

Molecular mimicry, in which peptides from pathogens share sequence or structural similarities with self-antigens, may also contribute to autoantibody production. Lekakh et al. found that autoantibodies with polyspecific activity in the serum of healthy donors were able to cross react with DNA and lipopolysaccharides (LPSs) of widespread species of bacteria including *E. coli*, *P. aeruginosa*, *Shigella boydii*, and *Salmonella* (Lekakh et al., 1991). CD<sup>+</sup> is classified as an autoimmune condition based largely on the presence of perinuclear anti-nuclear cytoplasmic antibodies (pANCA) in patients with the disease. Yet recently two major species of proteins immunoreactive to pANCA were detected in bacteria from anaerobic libraries, implicating colonic bacteria as a possible trigger for the disease-associated immune response.

We previously discussed how factors other than calorie consumption may contribute to the weight gain often associated with autoimmune or inflammatory conditions. Fetissov et al. studied healthy women for the presence of IgG or IgA autoantibodies directed against 14 key regulatory peptides and neuropeptides, including ghrelin, leptin, vasopressin, and insulin (Fetissov et al., 2008). They found numerous cases of sequence homology among these peptides and the protein structures of over 30 microbes including *Lactobacilli*, *H. pylori*, *E. coli*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes*, suggesting that the “autoantibodies” were actually the result of molecular mimicry. In the presence of certain pathogenic bacterial species, the production of IgG autoantibodies directed against ghrelin was upregulated, suggesting a complex interplay between autoantibody levels and microbial antigens. This suggested that these so-called “autoantibodies” might not only have physiologic implications in pathways that regulate hunger and satiety but also represent a key link between the gut and the brain.

An increasing number of studies also show that what are currently perceived as autoantibodies can often be detected in the so-called healthy individuals years before the full presentation of an autoimmune disease state. Many researchers now espouse that early detection of these antibodies can help predict whether or not



such a “healthy” person will develop an autoimmune disease. For example, in an 8-year prospective study, Swaak et al. examined the diagnostic significance of anti-double-stranded deoxyribonucleic acid (anti-dsDNA) determination in a group of 441 patients without systemic lupus erythematosus (SLE) whose sera were found to contain antibodies to dsDNA on routine screening (Swaak and Smeenk, 1985). Within 1 year, 69% (304) of these patients fulfilled the preliminary American Rheumatism Association (ARA) criteria for SLE. Eighty-two of the remaining 137 patients were followed up for several years. At the end of the study, 52% of these patients had also developed SLE. The team concluded that about 85% of patients without SLE with anti-dsDNA in the circulation would develop SLE within a few years.

Another recent study of blood from 441 healthy Portuguese blood donors found autoantibodies for rheumatoid factor, anticyclic citrullinated peptides, anti-mitochondria, anti-*Sacharomyces cerevisiae*, ANA, anti-TTG, and anti-Beta2-glycoprotein (Tavares-Ratado et al., 2009). More than 30% of the blood contained one or more of the antibodies, 4% exhibited two antibodies, and nearly 1% had three or more antibodies present. It is clear that sub-clinical autoimmune disease is much more common than previously thought.

This gradual presentation of an increasing number of the so-called “autoantibodies” in the years before a patient meets the official criteria for an autoimmune diagnosis supports the model of successive infection described earlier – pathogenic components of the microbiota gradually accumulate over the course of a lifetime until bacterial, viral, and phage load reaches a level at which a diagnosis can be made. It also supports the contention that individuals perceived as “healthy” may still harbor and accumulate pathogenic microbes that will eventually lead to an inflammatory diagnosis, or a process associated with “aging.” Indeed, it is possible that any antibodies that damage “self” do so as an unintended polyspecific consequence of their activity against the metagenomic pathogens.

## Therapies in the Era of the Metagenome

At the 2008 International Conference on Metagenomics in La Jolla, CA, James Kinross of the Imperial College of London began his speech with the following statement: “We surgeons have been operating on the gut for literally thousands of years and the microbiota has just been this extraordinary elephant in the room. We seem to have completely ignored the fact that we’ve co-evolved with thousands of bacteria over millions of years and that they somehow may be important to our health. As doctors, we routinely do terrible things to the microbiota and I’m sure this has implications for our health.”

Although most physicians are undoubtedly well intentioned, Kinross is correct in that many clinicians are generally not offered training that would keep them up to date with advances in metagenomics. The result is that many doctors still believe that nonmucosal surfaces of the body are largely sterile and that bacteria and other pathogens are not driving factors in the autoimmune processes. Instead, the standard

of care for patients with autoimmune disease continues to be corticosteroids and TNF-alpha blocking medications. According to a 2008 report, TNF-alpha inhibitors accounted for 80% of RA drug sales in the United States, France, Germany, Italy, Spain, the United Kingdom, and Japan. Use of these immunosuppressants is still grounded in the theory that autoimmune disease results from an overly exuberant immune response and these drugs are administered without consideration for the presence of a metagenome. Whether helpful or harmful, there is no question that by dramatically slowing the immune response, such therapies must necessarily and profoundly affect the composition, development, and stability of the human microbiota.

Despite the copious use of these immunosuppressant drugs in autoimmune conditions, they provide, at best, short-term palliation. Gottlieb et al. showed that steroid use causes relapse in sarcoidosis (Gottlieb et al., 1997). Additionally, there are no definitive studies showing corticosteroids improve long-term prognosis in the treatment of chronic inflammatory illness, nor is there any demonstrated reduction in mortality. Van den Bosch and Grutters write, "Remarkably, despite over 50 years of use, there is no proof of long-term (survival) benefit from corticosteroid treatment" (Grutters and van den Bosch, 2006). On the contrary, one of the side effects of TNF-alpha inhibitors is an increased risk of tuberculosis. Several studies have shown that TNF-alpha production is required for the proper expression of acquired specific resistance following infection with *M. tuberculosis* (Allie et al., 2008; Arend et al., 2003). So if we inhibit TNF-alpha expression, we would expect a long-term increase in the prevalence of not only tuberculosis, but also in any of the autoimmune or inflammatory diseases already associated with chronic forms of mycobacteria and other bacteria (Bull et al., 2003; Burnham et al., 1978).

The failure of these first-line therapies to cure "autoimmunity," and the range of detrimental side effects associated with their use, suggests that slowing the immune response of patients with autoimmune disease is counterproductive, allowing microbial populations to develop unchecked. Now that autoimmune conditions are more widely understood as illnesses in which myriad pathogens may trigger or drive the disease process, efforts to target the root cause of autoimmune disease should instead be targeted toward activating the innate immune response, not suppressing it.

Our own work (Perez, 2008) offers an example of the results of stimulating rather than suppressing the innate immune response of patients with autoimmune disease. Over the past 7 years, we have observed the effects of an experimental therapy for autoimmune disease that uses the VDR agonist olmesartan to reverse pathogen-induced VDR dysregulation. Subjects are also administered sub-inhibitory bacteriostatic antibiotics, which weaken bacterial ribosomes so that pathogens can more easily be targeted by the reactivated immune system. Nearly all of the hundreds of patients to start the therapy reported the predicted increase in specific symptoms of their autoimmune diagnosis. After months, or sometimes years, of dealing with these symptomatic flares, the very symptoms that waxed and waned in synchronism with antibiotic administration began to disappear, resulting in improvement and, in many cases, eventual resolution of the disease process. This response has been noted in the widely varying diagnoses of sarcoidosis, rheumatoid arthritis,

lupus, Type 2 diabetes, uveitis, Hashimoto's thyroiditis, ankylosing spondylitis, chronic fatigue syndrome, and fibromyalgia among others. The often dramatic elevations in disease activity observed among study subjects – particularly during the early stages of therapy – cannot be attributed to side effects of the protocol medications, as individually the drugs are well known and unremarkable (Schwocho and Masonson, 2001). Additionally, when healthy individuals have been administered the same medications they do not suffer any similar symptoms.

The most viable hypothesis for these temporary surges in disease symptoms and inflammatory markers is that treatment medications allow the immune system to mount an effective attack on an intracellular microbiota, such as the microbiota observed by Wirostko et al. It is reasonable to expect that when intraphagocytic pathogens are killed, some of the host cells will also undergo apoptosis, phagocytosis, or simply disintegration, leading to an increase in inflammation. For over 100 years, researchers have noted that the death of acute and persistent pathogens is accompanied by a surge in inflammation. They have attributed the temporary rise in inflammation to an increase in endotoxin and cytokine release upon bacterial death. Known as the Jarisch–Herxheimer reaction, or immunopathology, this phenomenon has been previously demonstrated after antibiotic administration in diseases including tuberculosis (Cheung and Chee, 2009), borreliosis (Vidal et al., 1998), tick-borne relapsing fever (Mitiku and Mengistu, 2002), multiple sclerosis (Kissler, 2001), Whipple disease (Peschard et al., 2001), and syphilitic alopecia (Pareek, 1977), among others. Zinkernagel also observed immunopathology in the mice he had infected with a persistent neuro-active virus (Zinkernagel et al., 2009). Similarly, immune reconstitution inflammatory syndrome (IRIS) is a condition seen in some cases of AIDS following the use of antiretroviral drugs. As the immune system begins to recover, it responds to previously acquired opportunistic infections with an overwhelming inflammatory response that, like the immunopathological reaction we observe, makes the symptoms of the infection temporarily worse (Shelburne et al., 2002). At this point in time, the exact species or forms of bacteria potentially killed by any one subject in our own study cohort remain unknown. As the focus of the HMP moves beyond the mucosal surfaces, and catalogs L-forms and other intracellular species within body tissues, a clearer picture of disease pathogenesis will emerge. However, as long as patients continue to report improvement and recovery, determining the exact nature of pathogens being targeted by the therapy has not been a high priority, given the limited resources currently allocated to this research team.

Some subjects in the cohort have reported drops in viral titers, suggesting that once the immune system is no longer burdened by the pathogenic components of the bacterial microbiota, it may regain the ability to target chronic viruses as well. This suggests that treatments that reverse immunosuppression caused by the bacterial microbiota might also prove useful in mitigating viral virulence.

Our research suggests that while some people report being “allergic” to certain bacteriostatic antibiotics, what they perceive as an “allergy” may actually be immunopathological reactions. For example, there are reports of minocycline “inducing lupus” (Geddes, 2007). A more logical explanation may be that certain

patients harbor persistent bacterial species that predispose for sub-clinical lupus. When minocycline is administered, some of these bacteria are killed, resulting in immunopathological reactions that are mistakenly interpreted as clinical manifestation of the disease.

What we have initiated needs further testing. However, the reports of profound immunopathological reactions in autoimmune subjects imply the need to re-examine whether palliative drugs actually provide long-term benefit for patients with autoimmune disease. Whether at the doctor's office or the health food store, patients with autoimmune conditions continually seek out palliative drugs or supplements that successfully reduce symptoms by lowering inflammation. Yet, if bacteria drive the pathogenesis of autoimmune inflammation, and chronic bacterial death invariably results in temporary increases in discomfort, then treatments that mitigate symptoms may well do so at the expense of proliferation in pathogenic components of the microbiota. Commonly used immunosuppressive compounds include vitamin D, which, although its immunosuppressive properties have now been identified (Arnsen et al., 2007), is now viewed as the ultimate inexpensive wonder drug (Holick, 2008). Frequent use of vitamin D, as well as other substances that slow immune activity, could at least partially account for the recently increased prevalence of nearly every autoimmune disease (Luque et al., 2006).

## **L-Form Bacteria: An Often Overlooked Component of the Microbiota**

Certain stages of the bacterial life cycle result in the loss of the cell wall. L-form bacteria are often less than 0.2  $\mu\text{m}$  in diameter (Domingue and Woody, 1997) and are therefore difficult to view with a standard optical microscope. Not only do these L-form variants fail to succumb to antibiotics that target the bacterial cell wall, but those antibiotics also encourage the formation of L-forms. "Treatment with penicillin does not merely select for L-forms (which are penicillin-resistant) but actually induces L-form growth," states Josep Casadesus of the University of Sevilla (Casadesus, 2007). In fact, researchers deliberately culture classical forms of bacteria in conjunction with various beta-lactam antibiotics in order to create L-forms (Mattman, 2000). The ability of the L-form to flourish in the face of treatment with the beta-lactam antibiotics points to a mechanism by which acute bacterial forms can mutate into latent mutants that may cause disease at a later time. Some researchers have deemed the conversion into the L-form state to be a universal property of bacteria (Gumpert and Taubeneck, 1983).

Joseleau-Petit et al. showed that classical forms of bacteria transform into the L-form only if they are denied the ability to form a normal cell wall (Joseleau-Petit et al., 2007). The beta-lactam antibiotics work toward this end by blocking the creation of penicillin-binding proteins (PBPs) – proteins responsible for forming the cross-linked chains associated with a peptidoglycan-derived cell wall. When the ability of the PBPs to create a full cell wall is blocked, the cells also become spherical and osmosensitive. Recently, Glover et al. performed the first systematic

genetic evaluation of genes and pathways involved in the formation and survival of unstable L-form bacteria (Glover et al., 2009). Microarray analysis of L-form versus classical bacterial colonies revealed many upregulated genes of unknown function as well as multiple overexpressed stress pathways shared in common with persister cells and biofilms. Dell’Era et al. also observed cell division and changes in gene expression in stable *L. monocytogenes* L-forms (Dell’Era et al., 2009).

Since the discovery of the L-forms in 1935 (Kleineberger-Nobel, 1951), they have been described in hundreds of publications. Yet because researchers are only just beginning to use molecular tools to study the L-form, they are still seldom factored into the mix of microbes that compose the human microbiome. However, over the years, L-forms have been implicated in dozens of diseases of unknown etiology, including RA, multiple sclerosis, sarcoidosis, glomerulonephritis, idiopathic hematuria, interstitial cystitis, rheumatic fever, and syphilis – as well as a large number of chronic and relapsing infections (Domingue and Woody, 1997; Mattman, 2000).

## **A Research Consideration: Men Are Not Tall Mice Without Tails**

The emerging role of the human microbiota implies a reconsideration of certain long-standing and frequently invoked models of disease. According to Javier Mestas of University of California, Irvine, “There has been a tendency to ignore differences and in many cases, perhaps, make the assumption that what is true in mice is necessarily true in humans. By making such assumptions we run the risk of overlooking aspects of human immunology that do not occur, or cannot be modeled, in mice” (Mestas and Hughes, 2004). Murine models are still used in an effort to understand most autoimmune and inflammatory conditions, despite the obvious differences between the murine and human immune systems.

For example, there are major differences in the Toll-like receptors. TLR1-9 exists in both mouse and man, although TLR8 detects single-stranded RNA in man and has no known function in the mouse. TLR10 exists in humans only; it is a degenerative pseudo-gene in the mouse. TLR11, 12 and 13 in mice do not exist in man and their function is not yet well defined.

Analysis of the human and murine VDR offers other examples of discord between man and mouse. Marshall’s molecular dynamics emulation showed that the drug olmesartan, a putative VDR agonist, binds into a different conformation in the murine VDR to that of *Homo sapiens* (Marshall, 2008), calling into question the whole concept of drug safety testing in murine models.

While the human VDR transcribes dozens of genes necessary for a robust innate immune response, including many key AMPs, the VDR does not similarly control the murine innate immune system.

The murine innate immune response is dependent on a cascade of nitric oxide functions in a manner yet to be fully understood (Bogdan, 2001). Although mice have VDRs, the homology differs, and they express different genes than the human

VDR. For example, the gene encoding the calcium-binding protein osteocalcin is “robustly” transcribed by the VDR in humans, but not in mice.

Brahmachary et al. showed that the rat VDR does not express the cathelicidin AMPs, marking an important difference in the way the two species target invading pathogens (Brahmachary et al., 2006). Gombart et al. recently expanded on the finding by providing evidence of an evolutionarily fixed, Alu-mediated divergence in steroid hormone nuclear receptor gene regulation between humans/primates and other mammals (Gombart et al., 2009). This divergence, which placed the cathelicidin pathway under VDR control only in humans and closely related primates, remained under purifying selection for the past 55–60 million years, and yet even cathelicidin in primates is not identical to that in man. Eventually, the pathway evolved to become a key component of a novel innate immune response unique to human infection. Because the murine VDR does not express cathelicidin, there is less of an evolutionary incentive for components of the murine microbiota to dysregulate its expression. This suggests that the survival mechanisms employed by the human and murine microbiotas may be very different. Thus, the intermingling of murine and human biologies in the literature hinders our ability to fully understand nuclear receptor control of the AMPs and other key aspects of innate immunity.

## Discussion

The prevailing theory of autoimmune disease, which dictates that the body creates autoantibodies that attack its own cells, was developed during an era when culture-based methods vastly underestimated the number of microbes capable of persisting in and on *Homo sapiens*. The advent of culture-independent tools such as 16S RNA sequencing, single cell sampling, and pyrosequencing has opened the door to an era of discovery. Rather than a sterile compartment, the human body is now known to teem with thousands of species of bacteria, viruses, and phages. In addition to persisting on the body’s external surfaces, these microbes survive in the blood and in many of the tissues, which become inflamed during autoimmune disease, suggesting that what were once thought to be “autoimmune” processes may instead result from the presence of persistent microbes. Metagenomics is allowing us to study these microbes in the tissues within which they naturally persist, where they can be examined in the context of other microbes in their community. A more exact understanding of how networks of microbes can interact to cause disease has superseded Koch’s postulates, which stipulate that a single microbe causes a single disease.

While diseases were once categorized largely on the basis of symptom presentation, they can now be classified based on their underlying genetics. Yet the expression of key human genes is continually altered by a plethora of microbial metabolites through an almost imponderable number of interactions. These metabolites, some of which are created by bacteria considered to be “friendly” or innocuous, can directly drive the pathogenesis of autoimmune disease by altering

the expression of genes such as ACE and PTN22, genes associated with diagnoses including rheumatoid arthritis, lupus, diabetes mellitus, myocardial infarction, renal tubular dysgenesis, and Alzheimer's. It is becoming apparent that autoimmune processes cannot be fully understood if the human genome is studied in isolation. An understanding of the interactions between the human genome and the metagenome calls for a more nuanced understanding of the microbiota. Classifying certain microbes as purely commensal may underrepresent the full spectrum of their actions. Indeed, harmless species of bacteria and viruses can easily acquire virulent plasmids via horizontal gene transfer or homologous recombination.

The microbiota has persisted in and on the human body for millennia. It has evolved to slow the host immune response in order to ensure microbial survival. Pathogens such as *M. tuberculosis*, *Borrelia*, EBV, and HIV have evolved to dysregulate the VDR nuclear receptor, inhibiting expression of the beta-defensin and cathelicidin AMPs along with TLR2. Flow-on effects from VDR dysregulation can further alter AMP expression via (at least) the alpha-thyroid, androgen, and glucocorticoid nuclear receptors. This may result in the immunosuppression and hormonal imbalances characteristic of many autoimmune diagnoses.

The bacteria that cause autoimmune disease likely accumulate over a lifetime, with individuals picking up pathogens with greater ease over time, as the immune response becomes increasingly constrained. Successive infection dictates that even people with the same autoimmune diagnosis are unlikely to present with identical clusters of symptoms and helps explain the high levels of comorbidity observed among these patients. Common autoimmune comorbidities include inflammatory conditions such as cardiovascular disease, along with mental diagnoses such as depression or anxiety, suggesting these conditions may also be driven by the microbiota. Thus, insights gained from studying microbial composition in autoimmune disease can accelerate research in other areas of medicine. Recently, several studies have shown the presence of "autoantibodies" in autism with antinuclear antibody seropositivity showing a significant positive association with disease severity, mental retardation, and electroencephalogram abnormalities. Rather than assign autism to the end of a growing list of autoimmune diagnoses, this knowledge might be better used as a basis on which to further explore the role that components of the microbiota may play in driving the pathogenesis of disease.

Analyzing autoimmune disease through the lens of metagenomics calls for a re-evaluation of the autoantibody. Polyspecific autoantibodies are increasingly being associated with elements of the microbiota, making it likely that the term "autoimmune" will soon lose its diagnostic utility. When a disabled immune system is forced to respond to the presence of a chronic microbiota, the resulting cascade of cytokines and chemokines will stimulate an adaptive immune response. The adaptive immune system will then proceed to generate antibodies to fragments of DNA generated by apoptosis or phagocytosis of infected cells. This is supported by studies showing that the so-called autoantibodies such as RO, La, dsDNA, and RF can be created in response to various bacterial and viral pathogens. Autoantibodies are often observed before a patient becomes fully symptomatic with an autoimmune diagnosis, reflecting the gradual accumulation of persistent microbes.



Rather than focusing on phenotypes and subsets of the metagenome, microbiome research may instead benefit from broader approaches geared toward understanding shared mechanisms of persistence. Translational medicine should aim at cutting through barriers among specialities, even between biologists and clinicians, so that more of the pieces of the emerging jigsaw of disease etiology can drop into place, and autoimmune disease patients can fully benefit from the insights gained from metagenomic science.

**Acknowledgments** The authors wish to acknowledge the assistance of Dr. Elena Kashuba for sharing her data and helping us prepare Fig. 12.2.

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