Autoimmune disease re-examined in light of metagenomic concepts

Amy D. Proal

This thesis is presented for the degree of

Doctor of Philosophy

of

Murdoch University

2012
School of Veterinary and Biomedical Sciences
Faculty of Health Sciences
Murdoch University
Perth, Western Australia
I declare that:

a) The thesis is my own account of my research, except where other sources are acknowledged.

b) The extent to which the work of others has been used is clearly stated in each chapter and certified by my supervisors.

c) The thesis contains as its main content work which has not been previously submitted for a degree at any other university.

Amy D. Proal

A note on formatting and style

This PhD thesis comprises a number of published research papers. These formatted documents are incorporated into this thesis along with additional text that has been provided to introduce and link the published work. It is hoped that the final amalgamation allows for the development of a cohesive body of research that can be easily followed.

The PhD thesis has continuous pagination, which can be seen at the bottom center of each page. For published documents, the original journal page numbers are also provided.
Acknowledgements

This thesis is dedicated to Paul Albert. For always believing in my potential even when I have been most challenged. For your love, which motivates me daily.

Mom, Dad and Sara:
Thank you for your constant love, support, and willingness to listen.

Dr. Trevor Marshall:
Thank you for your support, encouragement, and guidance. You have given me the chance to venture out into the field and learn much from interacting with others at conferences. Thank you for teaching me to continually pursue the alternative hypothesis.

Dr. Cassandra Berry:
Thank you for taking a chance on me as a student, even when my own hypotheses may not completely support your own. For being my advocate even while on the other side of the globe.

Dr. Douglas Eagles:
Thank you for going beyond the call of duty to support my research, even in the early days. You will always be one of my greatest role models.
Abstract

The concept of autoantibodies was developed at a time when, due to the limitations of culture-based techniques, the human body was considered to be largely sterile. However, over the past few years, researchers in the emerging field of metagenomics have developed molecular tools that instead allow microbes to be identified by their genomic fingerprints. These tools have opened a door to an era of tremendous discovery. *Homo sapiens* has been shown to harbor thousands of species of microbes in tissue and blood that were previously undetectable. Today it is estimated that around 90% of the cells in the human body are microbial, and that the genes of these microbes outnumber our own by a factor of at least 10:1. The genomes of intracellular microbes can directly interact with our own genomes, meaning that humans may be best described as superorganisms. When populations of these microbes interfere too much with the metabolism of *Homo sapiens*, the resulting changes in the proteome can lead to disease. This suggests that the inflammation observed in "autoimmune" disease may instead result from an effort by the innate immune system to target pathogens and restore microbial homeostasis. Many intracellular microbes survive by dysregulating the expression of genes and antimicrobials via key nuclear receptors. The VDR nuclear receptor plays a critical role by expressing cathelicidin and TLR2, the primary intracellular defenses. It appears that the pathogens that cause autoimmune disease accumulate during a lifetime, with individuals increasingly accumulating microbes as the innate immune response becomes incrementally compromised. One reason that autoimmune disease is more common in women may be that they have an additional site of VDR expression, in the cycling endometrium. Thus, they may more easily acquire microbial loads than their male counterparts. The interaction of many different microbes acting in concert is more likely to cause a particular autoimmune condition rather than, as Koch suggested, a single organism. This helps account for the high levels of comorbidity observed amongst patients with autoimmune conditions. Autoantibodies are increasingly being identified as the body's response to specific pathogens, with collateral
damage from these antibodies exacerbating the disease process. The possibility that microbes drive the autoimmune disease state calls for a re-evaluation of how these diseases are routinely treated. While the standard of care for autoimmune disease remains the use of medications that slow the immune response, treatments aimed at eradicating pathogens would attempt instead to stimulate the body's antimicrobial defenses. We have collaborated with American and international clinicians to research a therapy designed to reactivate the innate immune response in patients with autoimmune disease. Our case series demonstrate that patients generally report symptomatic improvement, but only after experiencing temporary increases in inflammation and disease symptoms. This is likely due to immunopathology - a reaction in which the release of cytokines and cellular debris accompany microbial death. Thus we must reconsider the long-term consequences of using immunosuppressive substances. For example, the secosteroid vitamin D reduces inflammation, but may do so at the expense of slowing the innate immune response and its ability to target underlying pathogens. Furthermore, the concept of vitamin D "deficiency" may itself be flawed. The low levels of 25-D in many patients with inflammatory conditions may be a result rather than a cause of the disease process. Conventional interpretation of other out-of-range metabolites must be similarly re-examined. This work offers a novel framework with which to understand and treat inflammatory disease, with broad implications across many disciplines. Efforts to further validate this model are needed, taking researchers down entirely new avenues of exploration.
# Table of Contents

Thesis declaration........................................................................................................................ iii
Acknowledgements..................................................................................................................... iv
Abstract....................................................................................................................................... v
Table of contents......................................................................................................................... vii
Abbreviations............................................................................................................................... xi
List of figures and tables............................................................................................................... xiv
List of presentations.................................................................................................................... xvii
List of publications...................................................................................................................... xviii
Ethical considerations................................................................................................................... xix

General introduction...................................................................................................................... 1
References........................................................................................................................................ 5

Chapter 1: Dysregulation of the vitamin D nuclear receptor may contribute to the higher prevalence of some autoimmune diseases in women. *Ann N Y Acad Sci.* 2009 Sep;1173:252-9 ............................................................................................................................. 6

Attribution....................................................................................................................................... 7
Abstract........................................................................................................................................ 8
Introduction..................................................................................................................................... 8
The Vitamin D Receptor is expressed in the human cycling endometrium....................... 9
Bacteria in autoimmune disease............................................................................................... 10
The human microbiome - a metagenome............................................................................... 10
VDR dysregulation by the microbiota...................................................................................... 11
The effects of VDR dysregulation ............................................................................................ 11
Secondary effects of VDR dysregulation on antimicrobial peptide expression................. 12
Elevated 1,25-D as a marker for autoimmune disease......................................................... 12
Pregnancy..................................................................................................................................... 13
Discussion........................................................................................................................................ 13
References......................................................................................................................................... 14
Summary and link to next chapter.............................................................................................. 16

Our therapeutic approach

Olmesartan appears to potentiate pulsed subinhibitory antibiotics.

Neurological comorbidities

Subclinical infection

Potential severity of immunopathology

Recently diagnosed patients

Surrogate outcomes for disease must be carefully chosen.

Markers of anemia

25-hydroxyvitamin D (25-D)

Blood pressure and creatinine

Blood urea nitrogen and creatinine

Immunostimulative therapies need further study.

Accepting discomfort

Blinding, randomization, and study design

Summary and link to General Discussion

References

Key Points

Translating science into practice

Moving away from reductionist approaches

Other considerations

Rethinking assumptions about the human microbiota

Continued support

Challenges in testing

Accepting discomfort

Blinding, randomization, and study design

Summary

References

General Discussion

Translating science into practice

Moving away from reductionist approaches

Other considerations

Rethinking assumptions about the human microbiota

Continued support

Challenges in testing

Accepting discomfort

Blinding, randomization, and study design

Summary

References
### Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-D</td>
<td>1,25-dihydroxyvitamin-D</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16S ribosomal RNA</td>
</tr>
<tr>
<td>1'H NMR</td>
<td>proton NMR</td>
</tr>
<tr>
<td>25-D</td>
<td>25-hydroxyvitamin-D</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AMPs</td>
<td>antimicrobial peptides</td>
</tr>
<tr>
<td>ANA</td>
<td>antinuclear antibody</td>
</tr>
<tr>
<td>anti-dsDNA</td>
<td>anti- double-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>anti-EBNA-1</td>
<td>anti-EBV nuclear antigen-1</td>
</tr>
<tr>
<td>anti-TTG</td>
<td>anti-tissue transglutaminase</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARA</td>
<td>American Rheumatism Association</td>
</tr>
<tr>
<td>ASCA</td>
<td>anti-Saccharomyces cerevisiae antibodies</td>
</tr>
<tr>
<td>B. anthracis</td>
<td><em>Bacillus anthracis</em></td>
</tr>
<tr>
<td>B. burgdorferi</td>
<td><em>Borrelia burgdorferi</em></td>
</tr>
<tr>
<td>B. cereus</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>B. fragilis</td>
<td><em>Bacteroides fragilis</em></td>
</tr>
<tr>
<td>BAK1</td>
<td>BRRI-associated receptor kinase 1</td>
</tr>
<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
</tr>
<tr>
<td>C. elegans</td>
<td><em>Caenorhabditis elegans</em></td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CFS</td>
<td>chronic fatigue syndrome</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>ChIP-Sequencing</td>
</tr>
<tr>
<td>CK</td>
<td>creatinine kinase</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CYP24</td>
<td>cytochrome P450C24</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>cytochrome P450C24A1</td>
</tr>
<tr>
<td>CYP27A1</td>
<td>cytochrome P450 27A1</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>25-Hydroxyvitamin D3 1-alpha-Hydroxylase</td>
</tr>
<tr>
<td>DDD</td>
<td>degenerative disc disease</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein–Barr virus</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ERB,</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>GCR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
</tr>
<tr>
<td>H. hepaticus</td>
<td><em>Helicobacter hepaticus</em></td>
</tr>
</tbody>
</table>
H. pylori  Helicobacter pylori
H. sapiens  Homo sapiens
H5N1  influenza A Virus, H5N1 Subtype
HAART  highly active antiretroviral therapy
HBD  human beta-defensin
hCAP18  human cationic antimicrobial protein 18
HHV-6  human herpesvirus-6
HIV  human immunodeficiency virus
HLA  human leukocyte antigen
HMP  Human Microbiome Project
HPV-16  human papillomavirus type 16
HRT  hormone replacement therapy
IGFBP-3  insulin-like growth factor
IgG  immunoglobulin G
IPA  indole-3-propionic acid
IRF8  interferon regulatory factor 8
IRIS  immune reconstitution inflammatory syndrome
ITP  idiopathic thrombocytopenic purpura
IU/L  international units per liter
Kd  kinetically determined dissociation constant
L. monocytogenes  Listeria monocytogenes
L1-L4  lumbar vertebrae 1-4
La  lupus anticoagulant antibodies
LCL  lymphoblastoid cell lines
LL-37  CAP18 lipopolysaccharide-binding protein
LTR  long terminal repeat
M. tuberculosis  Mycobacterium tuberculosis
mg  milligram
mg/L  milligrams per liter
MHC  major histocompatibility complex
mm/hr  millimeters per hour
mmHg  millimeters of mercury
mRNA  messenger ribonucleic acid
MS  multiple sclerosis
Mtbd  Mycobacterium tuberculosis
MTSS1  metastasis suppressor protein 1
NASA  National Aeronautics and Space Administration
NF-kappaB  nuclear factor-kappaB
NIH  National Institutes of Health
nmol/L  nanomoles per liter
NOD  nonobese diabetic
OA  osteoarthritis
P. aeruginosa  Pseudomonas aeruginosa
PBC  peripheral blood cells
PBP  penicillin-binding protein
PKA  protein kinase A
pmol/L  picomoles per liter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTN22</td>
<td>protein tyrosine phosphatase, non-receptor type 22 (lymphoid)</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RCTs</td>
<td>randomized controlled trials</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SARS</td>
<td>severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNPs</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>T3</td>
<td>triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TACO</td>
<td>transfusion-associated circulatory overload</td>
</tr>
<tr>
<td>TBC</td>
<td>tonsil B cells</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TLR2</td>
<td>toll-like-receptor 2</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
</tbody>
</table>
List of figures and tables

Chapter 1

Figure 1. Comorbidity of Hashimoto’s thyroiditis with other autoimmune diagnoses

Table 1. Affinities of native ligands and 1,25-D for various nuclear receptors

Figure 2. The Thyroid alpha receptor and its native ligand, T3 [PDB:2H77], with 1,25-D superimposed in the ligand binding pocket. Note how 1,25-D displaces T3 from binding to the key receptor residues. Calculated Kd is 8.41 for 1,25-D and 7.20 for T3.

Chapter 2

Figure 1. Patients with a given diagnosis.

Table 1. Selected research of serum values for 1,25-D.

Figure 2. 25-D vs. 1,25-D in a cohort of 100 patients.

Chapter 3

Figure 1. The secosteroids 25-hydroxyvitamin D (yellow) and 1,25- dihydroxyvitamin D (purple). Note that although the secosteroids have nearly identical structures, 25-D lacks the extra hydroxyl group, serving to stabilize the helices of the VDR and activate it[40]. The two
metabolites have nearly identical affinities for the VDR: 1,25-D has an estimated Kd of 8.48 while that of 25-D is 8.36.

Figure 2. Depiction of effect of vitamin D on chronic disease

Chapter 4

Figure 1. Relationships between diseases and genes, an excerpt from the shaded orange. Other inflammatory conditions are shaded red.

Chapter 5

Figure 1. Bacterial species identified by 16S rRNA gene sequencing of clones from 10 prosthetic hip joints

Figure 2. Nuclear receptors mRNA expression is downregulated upon infection of B cells with EBV

Figure 3. 25-D vs. 1,25-D in a cohort of 100 autoimmune patients

Table 1. Affinities of native ligands and 1,25-D for various nuclear receptors

Figure 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)

Figure 5. Co-morbidities 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.

Chapter 6

Figure 1. ANAs in a 58-year-old female with rheumatoid arthritis. ANA, anti-nuclear antibody.

Figure 2. BASDAI, ESR and CRP in a 50-year-old male with ankylosing spondylitis. BASDAI, bath ankylosing spondylitis disease activity index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Figure 3. Kidney metabolites in a 56-year-old male with sarcoidosis. BUN, blood urea nitrogen; GFR, glomerular filtration rate.
List of presentations

**Murdoch University, Perth, Western Australia, November 2011**

Gave invited lecture — “Autoimmune disease and the human metagenome”

**International Congress of Antibodies, Beijing, China, May 2009**

Gave invited lecture — “Antibodies and infection in the era of metagenome”

**International Congress on Autoimmunity, Porto, Portugal, Sept. 2008**

Gave invited lecture — “Vitamin D induced dysregulation of nuclear receptors may account for prevalence of some autoimmune diseases in women”

**Understanding Aging, UCLA, June 2008**

Presented poster — “VDR nuclear receptor competence in diseases of the aging”

**Days of Molecular Medicine, Karolinska, Sweden, Apr. 2008**

Presented poster — “Molecular mechanisms driving cognitive dysfunction in women with Chronic Fatigue Syndrome: examining the role of the endometrium, the nuclear receptors, and the antimicrobial peptides.”
List of publications


Ethical considerations

No patient interventions were initiated by Amy Proal. All patient care was performed by collaborating physicians as an accepted part of their practice of medicine, licensed under the laws of their respective countries of residence. Data analyzed by Amy Proal came from two sources. The majority of data was published publicly and willingly by patients of collaborating physicians via the Internet community websites operated by the Autoimmunity Research Foundation. Written consent was obtained from each member when they initially joined the discussion websites, and all data contributed by members describing their progress while using the interventions chosen by their physicians, was published willingly, publicly and openly by each individual. Additionally, papers published in joint authorship with licensed physicians occasionally discussed data supplied by those physician-authors, collected according to law, with full disclosure to, and permission from, their patients.
General introduction

In 2010, Sapkota used 16S rRNA-based taxonomic microarray to show that bacteria persisted in five commonly smoked brands of cigarettes. Such genomic sequencing tools as well as pyrosequencing and single cell sampling techniques can identify microbes by their genetic fingerprints and have proven vastly more effective than the culture-based techniques used for most of the past century. In fact, Sapkota’s method for identifying bacteria proved so powerful that she identified fifteen classes of bacteria and a broad range of pathogenic organisms in every cigarette tested – the vast majority of which have never been identified by in vitro technologies. While cigarette smoking has been linked to disease for some time, results like this imply that we must re-examine the hypotheses that underpin most of our research. By allowing for the identification and characterization of microbes in tissues once considered sterile, these new molecular tools allow much more compelling explanations for how disease develops and proliferates.

Autoimmune disease is widely understood to be a form of illness in which the adaptive immune system loses tolerance and begins to create autoantibodies against self. Over the past decades, researchers have characterized the relentless and long-standing inflammation that defines these diseases and their relapsing/remitting nature, but have not conclusively proven causation.

Numerous studies point to the chronic presence of microbes in patients with autoimmune disease, suggesting that they could play a role in the disease process. However, the autoimmune community continues to rely heavily on culture-based methods for microbial detection rather than use molecular tools. Thus, microbial prevalence and diversity are greatly underestimated in vivo. Tissue, blood, and cells continue to be regarded as largely sterile. In addition, historical assumptions still dominate the study of any identified
Researchers are expected to adhere to Koch’s postulates and consequently the notion of “one microbe, one disease” continues to hold sway.

Yet at the same time, researchers in another disparate field are actively re-defining the human/microbe relationship. That field is metagenomics - a specialty in which researchers perform genomic analysis of the microorganisms present in a specific habitat (a microbiome). In 2007, the NIH Human Microbiome Project was initiated, allowing dozens of research teams to identify previously undetected microbes and explore their ability to directly interact with the human genome. Some commentators have gone so far as to refer to the human body as a superorganism "whose metabolism represents an amalgamation of microbial and human attributes." Thus, metagenomic studies examine the metatranscriptome, or the expressed genetic information of an entire ecosystem.

It is now accepted that over 90% of cells in the human body are bacterial, fungal, or otherwise non-human in origin. Only a fraction of these microbes have been characterized, much less identified. The sheer number of non-human genes represented by the human microbiota – 1,000,000+ compared to the meager 23,000 in the human genome – implies we have just begun to fathom the full extent to which microbes impact the human condition in both health and disease.

Yet, as discussed above, most researchers in the field of autoimmunity have yet to apply these new findings to their work. This thesis represents an effort to cross-pollinate research from the field of autoimmunity with the copious data emerging from metagenomic studies.

We expound a novel model, a pathogenesis for autoimmune disease that describes how the genomes of many intracellular pathogens can interact directly with the human genome in
order to cause the catastrophic metabolic dysbiosis associated with the autoimmune disease state.

This pathogenesis centers on how communities of intracellular microbes can dysregulate gene expression by key nuclear receptors, particularly the vitamin D nuclear receptor (VDR), in order to promote their survival. Evidence is presented showing that microbial communities may act in concert to drive the inflammation characteristic of the autoimmune disease state and even the production of what are currently considered “autoantibodies.” These pathogens are acquired gradually over a lifetime so that the mix of species acquired determines which autoimmune symptoms and syndromes a person may eventually develop.

Chapter 1 introduces the VDR and its vital role in controlling components of the innate immune response including expression of TLR2 and the beta-defensin and cathelicidin antimicrobial peptides. It also expresses important genes involved in autoimmune and inflammatory disease processes (see Figure 1). The chapter posits in silico research showing that the sulphonolipid capnine, created by Lysobacter, likely dysregulates the receptor in order to promote its survival. By chapter 4, more detailed in vitro data is presented showing that Mycobacterium tuberculosis, Borrelia, and Epstein-Barr virus also dysregulate the VDR. Because disabling the innate immune system is such a logical pathogenic survival mechanism, other yet to be characterized microbes almost certainly persist in a similar fashion. Chapters 1 and 2 focus on the flow-on effects of such
dysregulation. Chapter 1 illustrates how VDR dysregulation may cause the active vitamin D metabolite 1,25-D to rise and subsequently affect expression of antimicrobial peptides expressed by other key nuclear receptors such as thyroid beta. This may impact women more severely as the cycling endometrium provides an added site of VDR expression and subsequently potential dysregulation in females. Indeed, chapter 2 presents data showing that of 100 patients with autoimmune disease, 81% present with 1,25-D levels above the “normal” range.

Chapter 3 explores how both 1,25-D and the inactive vitamin D metabolite 25-D affect transcription by the VDR and subsequently activity of the innate immune response. The mechanisms by which vitamin D palliates autoimmune symptoms and the concept of vitamin D deficiency are both re-evaluated in a new light. A novel model of vitamin D metabolism is described in which the low levels of 25-D often observed in patients with autoimmune disease are a result rather than a cause of the inflammatory disease process.

Chapter 4 introduces the novel concept of “successive infection.” Patients who present with autoimmune disease acquire pathogens in numerous ways including, but not limited to, childhood infection, vaccines, blood transfusions, and parental exposure (such as microbes in the sperm and egg). Successive infection dictates that, because many of these pathogens likely slow AMP expression via the nuclear receptors, such patients become increasingly immunocompromised. This creates a snowball effect in which each pathogen that decreases immune activity makes it easier for the host to pick up other pathogens and so on. Chapter 5 builds on this hypothesis, supporting it with novel data and describing in detail how the process may account for the high levels of co-morbidity and familial aggregation observed among patients with autoimmune disease. Chapter 5 also presents substantial data supporting the hypothesis that “autoantibodies”, often polyspecific, are created when the
innate immune system responds to the microbiota and a cascade of cytokines and chemokines stimulate the adaptive response. Weaknesses associated with a Mendelian model of inheritance in autoimmune disease are also discussed.

Chapter 6 introduces a therapeutic model for autoimmune disease that has formed the basis of our collaboration with United States-based and international physicians during the past eight years. The putative VDR agonist olmesartan is used to correct VDR dysregulation and prime the immune system to kill the intracellular pathogens driving the autoimmune disease process. Case series and histories are presented that examine the effects of the treatment in a variety of autoimmune diagnoses, most showing improvement and/or reversal of disease symptoms. Unfortunately increased microbicidal activity results in immunopathology - a temporary rise in symptoms due to apoptosis and toxin release. Challenges associated with managing immunopathology are discussed.

References


Chapter 1: Dysregulation of the vitamin D nuclear receptor may contribute to the higher prevalence of some autoimmune diseases in women

Attribution

AP developed the concept, reviewed the literature, wrote the manuscript, interpreted the findings, and helped design the figures. PA reviewed the literature, edited the manuscript and helped design the figures. TM supervised and critically revised the manuscript. Thank you to Janet Raty for the graphic design in Figure 2. All authors critically reviewed and approved the final version.

AP: 75%
Dysregulation of the Vitamin D Nuclear Receptor May Contribute to the Higher Prevalence of Some Autoimmune Diseases in Women

Amy D. Proal,a Paul J. Albert,b and Trevor G. Marshallc

aGeorgetown University, Washington, DC, USA
bWeill Cornell Medical College, New York, NY, USA
cMurdoch University, Perth, Australia

Researchers have noted that the incidence of autoimmune diseases, such as Hashimoto’s thyroiditis, is markedly higher in women than in men, but to date the reason for this disparity has been unclear. The vitamin D nuclear receptor (VDR) is expressed in the human cycling endometrium. Because the VDR controls expression of the cathelicidin and β-defensin antimicrobial peptides (AmPs), dysregulation of the receptor greatly compromises the innate immune response. Increasing evidence indicates the presence of a chronic, intraphagocytic, metagenomic microbiota in patients with autoimmune disease that may survive by dysregulating the VDR. VDR dysregulation, in turn, prevents the breakdown of the active vitamin D metabolite 1,25-hydroxyvitamin D (1,25-D) by CYP24. In silico data suggest that when 1,25-D rises above its normal range, it binds the α/β thyroid receptors, the glucocorticoid receptor (GCR), and the androgen receptor (AR), displacing their native ligands and causing an array of hormonal imbalances. IGF3 is displaced from α-thyroid, thyroiditis may result. Because the VDR, GCR, and AR also express multiple families of AmPs, expression of these natural antibiotics further wanes in response to dysregulation by 1,25-D. The end result is a system-wide drop in AmP expression that may allow pathogens to spread with greater ease. Because women have an extra site of VDR expression in the endometrium, the drop in AmP expression associated with nuclear receptor dysregulation may disproportionately affect them. This would cause women to accumulate higher bacterial loads than their male counterparts, particularly during early pregnancy when 1,25-D levels rise by 40%.

Key words: vitamin D receptor; metagenomic microbiota; 1,25-dihydroxyvitamin D; antimicrobial peptides; Hashimoto’s thyroiditis; autoimmune disease; pregnancy; vitamin D; olmesartan; women

Introduction

Systemic lupus erythematosus (SLE) and multiple sclerosis (MS) were first recorded over 100 years ago. Even at that time, it was noted that the diseases affect more women than men.1 Today it is estimated that autoimmune disease affects approximately 8% of the population, 78% of whom are women.2 Sex distribution in autoimmune disease, such as rheumatoid arthritis (RA), MS, and myasthenia gravis is around 60–70%. The most striking sex differences are observed in Sjogren’s syndrome, SLE, and scleroderma; these sex differences come from a spectrum of diagnoses in which the patient population is >80% women.1

Autoimmune thyroid diseases, such as Hashimoto’s thyroiditis, fall into the latter category. Beeson has reported that approximately 85% of patients with Hashimoto’s
thyroiditis are women. This rate of incidence is confirmed by data obtained from a retrospective trial in which a vitamin D nuclear receptor (VDR) agonist and bacteriostatic antibiotics are used to treat patients with various autoimmune diagnoses. While members of both sexes were allowed to participate in the trial, out of 100 subjects with autoimmune disease surveyed, 24 had Hashimoto’s thyroiditis and only three of them were men (see Fig. 1) (J.C. Waterhouse, personal communication, 2008).

Evidence for important interplays between the endocrine and immune systems has launched the new field of neuroimmunoendocrinology, which has attracted the interest of scientists and clinicians alike. Since autoimmune diseases often show preference for one sex, attention has been given to the possible role of sex hormones in affecting the disease process. The sex hormones activate or repress the activity of specific nuclear receptors, which form homodimers and heterodimers that directly bind DNA in order to regulate the expression of genes. Given the widespread relevance of the superfamily of nuclear receptors to almost all aspects of normal human physiology and the role they play in the etiology in human disease, a detailed understanding of these systems has major implications not only for human biology but also for the understanding and development of new therapies.

However, the majority of the body’s nuclear receptors are not activated by sex hormones. The potential of gender-related differences in the expression of these nonandrogenic nuclear receptors to affect the autoimmune disease process has received less attention. This chapter focuses on how differential expression of the VDR in females may contribute to the higher prevalence of autoimmune disease in women. It also examines how VDR dysregulation may impact the autoimmune disease process in both sexes.

The Vitamin D Receptor Is Expressed in the Human Cycling Endometrium

As discussed above, sex hormone expression differs between males and females. But, the active vitamin D metabolite 1,25-hydroxyvitamin D (1,25-D) and its target nuclear receptor, VDR, are also expressed in different quantities in males and females. Both sexes express the VDR in the keratinocytes, macrophages, and body tissue. However, Vigano’s recent work shows that 1,25-D and the VDR are expressed in the human cycling endometrium, meaning that women possess an extra site of VDR gene expression when compared to their male counterparts. Because the VDR plays a vital role in activating the innate immune response, this gender-based difference may have far-reaching consequences.

The innate immune response serves as the body’s first line of defense against infection. The VDR is activated by 1,25-D to directly induce expression of the cathelicidin and β-defensin antimicrobial peptides (AmPs). Furthermore, 1,25-D activates the VDR to transcribe (or repress) at least 913 genes. Several of these genes expressed by the VDR in the endometrium may well play a role in regulating events related to pregnancy or the menstrual cycle. They may also protect the fetus from infection.
Bacteria in Autoimmune Disease

A recent increase in autoimmune incidence led Rose to express concern over the possible role that infection might play in exacerbating autoimmune disease, particularly in women.2 Additionally, the Centers for Disease Control and Prevention has written that chronic infectious agents are emerging as notable determinants, not just complications, of chronic disease—stressing that infectious agents likely determine more cancers, immune-mediated syndromes, neurodevelopmental disorders, and other chronic conditions than currently appreciated.12

Rook provided evidence that several diseases usually regarded as “autoimmune” or “idiopathic,” including RA, Crohn’s disease, ulcerative colitis, sarcoidosis, and psoriasis, may be caused by infection with slow-growing bacteria.13 Similarly, Relman demonstrated evidence of persistent infection in sarcoidosis, various forms of inflammatory bowel disease, RA, SLE, diabetes mellitus, and primary biliary cirrhosis.14

Wirostko described persistent bacterial biofilm-like inclusions inside the phagocytes (monocytes, macrophages, neutrophils) of patients with Crohn’s disease,15 sarcoidosis,16 and juvenile RA.17 Serological evidence for bacterial infection has been demonstrated in patients with Hashimoto’s thyroiditis.18-19 Waterhouse et al. showed that 81% of a group of 54 patients representing 20 different autoimmune diagnoses reported continual improvement after treatment durations of 18–53 months with a VDR agonist and antibiotics—further pointing to bacteria as a causative agent in autoimmune disease.20

The Human Microbiome—a Metagenome

Recent advances in molecular techniques now allow for the detection of bacterial genomes of organisms that cannot be grown in culture. The scientific community is just beginning to comprehend the full impact of unculurable microbes upon human disease. The global initiative known as the Human Microbiome Project currently estimates that the microorganisms that live inside or on Homo sapiens outnumber somatic and germ cells by a factor of 10.21 To this point, only approximately 1% of this microbiota has been characterized and identified.22 The combined genetic contributions of these microbes—in excess of 100,000 protein-coding genes—provide traits not encoded in our own genomes.23 Some of these traits may well lead to autoimmune disease. Researchers affiliated with the Human Microbiome Project aim to use an array of molecular sequencing techniques to characterize the full Homo sapiens microbiota over the coming years.23

Bacteriologists are increasingly examining how the metagenome of complex microbial communities may contribute to disease. Koch’s postulates, which require that a single pathogen cause a single disease state, are being re-examined.24 This suggests that autoimmune disease results when patients concurrently accumulate a variety of different pathogenic forms, such as those that exist in a persistent metagenomic biofilm or in intracellular communities where they are better protected from the host immune response.20

The human body, once considered to be sterile, exists in symbiosis with the human microbiome. Recent studies show that chronic pathogens persist in the endometrium. Eighteen different taxa of microbes were recently identified in the amniotic fluid of women who gave birth prematurely.25 Mycobacterium tuberculosis and influenza HSN1 have been shown to cross the placental barrier.26,27 Infection with Shigella has been proposed as an explanation for the etiopathogenesis of endometriosis,28 and invasion of the endometrium by bacteria has been implicated in implantation failure, spontaneous abortion, and preterm birth.29
**VDR Dysregulation by the Microbiota**

While the expression of the VDR in the endometrium should put a healthy woman at an advantage by strengthening her ability to fight infectious agents, a dysregulated VDR leads to a state in which women are less able to mount an effective innate immune response. Among other compounds, bacterial ligands are capable of dysregulating the VDR. For example, the sulfonolipid capnine, produced by gliding biofilm bacteria, is a strong VDR antagonist. Because the creation of a VDR-dysregulating ligand provides a persistent pathogen with an evolutionary advantage, it is quite possible other bacteria have developed equivalent survival mechanisms. If this is the case, a chronic microbiota capable of dysregulating the VDR may well be perverting what the body intends as a protective environment during pregnancy and menstruation into one that allows disease to flourish.

The likelihood of a VDR-dysregulating microbiota in autoimmune disease is strengthened by the data collected by Waterhouse et al. in which subjects were routinely administered the VDR agonist olmesartan in conjunction with bacteriostatic antibiotics. He reported bacterial death resulting from the release of endotoxins and inflammatory cytokines, causing patients to experience an exacerbation in disease symptoms caused by immunopathology, sometimes referred to as the Jarisch–Herxheimer reaction. Patients administered the antibiotics without olmesartan experienced weak, sometimes negligible, increases in immunopathology. By contrast, when the same patients took the antibiotics in conjunction with olmesartan, immunopathology often became so strong that it had to be carefully controlled by palliative measures. That this dramatic change in immunopathology correlates with administration of a VDR agonist adds weight to the hypothesis that VDR dysfunction is central to the pathogenesis of autoimmune disease.

**The Effects of VDR Dysregulation**

Not only does VDR dysregulation decrease cathelicidin and β-defensin expression, it opens a number of other pathways leading to hormonal imbalance. The activated VDR expresses CYP24, the enzyme primarily responsible for breaking 1,25-D down into the inactive vitamin D metabolites. This exerts a feedback control on the maximum level that 1,25-D will attain. In silico modeling demonstrates that besides activating the VDR, 1,25-D also has a strong affinity for several of the body’s other nuclear receptors. This indicates that at high concentrations it can displace their native ligands. Table 1 shows, for example, that 1,25-D has a very high affinity for the α-thyroid receptor, suggesting that it can keep triiodothyronine (T3) out of the binding pocket (see Fig. 2). β-Thyroid is similarly affected. If 1,25-D prevents T3 from activating the thyroid receptors, genes with α-thyroid promoters will no longer be transcribed. The resulting thyroid disease would explain why increasing levels of exogenous thyroid hormone are necessary to maintain thyroid homeostasis as the disease progresses. Furthermore, since the type 1 nuclear receptors work as a group, if transcription by the α-thyroid receptor is dysregulated, a cascade of metabolic dysfunction will result. It is instructive to note that excessive 1,25-D also potentially interferes with several of the body’s other nuclear receptors. Table 1

---

**TABLE 1. Affinities of Native Ligands and 1,25-D for Various Nuclear Receptors**

<table>
<thead>
<tr>
<th>Nuclear receptor ligand</th>
<th>Native ligand</th>
<th>Native ligand (Kd)</th>
<th>1,25-D (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Thyroid</td>
<td>T3</td>
<td>7.20</td>
<td>8.41</td>
</tr>
<tr>
<td>β-Thyroid</td>
<td>T3</td>
<td>7.18</td>
<td>8.44</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Cortisol</td>
<td>7.36</td>
<td>8.12</td>
</tr>
<tr>
<td>Androgen</td>
<td>Testosterone</td>
<td>7.38</td>
<td>8.05</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Progesterone</td>
<td>7.53</td>
<td>8.09</td>
</tr>
</tbody>
</table>
shows high Kd values for the glucocorticoid, androgen, and progesterone receptors.

Secondary Effects of VDR Dysregulation on Antimicrobial Peptide Expression

If 1,25-D is able to dysregulate the nuclear receptors, it would have detrimental effects on system-wide AmP production. Just as the VDR expresses cathelicidin and β-defensin, other nuclear receptors also express AmPs. Brahmachary has shown that the glucocorticoid receptor, the androgen receptor, and the vitamin D receptor, are in control of 20, 17, and 16 families, respectively, out of the 22 analyzed. Thus, VDR dysfunction causes flow-on effects via glucocorticoid, thyroid, androgen, and other nuclear receptors, which potentially disable the bulk of the body’s production of AmPs.

Consequently, there is a strong relationship between hormonal dysfunction and autoimmune disease. Indeed, most of the patients with Hashimoto’s thyroiditis in the study reported by J.C. Waterhouse (personal communication, 2008) had also been diagnosed with other inflammatory or autoimmune diseases. Only 8% of subjects with Hashimoto’s thyroiditis had Hashimoto’s thyroiditis alone. Similarly, autoimmune thyroiditis has been reported in an elevated percentage of fibromyalgia patients. Smith has described a proven association between Hashimoto’s thyroiditis and Addison’s disease, type 1 diabetes mellitus, pernicious anemia, celiac disease, dermatitis herpetiformis, MS, RA, SLE, and systemic sclerosis. Sloka found that in nearly every subject studied, hypothyroidism caused by autoimmune thyroid disease showed a tendency to be more severe and more often present in patients with MS. Both men and women suffering from MS have been shown to manifest low-serum-T3 concentrations.

Because women have an extra site of VDR gene transcription—the endometrium—it is likely that a greater variety of genes are expressed by the female VDR. Thus, as women age, they may well be disproportionately affected by VDR dysfunction, particularly when it comes to AmP expression. It is likely they would accumulate heavier bacterial loads than their male counterparts. This might contribute to the higher incidence of autoimmune disease among females.

Elevated 1,25-D as a Marker for Autoimmune Disease

When active, transcription of CYP24 by the VDR keeps 1,25-D levels in the normal
range. If the VDR is disabled by disease and unable to express CYP24, patients should display higher than normal levels of 1,25-D. Studies on Crohn’s disease, ulcerative colitis, RA, Sjogren’s, and other autoimmune diagnoses confirm a higher than normal level of 1,25-D among study subjects. Blaney reported 1,25-D levels well above the accepted range in the majority of his cohort of 100 patients with autoimmune disease.

Yet data on 1,25-D levels in autoimmune disease remain relatively scarce because most clinicians test only the inactive vitamin D metabolite 25-hydroxvitamin-D (25-D) when determining vitamin D status. Low levels of 25-D have been tied to a higher incidence of autoimmune disease, leading to the consensus that vitamin D “deficiency” may be a risk factor for autoimmune disease. However, the low levels of 25-D often observed in autoimmune disease must also be viewed in light of data advanced by Marshall, Blaney, and others in which low 25-D levels are the result of the autoimmune disease process rather than part of its cause. According to this model, the likely pathway for the downregulation 25-D arises directly from the elevation of 1,25-D. Reduced gene expression by the pregnane X nuclear receptor (PXR) inhibits expression of CYP27A1 and thus downregulates conversion of vitamin D into 25-D.

It is clear that both 25-D and 1,25-D must be measured in patients with autoimmune disease, as the presence of inhibited 25-D expression or excessive 1,25-D expression both act as reliable markers of the disease process and are best interpreted in relation to one another.

Discussion

The confluence of in silico, in vivo, and in vitro data has elucidated a pathway in molecular biology that can potentially contribute to an understanding of the higher incidence of autoimmune disease observed among women. Dysregulation of the VDR by a chronic intraphagocytic microbiota would cause significant hormonal disruption by allowing 1,25-D to accumulate and displace native ligands from α-thyroid, glucocorticoid, androgen, and other nuclear receptors. By reducing the ability of these same nuclear receptors to express AmPs, accumulating 1,25-D would also cause a system-wide drop in AmP expression, allowing pathogens to proliferate. Because 1,25-D is expressed in the human cycling endometrium and rises by 40% during early pregnancy, women are disproportionately affected by the potential drop in AmP expression associated with VDR dysregulation and likely accumulate a more diverse microbiota than their male counterparts.

The advent of highly parallel DNA sequencers, high-throughput mass spectrometers, and other molecular techniques is ushering microbiology into a new era—steering focus away from the properties of isolated organisms to the manner in which a microbiota can act as metagenome when causing disease.

Pregnancy

In MS and RA, women experience periods of palliation during gestation only to become increasingly symptomatic after giving birth. Because 1,25-D production rises by 40% in the early pregnant decidua, its ability to dysregulate the nuclear receptors and the AmPs they express is particularly prevalent during this time. If a woman’s VDR expression has already become dysfunctional because of pathogen-induced 1,25-D dysregulation, the 40% surge in 1,25-D during pregnancy would result in additional substantial immunosuppression. Under such conditions, immunopathology would decrease, resulting in symptomatic relief. When the surge in 1,25-D disappears after pregnancy, AmP expression and immunopathology should increase, leading to exacerbation of disease symptoms.

Measuring both 25-D and 1,25-D may help resolve the anomalies in symptomatic presentation among MS, RA, and lupus.
Researchers affiliated with the Human Microbiome Project are beginning to characterize the milieu of unidentified bacterial organizations that persist in *Homo sapiens*.

Their findings have the potential to greatly expand our understanding of how chronic pathogens contribute to autoimmune disease. The potential role of persistent pathogens in autoimmune disease mandates reconsideration of the use of corticosteroids as a first-line treatment for many autoimmune diseases. Corticosteroids effectively reduce the ability of the immune system to respond to pathogens, including persistent microbiota, which is counterproductive to recovery. Waterhouse et al. report that antibacterial therapy can induce recovery from a variety of autoimmune diseases further cautions against the overuse of corticosteroids.

Scientists and clinicians should be encouraged to test both 25-D and 1,25-D in their subjects. A low 25-D and a high 1,25-D are both useful markers of the disease process. Low levels of 25-D are more likely a result rather than a cause of disease progression.

While this model provides novel insight into the manner in which pathogens may dysregulate the innate immune response and potentially contribute to the autoimmune disease process, much more research is needed. The relationship between nuclear receptors and the Amps they express has been sorely underexplored. Potential Amp expression by the estrogen or progesterone receptors has yet to be studied, leaving a gap in our understanding of how fluctuations in estrogen and progesterone during pregnancy and menstruation may also affect the female immune response.

**Conflicts of Interest**

The authors declare no conflicts of interest.

**References**

Summary and link to next chapter

In this first chapter, "Dysregulation of the Vitamin D Nuclear Receptor may contribute to the higher prevalence of some autoimmune diseases in women", we have explained the importance of the Vitamin D Receptor (VDR). The receptor expresses at least 913 genes, several associated with autoimmune disease and cancer. In addition, the VDR controls key aspects of the innate immune response including expression of cathelicidin, beta-defensins, and TLR2. We have introduced the major concept that the VDR is linked to health status and have shown how pathogens are able to dysregulate the VDR in order to gain a survival advantage leading to the development of a pathogenic microbiota.

Autoimmune disease is more common in women than in men. The reason for this discrepancy remains unclear. However, several mechanisms whereby this pathogen-induced VDR dysregulation may contribute to the higher incidence of autoimmune disease in women were explored in Chapter 1. Specifically, we illustrated how VDR dysregulation may cause the active vitamin D metabolite 1,25-dihydroxvitamin D (1,25-D) to rise, as it is not broken down by CYP24, and subsequently affect expression of antimicrobial peptides (AMPs) expressed by other key nuclear receptors such as β-thyroid. This may impact women more severely as the cycling endometrium provides an additional site of VDR expression and subsequently potential dysregulation in females. Differential expression of the VDR in females may contribute to greater prevalence of autoimmune disease in women. Pregnancy associated with a 40% rise in 1,25-D levels correlates with a decrease in AMP expression, allowing for VDR dysregulation and microbiota survival advantage. Importance is placed on measuring levels of both 25-D and 1,25-D in patients as biomarkers of disease.

We hypothesize that higher than normal levels of 1,25-D occur in patients with autoimmune disease due to dysregulation of the VDR by microbes. Building on this hypothesis, we
studied the 1,25-D levels of 100 Vancouver, Canada-based patients with a variety of autoimmune diagnoses, presented in Chapter 2, "Vitamin D metabolites as clinical markers in autoimmune and chronic disease".
Chapter 2: Vitamin D metabolites as clinical markers in autoimmune and chronic disease

Attribution

AP interpreted the findings, wrote the manuscript, and reviewed the literature. GB developed the concept, revised the manuscript, and collected the data. PA performed the statistical analysis, designed the figures, and reviewed the literature. All authors critically reviewed and approved the final version.

AP: 50%
Vitamin D Metabolites as Clinical Markers in Autoimmune and Chronic Disease

Greg P. Blaney, a Paul J. Albert, b and Amy D. Proal c

a Stillpoint Centre, Vancouver, British Columbia, Canada
b Weill Cornell Medical College, New York, NY, USA
c Georgetown University, Washington, DC, USA

Recent research has implicated vitamin D deficiency (serum levels of 25-hydroxyvitamin D <50 nmol/L) with a number of chronic conditions, including autoimmune conditions such as multiple sclerosis, lupus, and psoriasis, and chronic conditions such as osteoporosis, osteoarthritis, metabolic syndrome, fibromyalgia and chronic fatigue syndrome. It has been assumed that low levels of 25-hydroxyvitamin D (25-D) accurately indicate vitamin D storage and vitamin D receptor (VDR)–mediated control of calcium metabolism and innate immunity. To evaluate this assumption, 25-D and 1,25-dihydroxyvitamin D3 (1,25-D) levels were measured in 100 Canadian patients with these conditions. Additionally, other inflammatory markers (CK, CRP) were measured. Results showed a strong positive association between these autoimmune conditions and levels of 1,25-D >110 pmol/L. However, there was little association with vitamin D deficiency or the other inflammatory markers, meaning that the results challenge the assumption that serum levels of 25-D are a sensitive measure of the autoimmune disease state. Rather, these findings support the use of 1,25-D as a clinical marker in autoimmune conditions. High levels of 1,25-D may result when dysregulation of the VDR by bacterial ligands prevents the receptor from expressing enzymes necessary to keep 1,25-D in a normal range.

Key words: autoimmune disease; 1,25-dihydroxyvitamin D3; 25-hydroxyvitamin D; C-reactive protein; creatinine kinase

Introduction

There is increasing interest in the role of vitamin D deficiency in a number of chronic health problems, including autoimmune diseases. 1–8 However, other studies have shown a deleterious or no beneficial effect of vitamin D supplementation on certain diseases. 9–18 The effects of vitamin D are the result of genomic and non-genomic actions mediated by the active form of vitamin D, termed calcitriol, which is also known as 1,25-dihydroxyvitamin D3 (1,25-D). Yet most of the studies evaluating vitamin D and its association with disease are based on 25-hydroxyvitamin D (25-D) serum levels and not 1,25-D.

The definition of deficiency of 25-D is variable. One author recently surveyed the literature and determined that 25-D deficiency begins at or below 80 nmol/L. 19 Still, a number of studies have found levels below that to be common in healthy subjects. 20–24

Because of these ambiguities, an evaluation of vitamin D metabolites was conducted on 100 Canadian patients residing in the Pacific Northwest who suffer from diseases that have been associated with vitamin D deficiency. 25 Two markers of inflammation, creatinine kinase 26 and C-reactive protein, were also measured. 27,28

Address for correspondence: Greg P. Blaney, M.D., 4419 W. 10th Ave., Vancouver, BC, Canada. Voice: 604-224-6583; fax: 604-224-6584. gregblaney@shaw.ca

Materials and Methods

Blood samples from 100 randomly selected patients presenting with clinical criteria indicating the presence of autoimmune and associated diseases were drawn and analyzed by Lifelabs, located in Burnaby, British Columbia, Canada.

Of the 100 patients, 26 were male and 74 were female and ranged in age from 20 to 67 years. Patients with classical autoimmune disease totaled 30: nine with metabolic syndrome, 43 with chronic fatigue syndrome/fibromyalgia, 12 with post-Lyme disease syndrome, 29 and six with osteoarthritis (see Fig. 1).

Patients were measured for the presence of four blood markers: elevated levels of C-reactive protein, elevated levels of creatinine kinase, deficient levels of 25-D and elevated levels of 1,25-D. Elevated levels of C-reactive protein were determined by a finding of 5 mg/L or greater. Elevated levels of creatinine kinase were determined by a finding of above 300 U/L (males), 200 U/L (females). 25-D deficiency was determined by the finding of levels at or below 50 nmol/L.

Samples to be tested for 1,25-D were refrigerated and then frozen within 12 h after withdrawal. 25-D was measured using the Diasorin LIAISON chemiluminescence immunoassay. 1,25-D was measured using the Diasorin radioimmunoassay.

There appears to be a lack of consensus as to the normal serum levels of 1,25-D (see Table 1) with various authors citing ranges from 39–110 pmol/L,\(^{30}\) 33–160 pmol/L,\(^{31}\) 60–156 pmol/L,\(^{32}\) 47–162 pmol/L,\(^{33}\) and 36–108 pmol/L.\(^{34}\) The threshold for elevated 1,25-D was selected as 110 pmol/L based on the observation that all healthy patients in a clinical care setting showed levels under this range.\(^{35}\)

Furthermore, levels of 1,25-D have been shown to drop below 110 pmol/L in patients participating in later stages of a therapy in which a VDR agonist and pulsed low-dose antibiotics are used to eliminate bacteria thought to cause the vitamin D dysregulation observed in autoimmune disease.\(^{35}\)

Results

Levels of 25-D ranging from a low of 20 nmol/L to 50 nmol/L were found in 26 patients (see Fig. 2). None had below-normal levels of 1,25-D (<40 pmol/L). Interestingly, 1,25-D rather than 25-D served as a more accurate measure of a chronic inflammatory disease state.

Elevated levels of 1,25-D ranging from 110 pmol/L to a high of 350 pmol/L were found in 85 patients. Of these patients, 19 had 25-D levels below 50 pmol/L. The mean level of 1,25-D observed in our sample (143.46 ± 45.56 pmol/L) was significantly higher than the laboratory threshold value of 110 pmol/L (\(P < 0.0001\) by one-sample \(t\)-test).

Levels of C-reactive protein higher than 5.0 mg/L were observed in 17 patients, with the highest being 62.67 mg/L.

Creatinine kinase above the normal reference range was observed in 12 patients, with the highest being 1109 U/L in a male and 562 U/L in a female.

In diagnosed autoimmune patients, 10 out of 30 were found to have 25-D levels <50 nmol/L while 27 out of 30 showed 1,25-D levels
TABLE 1. Selected Research of Serum Values for 1,25-D

<table>
<thead>
<tr>
<th>Population</th>
<th>Age</th>
<th>Value of Serum 1,25-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 normotensive male industrial employees with no history of disease[^30]</td>
<td>25 to 64 years (range)</td>
<td>39–110 pmol/L</td>
</tr>
<tr>
<td>173 subjects at high and moderate risk for coronary heart disease[^31]</td>
<td>Two groups analyzed: 63.3 ± 7.1 years; 29.5 ± 10.6 years</td>
<td>33–160 pmol/L</td>
</tr>
<tr>
<td>1,903 chronic kidney disease patients who were not prescribed vitamin D[^32]</td>
<td>70.1 (mean)</td>
<td>60–156 pmol/L</td>
</tr>
<tr>
<td>10 healthy Inuit children[^34]</td>
<td>5 to 17 years (range)</td>
<td>36–108 pmol/L</td>
</tr>
<tr>
<td>1,384 premenopausal and 1,084 postmenopausal women[^40]</td>
<td>33.1 ± 10.1 years (premenopausal); 65.4 ± 8.1 years (postmenopausal)</td>
<td>35.5 pg/mL (mean for premenopausal women); 39.1 pg/mL (mean for postmenopausal women)</td>
</tr>
</tbody>
</table>

>110 pmol/L. Five out of 30 had C-reactive protein levels >5.0 mg/L. Five out of 30 had creatinine kinase levels above normal. Levels of 25-D below the normal range were observed in two out of nine patients with metabolic syndrome, while six had elevated levels of 1,25-D[^36,37]. Of the nine patients, five showed elevated levels of C-reactive protein, while none showed elevated creatinine kinase levels.

Of 43 patients with chronic fatigue syndrome/fibromyalgia[^38,39], 10 had <50 nmol/L of 25-D while 38 had >110 pmol/L of 1,25-D. Eight patients had elevated levels of C-reactive protein, while only one had an elevated level of creatinine kinase.
Post-treatment Lyme Disease syndrome patients may well represent a subcategory of CFS. Of 12 patients tested three showed 25-D levels <50 nmol/L. Nine showed 1,25-D levels >110 pmol/L. Two patients had C-reactive proteins >5 mg/L, while three had above-normal creatinine kinase levels.

In six patients with osteoarthritis (OA) and degenerative disc disease (DDD), only one patient had <50 nmol/L 25-D. However, all six patients had >110 pmol/L of 1,25-D. None had elevated levels of C-reactive protein. Two had elevated levels of creatinine kinase.

**Discussion**

These findings show that vitamin D deficiency was not as common as speculated in Canadian adults living in the Pacific Northwest and was not found in the majority of patients with diseases previously associated with vitamin D deficiency. However, elevated levels of serum 1,25-D were found in 85% of patients examined.

Technically, high levels of 1,25-D down-regulate, via the pregnane X receptor (PXR) nuclear receptor, the amount of vitamin D converted into 25-D, resulting in low levels of 25-D in patients with autoimmune disease. That 25-D levels remained above range in the majority of our cohort suggests that subjects were supplementing with vitamin D or simply eating a plethora of foods that are now artificially fortified with the secosteroid/hormone. So long as this confounding variable continues to exist, reliable data on how 25-D may be affected by the disease process itself and is likely to remain inconclusive.

Vitamin D supplementation leads to the formation of 25-D rather than 1,25-D. High levels of 25-D do not appear to prevent inflammatory disease, as 34 out of the 100 patients tested had 25-D levels above 80 nmol/L. This is consistent with other poor results of vitamin D supplementation in the treatment of inflammatory disease, particularly over long periods of time.

Certain studies have documented a therapeutic effect of vitamin D over the short term; Lappe et al. showed that consumption of vitamin D appears to be protective against colorectal cancer in a four-year trial. However, a similar study done on a larger cohort that lasted only an additional three years found no such effect. In fact, studies in which patients had been consuming vitamin D over the course of decades have shown a negative effect of vitamin D supplementation.

Also, it is well documented that those who supplement with vitamin D are qualitatively different than those who do not, having higher socioeconomic status, better education, stronger interest in health education, and presumably access to higher quality health care. This should give one further pause when assessing observational epidemiological studies on vitamin D supplementation.

In contrast, 1,25-D appears to be a highly sensitive clinical marker in diagnosis of autoimmune and associated diseases. It may be fruitful to consider why levels of 1,25-D are elevated in patients with autoimmune diagnoses. One possibility is that the vitamin D receptor (VDR) becomes dysregulated when exposed to sufficient quantities of substances created by bacteria that antagonize or otherwise inhibit the VDR. One such substance is the sulfonolipid ligand capnine. The protease caspase-3, which is up-regulated by *P. aeruginosa* and *H. pylori*, has a similar effect on the VDR, effectively inactivating it by cleaving it. The persistent and difficult-to-culture bacteria that create these substances may play a role in the pathogenesis of autoimmune and related diseases.

As bacterial ligands compromise the activity of the VDR, the receptor is prevented from expressing CYP24, an enzyme that breaks the 1,25-D down into its inactive metabolites. This allows 1,25-D levels to rise without a feedback system to keep them in check, resulting in the elevated levels of the hormone as observed in our cohort. Acquired hormone resistance has also been recognized with insulin, thyroid, steroid, and growth hormone releasing
Elevated levels of hormones are seen in some of these conditions.54

Consistent with the hypothesis is that although 85 out of 100 patients in our cohort had a 1,25-D higher than 110 pmol/L and a significant number (38) had levels greater than 160 pmol/L, there were no apparent clinical manifestations of hypercalcemia.

This suggests that although 1,25-D rises in inflammatory disease, it is unable to actually bind to the VDR and drive the expression of genes associated with calcium absorption. This could result because the receptor is already antagonized by bacterial ligands.

This model is supported by data collected from a trial in which patients with autoimmune diagnoses used a VDR agonist to restore VDR activity. Over the course of therapy, 1,25-D levels dropped into a normal range as inflammation decreased. This suggests that tracking 1,25-D levels may also serve as a valuable clinical marker of therapeutic response and efficacy of treatment modalities for autoimmune disease.

Given the potential benefits of serum 1,25-D as a clinical marker both in the diagnosis and monitoring of treatment response, further research is warranted. If elevated levels of 1,25-D continue to be associated with an inflammatory disease state, 1,25-D could be used as a reliable marker of the autoimmune disease process.

Due to the length of this chapter, we are unable to summarize all of the cutting-edge issues that surround this research. For this reason, we refer to the following recent literature on this subject.55–59

Conflicts of Interest

The authors declare no conflicts of interest.

References


D levels or improved by treatment with vitamin D. *J. Clin. Rheumatol.** 14: 12–16.


Summary and link to next chapter

Chapter 2, "Vitamin D metabolites as clinical markers in autoimmune and chronic disease", explored how both 1,25-D and the inactive vitamin D metabolite 25-D affect transcription by the VDR and subsequent activity of the innate immune response. The mechanisms by which vitamin D palliates autoimmune symptoms and the concept of vitamin D deficiency are both re-evaluated in a new light. A novel model of vitamin D metabolism is described in which the low levels of 25-D often observed in patients with autoimmune disease are believed to be a result rather than a cause of the inflammatory disease process.

We presented data showing that 85 of 100 patients with autoimmune disease present with 1,25-D levels above the normal range (>110pmol/L). However, low 25-D levels were not as common as expected in patients with autoimmune disease, possibly confounded by factors such as Vitamin D supplementation.

Many studies have noted that the secosteroid 25-D palliates the symptoms of autoimmune disease. However, the mechanisms behind such palliation remain unclear. Chapter 3 "Vitamin D: The alternative hypothesis" introduces a model that explores 25-D's actions in autoimmune disease at the molecular level.
Chapter 3: Vitamin D: the alternative hypothesis

Attribution

AP wrote the manuscript, interpreted the findings, developed the concept, and reviewed the literature. PA wrote the manuscript, designed Figure 2, and reviewed the literature. TM supervised and designed Figure 1. All authors critically reviewed and approved the final version.

AP: 55%
Vitamin D: The alternative hypothesis

Paul J. Albert a, *, Amy D. Proal b, Trevor G. Marshall c

a Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, United States
b Georgetown University, 37th and O Streets, NW, Washington, DC 20057, United States
c Murdoch University, Perth, Australia

ABSTRACT

Early studies on vitamin D showed promise that various forms of the “vitamin” may be protective against chronic disease, yet systematic reviews and longer-term studies have failed to confirm these findings. A number of studies have suggested that patients with autoimmune diagnoses are deficient in 25-hydroxyvitamin D (25-D) and that consuming greater quantities of vitamin D, which further elevates 25-D levels, alleviates autoimmune disease symptoms. Some years ago, molecular biology identified 25-D as a secosteroid. Secosteroids would typically be expected to depress inflammation, which is in line with the reports of symptomatic improvement. The simplistic first-order mass-action model used to guide the early vitamin studies is now giving way to a more complex description of action. When active, the Vitamin D nuclear receptor (VDR) affects transcription of at least 913 genes and impacts processes ranging from calcium metabolism to expression of key antimicrobial peptides. Additionally, recent research on the Human Microbiome shows that bacteria are far more pervasive than previously thought, increasing the possibility that autoimmune disease is bacterial in origin. Emerging molecular evidence suggests that symptomatic improvements among those administered vitamin D is the result of 25-D’s ability to temper bacterial-induced inflammation by slowing VDR activity. While this results in short-term palliation, persistent pathogens that may influence disease progression, proliferate over the long-term.

© 2009 Elsevier B.V. All rights reserved.

Keywords: 25-hydroxyvitamin D (25-D) 1,25-dihydroxyvitamin D3 (1,25-D) Vitamin D Antimicrobial peptides VDR

1. Introduction

Some researchers claim vitamin D is immunosuppressive; others argue it activates the immune system. Advocates for
vitamin D supplementation insist that various forms of the “vitamin” can be protective against chronic disease, but longer-term studies have failed to confirm these findings [1]. Even among those who support widespread supplementation with the substance, there is wide acknowledgement that the understanding of vitamin D metabolism is “imprecise” [1].

L.R. Karhausen wrote, “Actually, there is no experience of causation: events do not wear their causal credentials on their faces [2.]” In this review, we discuss how the understanding of vitamin D metabolism in chronic disease may be approaching “black box epidemiology” [2].

We hope to show that although vitamin D is currently viewed in a beneficial light, explanations for how it provides a benefit are simplistic and imprecise. We will address liabilities of the disease/deficiency model for vitamin D and summarize an alternative theory that, if valid, would necessitate rethinking systematic supplementation with vitamin D.

2. Black box epidemiology

Black box epidemiology is a focus on risk factors related to disease outcome without satisfactorily understanding pathogenesis [3]. This is the case with autoimmune disease, of which there remains widespread debate about what causes the majority of these common illnesses [4].

There are a number of examples of how incomplete understanding of the causative factors of disease can have unfortunate ramifications. Double-blind and/or randomized controlled trials (RCTs), as recently as 15 years ago, erroneously showed women taking combined hormone replacement therapy (HRT) had a lower-than-average incidence of coronary heart disease (CHD). This led doctors to propose HRT was protective against CHD.

As it was learned, those with higher socioeconomic status were more likely to use HRT [5]. The increased incidence in CHD caused by HRT could have been mitigated by other factors also associated with elevated status: better medical care, healthier eating habits, etc. Although we don’t know the mechanism by which HRT causes CHD, studies suggest, given its widespread use, tens of thousands of women died prematurely or suffered strokes or cancer. One commentator asked provocatively, “Is this the death of observational epidemiology?” [5].

RCTs are not without liabilities—especially not when an intervention generates a short-term benefit, but is harmful over the long-term. Multiple studies found the combination therapy of fenfluramine and phentermine (fen-phen) improved various physiological measures of health—raising HDL cholesterol, lowering triglycerides [6,7] and reversing obesity over the short-term. Yet, when researchers finally gathered data on patients who had been taking the drug for longer periods of time, it became clear fen-phen caused pulmonary hypertension and valvular dysfunction [7].

The entire class of steroids seems to be especially problematic. The first-line treatment for many autoimmune diagnoses, the corticosteroid Prednisone, may temporarily reduce symptoms of disease, but long-term use dramatically increases the odds of disease relapse [8]. This finding, as we will see, may be true for the secosteroid 25-D.

3. The vitamin D receptor and the vitamin D metabolites

People obtain vitamin D through diet, supplements, and exposure to sunlight. Vitamin D2 is found in plants and fungi and vitamin D3 in meats. Vitamin D3 is also produced endogenously when the eyes and skin are exposed to ultraviolet light. Both vitamins D3 and D2 are hydroxylated in the liver, becoming the secosteroid 25-hydroxyvitamin-D (25-D). Under hormonal control mechanisms, the enzyme 1-alpha-hydroxylase further hydroxylates 25-D into the main biologically active hormone/secosteroid, 1,25-dihydroxyvitamin-D3 (1,25-D). 25-D and 1,25-D serve as the native or endogenous ligands for the vitamin D receptor (VDR), a nuclear receptor found in immune and other cell types [9].

The VDR is responsible for transcribing 913 genes and probably many more [10]. Directly and/or indirectly, the vitamin D endocrine system regulates 3% of the human genome [11]. The VDR transcribes the beta-Defensin and cathelicidin antimicrobial peptides, broad-spectrum bacteria which target pathogens [12]. When active, the VDR also transcribes TLR2, which recognizes gram-positive bacteria.

Recent vitamin D studies seem to address two broad observations regarding 25-D. First, serum levels of 25-D tend to be significantly lower in patients with autoimmune disease [13]. Second, subjects given vitamin D, even in controlled studies, often seem to have lower rates of autoimmune disease and fewer markers of inflammation [14].

These observations have led people to assume that supplemental vitamin D is beneficial, because it decreases inflammation and autoimmune disease symptoms. Therefore, many researchers suggest, some more strongly than others, that regular and systematic supplementation with vitamin D alleviates autoimmune disease [9]. We will call this view the deficiency/disease model. However, these same observations can be interpreted differently. Low 25-D levels in autoimmune disease may be a result of the disease process itself, and the drop in inflammation among individuals taking the secosteroid may stem from its ability to slow immune function. We will call this view the alternative model. Efforts to determine which of these models is correct must examine how 25-D affects the VDR.

4. Liabilities of the deficiency/disease model

Vitamin D’s mode of action at the molecular level remains a matter of debate among those who espouse the deficiency/disease model. Autoimmune diagnoses are widely explained to be illnesses in which the immune system and subsequently the VDR are overactive [15]. These researchers argue additional vitamin D calms the immune response, presumably by deactivating the VDR. Shoefield et al note, “Vitamin D has multiple immunosuppressant properties [15].”

In contrast, cancer, arteriosclerosis, and other inflammatory diagnoses are often characterized as illnesses in which the immune system fails to function adequately, suggesting decreased VDR activity. Such research theorizes that additional vitamin D activates the VDR after being converted into 1,25-D [16]. Researchers have even tried to treat cancer by inducing autoimmune disease [17].
Autoimmune diseases and cancers are not mutually exclusive. There is co-morbidity between the diseases, and they share some physiological biomarkers. People with the autoimmune diagnosis Crohn’s disease are much more likely to get certain forms of cancer, including colon/rectal cancer [18].

If a patient presents with both cancer and Crohn’s, how can vitamin D alleviate symptoms associated with both diseases if it is expected to activate the VDR in one disease state and slow its activity in another? Thus, the deficiency/disease model for vitamin D metabolism leaves us without a clear model for how the secosteroid works at the molecular level and contradictory assumptions for how it exerts a beneficial effect.

5. Insights emerging from the molecular biology

Recent molecular and clinical research forms the basis for an alternative model of vitamin D metabolism, one that fully accounts for clinical observations in autoimmune disease. If valid, this theory significantly undermines any rationale for giving supplemental vitamin D to patients with autoimmune diagnoses. According to the alternative model, low levels of 25-D in patients with autoimmune disease are a result rather than a cause of the disease process. Secondly, the reduction in inflammation, clinical disease markers, and disease symptoms in patients taking supplemental vitamin D result from temporary suppression of the innate immune response.

As previously discussed, research indicates the VDR is ultimately a control system for the innate immune response. In silico simulations show that while 1,25-D possesses the residue necessary to agonize the VDR, 25-D does not (Fig. 1) [19]. That the two main forms of vitamin D alternately activate or deactivate a receptor at the heart of several critical feedback pathways makes sense from an evolutionary viewpoint. Indeed, 25-D and 1,25-D share an almost identical affinity for the receptor [19]. The body regulates the production of 1,25-D, and, in turn, the VDR, through a series of intricate and carefully controlled feedback pathways, mechanisms that belie the simplicity of the deficiency/disease model.

Understanding the alternative model for vitamin D requires an appreciation for how the human microbiota has evolved to slow the innate immune response in order to facilitate its survival. Molecular data shows certain members of the microbiota create ligands that block the transcriptional pathways set in motion by an active VDR [20].

Relatively little is known about the 90% cells persisting in Homo sapiens that are non-human; the genomes of only a fraction of such microbes have been sequenced. While some of these bacteria may contribute to well-being, others may be pathogenic. Persistent and unique communities of microbes have been detected in subjects with diseases ranging from autism [21] to obesity [22].

The innate immune system responds to chronic pathogens by secreting cytokines and chemokines in an effort to clear them from the body. If it fails, the result may be a disease stalemate that accounts for the chronic inflammation observed in autoimmune disease. Furthermore, as the microbiota continues to dysregulate the VDR, transcription of key enzymes is thwarted. VDR production of CYP24A1 decreases, allowing 1,25-D to rise without a feedback system to check it. As the hormone/secosteroid rises above a normal range, it down-regulates, via the PXR nuclear receptor, the amount of vitamin D converted into 25-D [19]. This results in the low levels of 25-D characteristic of autoimmune diagnoses.

5.1. Explanation for effects of vitamin D supplementation

Substances capable of slowing VDR activity also reduce the innate immune response and subsequently the inflammation associated with bacterial death. Since 25-D antagonizes the VDR, it follows that as the secosteroid and bacterial ligands accumulate, the innate immune system is less able to effectively target pathogens—including those that may further dysregulate the VDR. In the short-term, cytokine and chemokine production by the innate immune system drops. Fewer endotoxins and less cellular debris are created by bacterial die-off, resulting in a decrease in inflammation and overall disease symptoms. Yet, over the long-term, the pathogens at the heart of the disease process spread with greater ease. In this respect, the vitamin D in food and supplements is not unlike corticosteroids—substances that ameliorate disease symptoms in the short-term but exacerbate them over time.

We all can appreciate that the absence of disease symptoms is not necessarily the same as the absence of disease. The adverse effects of immunosuppressants sometimes take decades to be realized. Users of anabolic steroids, which are immunosuppressive [23], feel well being and euphoria when taking the drugs. However, researchers have
documented higher rates of cardiovascular disease in former users [24]. Use of corticosteroids, a first-line treatment for many autoimmune diagnoses, significantly increases relapse by a striking margin [8]. There are no studies that show that corticosteroids improve long-term prognosis in the treatment of illness. One author writes, “Remarkably, despite over 50 years of use, there is no proof of long-term (survival) benefit from corticosteroid treatment [25].”

According to the alternative model, true recovery from autoimmune disease involves an activated immune response and a corresponding spike in symptoms due to bacterial die-off—a phenomenon known as immunopathology [26]. Symptom exacerbation in the face of an activated immune response occurs in other diseases including AIDS, in which patients exhibit Immune Reconstitution Inflammatory Syndrome after beginning antiretroviral therapy aimed at targeting opportunistic infections. Syphilis, sarcoidosis [27], and a number of additional diseases [26] also induce immunopathological-type reactions during periods where the immune system succeeds in targeting chronic pathogens.

Consequently, if patients with autoimmune disease succeed in killing bacteria associated with their disease state, their symptoms should be expected to escalate, at least in the short-term, as cytokines and endotoxins are generated [28]. Conversely, in cases in which the immune response has been suppressed by supplementation with an immunosuppressant such as the secosteroid 25-D, one would expect to see fewer clinical manifestations of disease in the short-term, yet more advanced disease in the long-term. At a certain point, depending on the clinical symptom or physiological markers of disease, patients supplementing with vitamin D would be expected to approach a “crossover point” when additional reduction of the immune response is eclipsed by the advancing disease (Fig. 2). This outcome has been demonstrated in longitudinal studies, with studies on sicker or older patients taking less time to realize the effect.

The Iowa Women’s Health study showed vitamin D intake indeed allows the innate immune system to succeed in other diseases including AIDS, in which patients exhibit Immune Reconstitution Inflammatory Syndrome after beginning antiretroviral therapy aimed at targeting opportunistic infections. Syphilis, sarcoidosis [27], and a number of additional diseases [26] also induce immunopathological-type reactions during periods where the immune system succeeds in targeting chronic pathogens.

Consequently, if patients with autoimmune disease succeed in killing bacteria associated with their disease state, their symptoms should be expected to escalate, at least in the short-term, as cytokines and endotoxins are generated [28]. Conversely, in cases in which the immune response has been suppressed by supplementation with an immunosuppressant such as the secosteroid 25-D, one would expect to see fewer clinical manifestations of disease in the short-term, yet more advanced disease in the long-term. At a certain point, depending on the clinical symptom or physiological markers of disease, patients supplementing with vitamin D would be expected to approach a “crossover point” when additional reduction of the immune response is eclipsed by the advancing disease (Fig. 2). This outcome has been demonstrated in longitudinal studies, with studies on sicker or older patients taking less time to realize the effect.

The Iowa Women’s Health study showed vitamin D intake indeed allows the innate immune system to succeed in targeting chronic pathogens.

**6. 1,25-D and inflammatory disease**

It is often assumed that administering supplemental vitamin D will stimulate 1,25-D production. Many also believe 1,25-D can be raised to very high levels in patients with autoimmune disease without exacerbating the disease state. There are several problems with these assumptions.

First, 1,25-D is generally already well above a healthy range in patients with autoimmune diagnoses due to the inability of CYP24A1 to break down the active metabolite. Unfortunately, since most researchers test only 25-D when determining vitamin D status, this elevation is frequently missed. One recent study of patients with autoimmune diagnoses residing in cloudy Vancouver found that only 15...

![Fig. 2. Depiction of effect of vitamin D on chronic disease.](image-url)
of 100 had serum values of 1,25-D below 110 pmol/L (46.2 pg/ml) [35]. Inappropriately high levels of 1,25-D, defined in another study as greater than 60 pg/ml, were likewise observed in 42% of patients with Crohn’s disease [36].

Furthermore, even if 1,25-D levels could be elevated by supplementation in autoimmune disease, the hormone/secosteroid would be unable to effectively bind the VDR since receptor binding pockets are already blocked by bacterial ligands. The inability of 1,25-D to activate the VDR in patients with autoimmune illness is supported by data showing that many subjects with autoimmune disease who present with higher than normal levels of 1,25-D do not develop hypercalcemia [35]. Of the aforementioned cohort of 100 patients with autoimmune disease, 85 of which had high 1,25-D, none had signs of hypercalcemia. Oncologists have noted a similar effect in cancer. An active VDR has been shown to inhibit growth of cancerous cells and induce apoptosis in tumors [37]. However, some cancer researchers have suggested the VDR loses sensitivity to 1,25-D as the disease progresses [37].

Third, up-regulation of 1,25-D in disease, even without additional vitamin D supplementation, already interferes with transcription by other receptors. Molecular research shows that excessively high concentrations of 1,25-D interfere with numerous hormonal pathways by displacing native ligands from nuclear receptors such as PPAR-gamma and alpha, the glucocorticoid receptor, and the androgen receptor [38]. Since these receptors also express antimicrobial peptides, when 1,25-D reaches unnaturally high levels, the innate immune system’s ability to eliminate pathogens is further thwarted.

7. Conclusion

Uncertainties resulting from epidemiological studies underscore the danger in recommending use of a substance when the exact manner in which it works to ameliorate disease is not fully understood. Ioannidis wrote, “…for many current scientific fields, claimed research findings may often be simply accurate measures of the prevailing bias [39].” The literature has been nearly unequivocal in its support for vitamin D supplementation in autoimmune disease, but the factors dictating the autoimmune disease process are not empirically self-evident. In this case, it is possible that the statistical analyses and studies on which they are based are misleading, and a reassessment may be warranted.

Definitive mechanisms by which vitamin D protects against autoimmune disease have yet to be identified. Some argue that low levels of 25-D exacerbate disease and can be remedied by additional consumption of vitamin D. This explanation may be overly simplistic. Researchers are recommending vitamin D supplementation at historically unprecedented levels. Yet, by most measures, rates of chronic diseases that ought to be reduced by such supplementation continue to escalate.

A reconsideration of the deficiency/disease model is warranted. The alternative model is based on the growing possibility that persistent bacteria drive autoimmune disease. Under such circumstances, 25-D, which inactivates the VDR, palliates symptoms over the short-term, but allows chronic pathogens to proliferate over time. If so, low 25-D in patients with autoimmune diagnoses is the result, rather than cause, of the disease process—further undermining any therapeutic benefit from vitamin D supplementation.

Take-home messages

- Prevailing theories of vitamin D are imprecise and suggest contradictory understandings of vitamin D metabolism.
- 25-hydroxyvitamin D is immunosuppressive.
- Supplementation of the secosteroid vitamin D temporarily alleviates signs and symptoms of chronic disease but leads to a long-term increase in morbidity.
- Molecular biology suggests that low levels of 25-D are a result rather than a cause of the autoimmune disease process.
- A microbiota of bacterial pathogens may survive in the human body by secreting proteins that antagonize the VDR and disable the innate immune response.
- Elevated levels of 1,25-D exist at the site of disease and are an indication that the innate immune system is responding to an infection.

References

HLA-E gene polymorphism associated with susceptibility to Kawasaki disease and formation of coronary artery aneurysms

Kawasaki disease (KD) is a pediatric systemic vasculitis of unknown cause for which a genetic influence is supposed. However matching susceptible genes to KD with specific clinical manifestations remains controversial. The purpose of the study performed by Lin Yj, et al. (Arthritis Rheum 2009; 60:604–610) was to identify possible genetic variants in the major histocompatibility complex (MHC) region that are associated with KD and the development of coronary artery aneurysms (CAAs) in a Taiwanese population. The 189 genetic variants covering the MHC locus were analyzed in an association study of a Taiwanese cohort of 93 KD patients and 680 unrelated healthy children matched for sex and age with the study patients. They found that eleven single-nucleotide polymorphisms (SNPs) were associated with the occurrence of KD. The SNP located at the 3'-untranslated region of HLA-E (rs2844724) was highly associated (P<1 x 10^-7). In addition, the frequency of the C allele was higher in KD patients without CAAs than in controls (P<0.001) due to a significantly increased frequency of the CC and CT genotypes. Plasma levels of soluble HLA-E were significantly higher in KD patients than in controls regardless of the presence of CAAs. Furthermore, there was a trend toward higher plasma levels of soluble HLA-E in KD patients with the CT and TT genotypes of the HLA-E gene polymorphism. Their results suggest that the HLA-E gene polymorphism may play a role in the pathogenesis of KD.

Ultrasonographic depiction of knee joint alterations in systemic lupus erythematosus

Inflammatory changes within the knee joint of systemic lupus erythematosus (SLE) patients are not routinely explored. Ossandon A, et al. (Clin Exp Rheumatol 2009;27:329–32) intended to evaluate inflammatory changes within the knee joint of SLE patients compared to Rheumatoid arthritis (RA) patients and healthy subjects (HS) by using ultrasonographic method (US). US findings were correlated with disease activity parameters. Twenty-six SLE patients were enrolled in the study, 25 RA patients and 15 HS were selected as controls. US was performed by two different experienced operators, using an Aguilent–HP Image point Hx machine equipped with a 10 MHz linear transducer. Power Doppler (PD) was used to determine local synovial perfusion (PFR). Twenty-six SLE patients were enrolled in the study, 25 RA patients and 15 HS were selected as controls. US was performed by two different experienced operators, using an Aguilent–HP Image point Hx machine equipped with a 10 MHz linear transducer. Power Doppler (PD) was used to determine local synovial perfusion (PFR). Knee joints were examined bilaterally. US findings, expressed after consensus of the 2 operators, were correlated to clinical and serological parameters of disease activity. Statistical analysis was performed by the EPISSTAT program. They found that in SLE, synovitis was found in 21 knees (40%), joint effusion in 12 (23%), synovial proliferation in 18 (33%), and positive PD signal in 5 (10%) and gastrocnemius-semimembranosus bursitis in 5 (10%). No erosions were detected. There was a significant difference respect to RA for synovitis (p<0.003), synovial proliferation (p<0.002) and positive PD signal (p<0.001). No correlation was found between US alterations and SLE disease activity parameters. In the HS group 1 patient showed mild synovial proliferation. This is the first study that investigates knee joint involvement in SLE by ultrasonography. US was able to depict inflammatory alterations in the articular tissues of SLE patients, revealing some common characteristics with RA, except for the presence of erosions. The authors concluded that US might be of help in the global evaluation of SLE patients with inflammatory joint involvement, providing relevant information to the clinician.
Controversy exists in relation to the benefits of vitamin D supplementation for health. In the chapter, "Vitamin D: the alternative hypothesis", we discussed how a dysregulated immune response may allow metagenomic pathogens that play a role in the autoimmune disease process to spread. Liabilities of the deficiency/disease model for vitamin D were presented and an alternative model was supported.

Our alternative model can be summarized by the following points:

1. Low levels of 25-D observed in many patients with autoimmune diagnoses are likely the result rather than the cause of the disease process. Under these conditions, the concept of "vitamin D deficiency" is no longer valid.

2. 25-D's immunosuppressive properties may result from its ability to slow activity of the VDR and subsequently the innate immune response. This allows for symptomatic improvement in the short term. However, over longer periods of time, any microbes contributing to the disease state are able to proliferate much more easily. This ultimately results in long-term relapse.

3. Most studies on vitamin D test only levels of 25-D and do not test levels of the active vitamin D metabolite, 1,25-D. This results in an incomplete picture of how vitamin D metabolism is altered by the autoimmune disease state.

In the next chapter, "Autoimmune disease in the era of the metagenome", we examine the microbiota/disease nexus in greater depth. We further explain how many microbes can
themselves slow activity of the VDR and other key receptors, thus controlling the innate immune response in order to gain a significant survival advantage.
Chapter 4: Autoimmune disease in the era of the metagenome

Attribution

AP developed the concept, reviewed the literature, wrote the manuscript, interpreted the findings, and helped design the figures. PA reviewed the literature, edited the manuscript and helped design the figures. TM supervised and critically revised the manuscript.

AP: 70%
Autoimmune disease in the era of the metagenome

Amy D. Proal a,*, Paul J. Albert b, Trevor Marshall c

a Georgetown University, United States
b Weill Cornell Medical College
c Murdoch University, Australia

A R T I C L E   I N   F O

Article history:
Received 12 January 2009
Accepted 9 February 2009
Available online xxx

Keywords:
Autoimmune disease
Metagenome
Vitamin D
Microbiota
Vitamin D receptor (VDR)
Metabolome

A B S T R A C T

Studies of autoimmune disease have focused on the characteristics of the identifiable antibodies. But as our knowledge of the genes associated with the disease states expands, we understand that humans must be viewed as superorganisms in which a plethora of bacterial genomes – a metagenome – work in tandem with our own. The NIH has estimated that 90% of the cells in Homo sapiens are microbial and not human in origin. Some of these microbes create metabolites that interfere with the expression of genes associated with autoimmune disease. Thus, we must re-examine how human gene transcription is affected by the plethora of microbial metabolites. We can no longer assume that antibodies generated in autoimmune disease are created solely as autoantibodies to human DNA. Evidence is now emerging that the human microbiota accumulates during a lifetime, and a variety of persistence mechanisms are coming to light. In one model, obstruction of VDR nuclear receptor-transcription prevents the innate immune system from making key antimicrobials, allowing the microbes to persist. Genes from these microbes must necessarily impact disease progression. Recent efforts to decrease this VDR-perverting microbiota in patients with autoimmune disease have resulted in reversal of autoimmune processes. As the NIH Human Microbiome Project continues to better characterize the human metagenome, new insights into autoimmune pathogenesis are beginning to emerge.

© 2009 Published by Elsevier B.V.

1. Introduction

A decade ago microbiologists were generally confident that most of the bacterial species capable of persisting in or on humans had already been identified. However, over the past...
few years this perception has changed dramatically. Advances in molecular genetic sequencing have revealed the presence of a vast human microbiota, much of which defies detection by culture-based methods.

_Homo sapiens_ was once thought to be the product of one genome. Now, humans are best described as superorganisms in which a multitude of microbial genomes persist in concert with our own [1]. The genomes interact by affecting translation, transcription, and DNA repair in the cytoplasm of infected cells. It is essential that we examine how both human and microbial metabolites (the Human Metabolome) alter the expression of key genes associated with the presentation and progression of autoimmune disease.

2. The human microbiota

According to the NIH, a mere 10% of the cells that comprise the organism known as _H. sapiens_ are human cells. The remaining 90% are bacterial in origin [2]. Thus, _H. sapiens_ is best described as a superorganism in which a large number of different organisms coexist as one [3]. Previously occult bacteria are being found in and on the human body. For example, hydrothermal vent bacteria were found in studies of hip joints during revision arthroplasty [4].

To date, only a fraction of the human microbiota has been genetically characterized and identified, leaving large gaps in our understanding of how it contributes to human health and disease. The NIH Human Microbiome Project aims to use molecular genetic sequencing to catalog the balance of the human microbiome over the coming years. This project has already succeeded in characterizing over 1000 novel bacterial genomes [2]. These genetic “fingerprints” allow for a better tracking and understanding of species and any metabolites they produce which might interact with the human genome.

Medicine is now comfortable with the bacterial populations that exist in the gut and areas of the body in contact with the external environment, such as the mouth, ears, nose and skin. Yet, components of the human microbiota also likely persist in many other body tissues, including those which become inflamed in autoimmune disease [5]. Such bacteria can persist inside the very cells of the immune system that are supposed to kill them [6], or in biofilm communities in which they are protected from the immune response by a self-created polymeric matrix [7].

These bacteria rapidly and frequently share their DNA with their fellow species – even distantly related species – through horizontal gene transfer. Homologous recombination further muddles genomic coherence. As a result, the diversity and the variability present in the human microbiota is much greater than anticipated [8]. Some argue that the number of microbes created through genetic recombination is so high that the concept of distinct bacterial species may become obsolete [9]. For example, researchers associated with the European Tract Meta Initiative seek to understand 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 [10]. Such efforts are shifting the focus of microbiology away from the search for single pathogens in a disease state [1]. Instead, an increasing number of researchers are exploring how components of the microbiota may cause disease by interacting together.

3. A metagenome

Because so many bacteria persist in _H. sapiens_, the microbiota is currently estimated to harbor millions of genes compared with the mere 31,897 [3] that comprise the human genome. In fact, the human genome is barely larger than that of the worm _C. elegans_ (23,399 genes) or that of the small flowering plant thale cress (29,388) [3].

Due to their small size, hundreds, or even thousands, of bacterial cells can fit inside a human cell [6]. The combined genetic contributions of these microbes inevitably provide myriad gene products not encoded by our own relatively small genomes. This means that the human genome is only one of the many genomes that affect _H. sapiens_ function. In reality, the organism we call _H. sapiens_ is controlled by a metagenome, a tremendous number of different genomes working in parallel.

Bacterial gene products can be very similar to our own. For example, the metabolism of glucose-6-phosphate by both the human body and _E. coli_ is nearly identical, so that remarkably similar metabolites are produced by both species [11]. With this in mind, the interaction between an _E. coli_ genome and the human genome, as they exchange nutrition and toxins, increases the complexity of transcription and translation for both species.

4. Bacteria alter the expression of genes that affect the progression of autoimmune disease

When analyzing a genetic pathway, we must study how bacterial and human genes interact, in order to fully understand any process related to the _H. sapiens_ superorganism. Some of these pathways contribute to the pathogenesis of autoimmune disease.

Fig. 1 illustrates some of these gene–disease relationships [12]. A number of autoimmune and inflammatory diseases are shown together with the genes that have been associated with each illness. Note that the gene ACE is related to myocardial infarction, renal tubular dysgenesis, Alzheimer’s, the progression of SARS, diabetes mellitus, and sarcoidosis. ACE has been shown to be down-regulated by a number of peptides created by _Lactobacillus_ and _Bifidobacteria_ [13], species of bacteria considered to be innocuous or “friendly.” No one would argue that these species aren’t present in the human body, yet there has been little study of how they affect chronic inflammatory disease.

For example, PTPN22 is related to rheumatoid arthritis, lupus, and diabetes mellitus. Yet PTPN22 has been shown to be up regulated as part of the innate immune response to mycobacteria [14]. Our population is facing a surge in latent tuberculosis and an increased prevalence of _Mycobacterium avium_. So it is extremely important that we look at how the presence of increased PTPN22 from latent infection, or any of the mycobacteria in the microbiota, might contribute to the autoimmune process.

5. Capnine and the persistence of the metagenome

Created by the gliding bacteria that are present in biofilm, the sulfonolipid Capnine provides a specific example of how a
bacterial metabolite could manipulate human gene expression in order to dramatically alter the progression of autoimmune and other chronic diseases. Capnine has the capacity to disrupt transcription by the VDR, one of the body’s most prolific nuclear receptors [15].

The VDR expresses at least 913 genes, many connected to autoimmune conditions and cancers [16]. The receptor also regulates expression of several antimicrobial peptides (AmPs) that play a vital role in allowing the innate immune system to recognize gram-positive bacteria [18, 19].

Thus, if Capnine was dysregulating the VDR, it would greatly hamper the innate immune response. VDR dysfunction would cause the active vitamin D metabolite 1,25-D to rise to excessively high levels where it could inhibit expression by the bulk of the body’s other nuclear receptors — including alpha thyroid, the glucocorticoid receptor, and the androgen receptor [20]. This would result in hormonal imbalances and also interfere with expression of the dozens of other AmPs expressed by these receptors. In vivo, the microbiota appears to gradually shut down the innate immune response over a person’s lifetime, resulting in the increased accumulation of chronic bacteria and other pathogens [21].

Eventually, genes from the accumulating microbial metagenome may determine a clinical disease symptomology such as an autoimmune diagnosis, or simply drive the inflammation associated with the aches and pains of aging [5]. This accumulation is an extremely logical evolutionary survival mechanism. Components of the human microbiome have evolved to dysregulate the VDR receptor that would otherwise activate a potent immune response against its presence. As Royston Goodacre comments in *Journal of Nutrition*, we are born with a genome that, aside from the genes of species that can survive in the womb and endometrium [22], is largely human. But we inevitably die with a genome that is at least 90% bacterial [3]. This shift towards an increasingly diverse microbiota over a lifetime is directly correlated with an increase in diseases and symptoms driven by inflammation.

6. Antibodies may be generated in response to microbial DNA

Autoimmune diseases are often regarded as illnesses in which the immune system creates antibodies against itself [23]. Yet now that *H. sapiens* is understood to be the product of multiple genomes, it is equally possible that the antibodies observed in autoimmune disease result from alteration of human genes and gene products by the bacterial metagenome.

It seems that autoimmune disease is largely the result of the adaptive immune system gone awry. However, when a disabled innate immune system is forced to respond to the chronic microbiota the resulting cascade of cytokines and chemokines will also stimulate an adaptive response. At this point, the adaptive immune system may likely create antibodies to fragments of DNA that have been generated by phagocytosis or apoptosis of infected cells [5]. Yet, until a much larger portion of the human microbiota has been characterized, correlation of such antibodies with specific components of the microbiota remains difficult.

7. The Human Metabolome is a product of its environment

The spectrum of metabolites found in *H. sapiens* is known as the Human Metabolome [3]. Although many bacteria produce substrates similar to those of their human hosts, others produce metabolites that differ from the byproducts of human metabolism. The human microbiota differs from person to person depending on the unique species of bacteria accumulated over a lifetime. This means that every person’s
health is distinctly influenced by the specific byproducts created by their particular microbiota. Changes in the metabolome pool are the downstream results of gene expression [3].

Some of the human and microbial metabolites in the H. sapiens superorganism will be manifested in serum and urine samples. For example, mass spectroscopy has been used to identify the non-human metabolites present in the urine of subjects living in three distinct populations—the United States, China, and Japan [24]. The study found that subjects in each population produced very different non-human metabolites [24]. Thus, genetic makeup, nutrition, healthcare, external toxins—factors associated with the acquisition of a particular microbiota—caused the three populations to become substantially different. That environmental factors drive the metabolome is supported by the observation that when five of the Japanese subjects moved to America, their metabolomes adapted to resemble those of the American population [24]. When evaluating the overall operation of H. sapiens, it is thus clear that the composition of the microbiota is far more important than regional variations in the human genome itself.

8. The microbiota can interfere with transcription and translation

Persistent bacteria including Francisella tularensis [25], Mycobacterium tuberculosis [26], Rickettsia massiliae [27], Brucella spp. [28], Listeria monocytogenes [29], Salmonella typhimurium [30] and others, use a variety of mechanisms to evade the immune response and survive inside macrophages and other phagocytic cells. Furthermore, various species of bacteria have been detected inside the cells of patients with juvenile rheumatoid arthritis [31], sarcoidosis [6], and other inflammatory diseases [32]. This suggests that disease-causing microbiota largely persists in the cytoplasm of nucleated cells, where it has access to both the DNA transcription and protein translation machinery of H. sapiens. For example, upon infecting a macrophage, Brucella spp. down-regulates genes involved in cell growth and metabolism, but up-regulates those associated with the inflammatory response and the complement system [33]. When Shigella persists within a macrophage it modulates numerous host signaling pathways, including those that inactivate mitogen-activated protein kinases [34]. According to one analysis, expressions of 463 human genes are changed during an infection with Mycobacterium tuberculosis [35].

Microorganisms are also capable of integrating their DNA with our own [36]. This result in alteration of the human DNA by the microbiota over time, potentially leading to genetic mutations associated with autoimmune diagnoses. Genetic haplotypes observed in autoimmune disease frequently have very low statistical significance, as would be expected based on knowledge that the metabolome varies from population to population and individual to individual.

In addition, host DNA repair mechanisms are susceptible to modification by the products of the metabolome. In fact, bacteria may hijack DNA repair mechanisms to generate genetic diversity without losing genomic stability [37,38]. If the rate of DNA damage or mutation by bacterial metabolites exceeds the capacity of cellular repair, the accumulation of errors can overwhelm the cell and result in early senescence, apoptosis or cancer [39].

9. Discussion

It is becoming apparent that the body of H. sapiens consists not only of the human genome, but also genomes of commensal bacteria, bacteriophages, and viruses. Consequently, the human genome can no longer be studied in isolation. Genes known to be associated with autoimmune conditions are susceptible to modification by the myriad pathogenic metabolites. Thus their activity in disease processes must be studied in the tissues in which they are expressed.

Commensal microbes that were thought to be solely beneficial to man are now known to create metabolites that interfere with the expression of genes associated with autoimmune disease. For example, peptides from of Lactobacillus and Bifidobacteria affect expression of the ACE gene. Those species that disable VDR gene expression secure their survival by suppressing key antimicrobial peptides. Their persistence may well cause the inflammation and antibody production thought to result from autoimmune processes. Species of pathogens that collect in an individual’s microbiota will affect disease presentation and progression. In particular as the innate immune response is compromised by the chronic infection, the body additionally loses its ability to stop the proliferation of opportunistic acute infectious agents.

Lifelong symbiosis between the human genome and persistent components of the bacterial metagenome does not simply result in modification of the metagenome. It results in the accumulation of microbial metabolites in the cytoplasm of infected cells that are capable of interfering with DNA repair and transcription activity. Thus genetic abnormalities such as those observed in autoimmune disease may well be the result of metagenomic activity.

The use of a VDR agonist and subinhibitory antibiotics has demonstrated the ability to restore VDR function and induce recovery in diverse autoimmune diagnoses [21,40]. This supports a biological description in which a persistent pathogenic component of the microbiota accumulates inside macrophages and other nucleated cells. The use of corticosteroids slows the immune system’s ability to target the cause of any chronic inflammation resulting from this persistent infection. This can at best result only in short-term palliation.

Take-home messages

- H. sapiens is a superorganism controlled by both the human genome and a microbial metagenome.
- Bacterial metabolites can up-regulate and down-regulate the expression of genes associated with autoimmune disease.
- The Human Metabolome varies greatly from person to person depending on microbiota composition. Thus, its ability to alter gene expression varies greatly depending on the individual.
- The microbiota can survive by slowing VDR Nuclear Receptor transcription, and subsequently the expression of ~913 genes key antimicrobials for the innate immune response.
- The microbiota can persist in the cytoplasm of nucleated cells where it has direct access to both the DNA transcription and to the protein translation machinery of H. sapiens.
- We must necessarily study how the metagenome and the human genome interact in order to fully understand any process related to autoimmune disease.

Please cite this article as: Proal AD, et al, Autoimmune disease in the era of the metagenome, Autoimmun Rev (2009), doi:10.1016/j.autrev.2009.02.016
References


Summary and link to next chapter

In the chapter, "Autoimmune disease in the era of the metagenome", we examined the nature of the human microbiota in detail and introduced the novel concept of “successive infection.” Patients who present with autoimmune disease acquire pathogens in numerous ways including, but not limited to, childhood infection, vaccines, blood transfusions, and parental exposure (such as microbes in the sperm and egg). Successive infection dictates that, because many of these pathogens likely slow AMP expression via the nuclear receptors, such patients become increasingly immunocompromised. This creates a snowball effect in which each pathogen that decreases immune activity makes it easier for the host to pick up other pathogens and so on.

We have introduced the concept of the human superorganism in which microbial and human genes continually interact in both health and disease. Human microbiota accumulates during our lifetime. We have reexamined how human gene transcription can be affected by microbial metabolites. We further expanded on this topic and discussed how antibodies generated in autoimmune disease may not be created solely in response to human DNA but may be primarily a response to the DNA of the microbiota. Finally, we briefly discussed a treatment aimed at targeting the VDR dysregulation.

In summary we have clarified the following points:

1. *Homo sapiens* is a superorganism controlled by both the human genome and a microbial metagenome.
2. Bacterial metabolites can up-regulate and down-regulate the expression of genes associated with autoimmune disease.

3. The Human Metabolome varies greatly from person to person depending on microbiota composition. Thus, its ability to alter gene expression varies greatly depending on the individual's specific microbiota.

4. The microbiota can persist in the cytoplasm of nucleated cells where it has direct access to both the DNA transcription and the protein translation machinery of *H. sapiens*. A special case is the nucleated phagocytic cells of the immune system.

5. We must necessarily study how the metagenome and the human genome interact to produce a spectrum of metabolites in order to fully understand any process related to autoimmune diagnoses.

In the next chapter, "Autoimmune disease and the human metagenome" we expand on all these points. We discuss the model of successive infection in greater depth. Furthermore, we provide a much more detailed description of where and how microbes that contribute to a pathogenic microbiota are acquired. We use our model of autoimmune disease to elucidate topics such as co-morbidity and familial aggregation.
Chapter 5: Autoimmune disease and the human metagenome

Attribution

AP developed the concept, reviewed the literature, wrote the manuscript, interpreted the findings, and helped design the figures. PA reviewed the literature, edited the manuscript and helped design the figures. TM supervised and critically revised the manuscript.

AP: 65%
Chapter 12
Autoimmune Disease and the Human Metagenome

Amy D. Proal, Paul J. Albert, and Trevor G. Marshall

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.
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 count-
ing than by the plating procedure. In 1949, Winogradsky confirmed Razumov’s
assessment and noted that many microbes are not satisfied with laboratory cultiv-
ation 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 bac-
terial 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 includ-
ing 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 bac-
teria 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 spe-
cific 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. Anelioviruses, 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-Zaatari et al., 1996), ankylosing spondylitis (Liu et al., 2001), chronic fatigue syndrome (Lombardi et al., 2009), rheumatoid arthritis, multiple sclerosis, Hashimoto’s
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 $^1$H NMR-based metabonomics 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 I 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 down-regulating 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
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’ 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...
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, 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)

<table>
<thead>
<tr>
<th>Nuclear receptor</th>
<th>Native ligand</th>
<th>Native ligand (Kd)</th>
<th>1,25-D (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid alpha</td>
<td>T3</td>
<td>7.20</td>
<td>8.41</td>
</tr>
<tr>
<td>Thyroid beta</td>
<td>T3</td>
<td>7.18</td>
<td>8.44</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Cortisol</td>
<td>7.36</td>
<td>8.12</td>
</tr>
<tr>
<td>Androgen</td>
<td>Testosterone</td>
<td>7.38</td>
<td>8.05</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Progesterone</td>
<td>7.53</td>
<td>8.09</td>
</tr>
</tbody>
</table>

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 HSN1 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...
Autoimmune Disease and the Human Metagenome

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.
12 Autoimmune Disease and the Human Metagenome

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 nonrelatives 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 decent (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). Possnett 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 (Possnett 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 rickettsial (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- 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, antimitochondria, 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; Arendt 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 subinhibitory 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 (Arnson 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 µm 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 specialties, 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.

References


Summary and link to next chapter

While previous chapters emphasized that an inflammatory disease state results from the interaction of both human and microbial genes, "Autoimmune disease and the human metagenome", further explored this topic. In Chapter 5, we built on our alternative model hypothesis, supported with novel data and described in detail how the process may account for the high levels of co-morbidity and familial aggregation observed among patients with autoimmune disease. Any one autoimmune disease is likely to be due to many different microbes within the metagenomic microbiota. We presented substantial data supporting the hypothesis that “autoantibodies”, often polyspecific, are created when the innate immune system responds to the microbiota and a cascade of cytokines and chemokines stimulate the adaptive immune response. Thus autoantibodies are produced in response to chronic infection with microbes.

Weaknesses associated with a Mendelian model of inheritance in autoimmune disease were also discussed. We showed how genetic predisposition for autoimmune disease is not necessarily Mendelian and questioned whether primers selected for most epidemiological studies are chosen without consideration for whether they might amplify DNA from the genomes of any intracellular microbes. Finally, Chapter 5 expanded on the therapy discussed in the previous Chapter "Autoimmune disease in the era of the metagenome," a therapy which attempts to stimulate rather than suppress the immune response in autoimmune disease.

In the next chapter, “Immunostimulation in the era of the metagenome”, we introduce a therapeutic model for autoimmune disease which has formed the basis of our past collaboration with American and international physicians. Unfortunately increased microbicidal activity results in immunopathology, a temporary rise in symptoms due to...
apoptosis and toxin release by destroyed pathogens. Challenges associated with managing such treatment-induced immunopathology will be discussed.
Chapter 6: Immunostimulation in the era of the metagenome

**Attribution**

AP developed the concept, reviewed the literature, wrote the manuscript, interpreted the findings, and helped design the figures. PA reviewed the literature, edited the manuscript and helped design the figures. TM supervised and critically revised the manuscript. GB and IL collected case data, and CB for edited the manuscript. All authors critically reviewed and approved the final version.

AP: 55%
REVIEW

Immunostimulation in the era of the metagenome

Amy D Proal1, Paul J Albert2, Greg P Blaney3, Inge A Lindseth4, Chris Benediktsson5 and Trevor G Marshall1

Microbes are increasingly being implicated in autoimmune disease. This calls for a re-evaluation of how these chronic inflammatory illnesses are routinely treated. The standard of care for autoimmune disease remains the use of medications that slow the immune response, while treatments aimed at eradicating microbes seek the exact opposite—stimulation of the innate immune response. Immunostimulation is complicated by a cascade of sequelae, including exacerbated inflammation, which occurs in response to microbial death. Over the past 8 years, we have collaborated with American and international clinical professionals to research a model-based treatment for inflammatory disease. This intervention, designed to stimulate the innate immune response, has required a reevaluation of disease progression and amelioration. Paramount is the inherent conflict between palliation and microbicidal efficacy. Increased microbicidal activity was experienced as immunopathology—a temporary worsening of symptoms. Further studies are needed, but they will require careful planning to manage this immunopathology.

Cellular & Molecular Immunology advance online publication, 31 January 2011; doi:10.1038/cmi.2010.77

Keywords: antimicrobial peptides; autoimmune disease; immune reconstitution inflammatory syndrome; immunopathology; innate immunity; metagenomics

INTRODUCTION

Ten years ago, the first draft of the human genome was published, opening a window into the detailed operation of the healthy human body, which, even today, is only just beginning to reveal its secrets. However, it is the subsequent understanding of microbial genomes, the emerging field of metagenomics, which is allowing us to start deciphering many of the secrets of human disease.

While the extent of the relationship between microbes and disease has yet to be fully characterized, provocative data are accumulating which suggests a complete re-examination of the factors driving chronic inflammatory disease. We can now begin reappraisal of key assumptions that have guided the assessment, management and treatment of autoimmune conditions.

THE HUMAN BODY IS AN ECOSYSTEM OF MICROBES

A decade ago, Chiller et al. concluded 'The skin is a poor media for bacteria given the large number of inherent defense mechanisms'.2 This assessment was undermined seven years later by Fierer et al.’s work, which found that the average human palm harbors at least 150 bacterial species—an order of magnitude greater than previous estimates.3 A 2009 Science study expanded on this understanding of microbial diversity in skin, showing that forearms and underarms, though located just a short distance apart, are as ‘ecologically dissimilar as rainforests are to deserts’.4

Until quite recently, efforts to characterize the human microbiota, such as those of Chiller, had to rely upon in vitro cultivation of microbial species. Today we understand that these conditions scarcely mimic those of the human body. In order to obtain his results, Fierer used 454 pyrosequencing, one of several novel molecular tools that today allow researchers to identify microorganisms based on their DNA signatures with a very high degree of accuracy. These tools, which also include 16S rRNA sequencing, shotgun sequencing and single-cell sampling, are revolutionizing microbiology, providing researchers with unprecedented capability to perform hypothesis-driven analyses of uncultured microorganisms. They have even allowed researchers to understand the interactions among individual microbes in communities within living tissues.

It is now understood that microbial cells vastly outnumber our own human cells, by a factor of at least 10 : 1. The genes of these microbes number in the millions, dwarfing the paltry 23 000 genes comprising the human genome.5 Many microbiologists have begun to replace the concept of ‘human being’ with a ‘human superorganism’ in an effort to reflect the emerging reality that the human genome is one of the myriad genomes dictating the human experience in both health and disease.6

Viruses (comprising the virome) and phages are also key components of the human microbiome. Like bacteria, many viruses have yet to be fully characterized by high-throughput genome sequencing. However, molecular analysis has revealed that nearly all humans acquire multiple persistent viruses within the first years of life, viruses that generally remain with them throughout life. A team led by Gordon recently analyzed the fecal virome of monozygotic twins and their mothers. This study found that 81% of the reads generated from this virome do not match any known viruses.7

In concert with a number of privately funded groups, two major multisite collaborations, the US-based NIH Human Microbiome Project and MetaHIT, an EU consortium, have begun the process of

1Murdoch University, Perth, WA, Australia; 2Weill Cornell Medical College, New York, NY, USA; 3Stillpoint Centre, Vancouver, BC, Canada; 44M-Klinikken, Oslo, Norway and 5AutoImmunity Research Foundation, Thousand Oaks, CA, USA.

Correspondence: AD Proal, 429 E. 65th St., apt. 14, New York, NY 10065, USA.
E-mail: amy.proal@gmail.com
Received 14 December 2010; accepted 15 December 2010
detailing the human microbiota. Thus far, the Human Microbiome Project has committed itself to collecting sequence data from several key body sites: the gastrointestinal tract, oral cavity, urogenital/vaginal tract, skin, respiratory tract and, to a lesser extent, the blood.

One of the primary goals of these studies has been to compare populations of microbes in healthy individuals with equivalent populations of microbes in their diseased counterparts. Such studies have quickly shown that patients with a given autoimmune or inflammatory diagnosis tend to present with microbial profiles that differ substantially from those of healthy subjects.

A 2008 study of psoriatic skin not only found 84 novel species never before known to persist in skin, but also doubled the proportion of microbes from the Firmicutes phylum in psoriatic patients, as compared to healthy controls. Distinct microbial profiles have been further demonstrated in obesity as well as inflammatory bowel conditions. For example, the presence of methanogenic bacteria has now been shown to be an independent predictor of higher body mass index in obese adults. Communities of bacteria in the gut of patients with diabetes mellitus type 2 were recently reported to differ substantially from those of their healthy counterparts. Using real-time quantitative PCR researchers determined that the proportions of phylum Firmicutes and class Clostridiales were significantly reduced in the diabetic group compared to the control group, among other differences. Further, the ratio of Bacteroidetes to Firmicutes as well as the proportion of Bacteroides–Prevotella to Clostridium cocoides–Eubacterium rectale group correlated positively and significantly with plasma glucose concentration but not with body mass index. Gophna et al. showed that Crohn’s patients had a significantly higher proportion of Proteobacteria and Bacteroidetes in their gut as compared to healthy subjects. Yap et al. showed that autistic children had several urinary metabolites that were highly significant as compared to controls. In a murine model, Lee and her California Institute of Technology colleagues recently found that specific intestinal bacteria have a significant role in affecting the nervous system during multiple sclerosis.

However, the human microbiome is not confined to mucosal surfaces. An increasing number of scientific teams are using molecular techniques to re-evaluate the sterility of internal body cavities—with eye-opening results. The amniotic fluid, previously considered completely sterile, was shown to harbor uncultivated, previously uncharacterized taxa of bacteria, the presence of which was robustly correlated with premature birth. Molecular characterization of prothetic hip joints removed from body tissues was found to harbor a plethora of diverse bacteria, including species such as hydrothermal hip joints removed from body tissues was found to harbor a plethora of diverse bacteria, including species such as hydrothermal.

COMMUNITIES OF MICROBES DRIVE AUTOIMMUNE DISEASE

Discrete pathogens such as human herpesvirus-6 (HHV-6), cytomegalovirus, Epstein–Barr virus (EBV) and Chlamydia pneumoniae, have been repeatedly identified in association with autoimmune disease. However, none of these microbes has been detected in 100% of patients with any single autoimmune disease state. The evidence for causality has been lacking. We now understand how these conditions can be polymicrobial in nature. Pathogens are capable of working in concert to cause disease and entire ecosystems of microbes can become dysregulated by the pathogenic genomes. These discoveries have caused a total reevaluation of Koch’s postulates, which, over a century ago, theorized that one microbe would cause one disease.

These and other findings additionally challenge the traditional view that a largely sterile human body can create antibodies against self. Instead it is becoming increasingly likely that, in autoimmune disease, the body is not targeting its own tissues, but is generating antibodies against fragments of these metagenomic communities of microbes.

One of the most effective survival mechanisms involves pathogens that enter host cells, especially the phagocytic cells. Earlier work has demonstrated that intracellular pathogens are indeed present in patients with a variety of autoimmune conditions. Intracellular microbes living within nucleated cells can interfere with DNA transcription and repair mechanisms, which allows them to create much of the systemic dysfunction often associated with autoimmune diagnoses.

An increasing number of studies are providing support for the view that ‘autoantibodies’ can be generated in response to the persistent presence of a pathogenic microbiota. While high titers of rheumatoid factor (RF) are associated with severe rheumatoid arthritis, they also appear in a number of other diseases including viral, bacterial and parasitic infections. Maturation of RF can be initiated by chronic infections. For example, patients with subacute bacterial endocarditis, which is frequently tied to the presence of Streptococcus, also often present with high levels of RF. A 2007 study examined blood sera from 88 patients with acute infections (41 bacterial, 23 viral, 17 parasitic and 7 rickettsial). Elevated titers of autoantibodies including annexin V, prothrombin, anti-Saccharomyces cerevisiae antibody, antinuclear antibody (ANA) or antiphospholipid antibodies were detected in about 50% of the subjects, with 34 individuals harboring elevated titers of at least two autoantibodies.

Many proteins from pathogens share significant sequence or structural similarities with human proteins, and these can 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 of widespread species of bacteria including Escherichia coli, Pseudomonas aeruginosa, Shigella boydii and Salmonella. Furthermore, since human antibodies are polyspecific, it is likely that some antibodies created to target pathogens may mistakenly target human proteins, causing ‘collateral damage’.

IMMUNOSTIMULATION

While the standard of care for chronic inflammatory disease remains the use of medications that slow the immune response, our bodies themselves seek to do the exact opposite; they strive to stimulate the immune system (immunostimulation) when they sense intracellular pathogens. Current exogenous intervention is focused on immunosuppression. It therefore seems prudent to re-evaluate the manner in which inflammatory conditions, including autoimmune conditions, are routinely treated.

A fully activated immune response should be capable of clearing common pathogens from the body, yet, in autoimmune disease, this does not appear to be the case. The answer may lie in the way that pathogens have evolved to slow the defenses of the innate immune system—the very branch of the immune response that would otherwise work to kill them. Indeed, some of these persistent pathogens have long been implicated in autoimmune disease.

One of the key mechanisms by which microbes achieve this immunosuppression is by subverting one of the body’s most prolific nuclear receptors, the vitamin D receptor (VDR). Defects in VDR signaling transduction have previously been linked to bacterial infection and chronic inflammation.

This is not surprising as the VDR is responsible for expression of several families of key endogenous antimicrobials, including

---

**Cellular & Molecular Immunology**
cathelicidin and the beta-defensins. These play a vital role in allowing the innate immune system to target intracellular pathogens. Avunnet and Rosenstein have argued that antimicrobial peptides 'seem to participate in every facet of it [modulating immunity] by boosting the immune response to prevent infection, and also by suppressing other proinflammatory responses to avoid uncontrolled inflammation'. Indeed, the activated VDR, which also increases CD14 and TLR2 synthesis, has been described as a critical regulator of the innate immune response. Perversion of VDR function would clearly ease pressure on intracellular microbial communities, thus making it an obvious evolutionary selection.

It should be noted that the antimicrobial peptides also play a role in mitigating the virulence of the virome and other non-bacterial infectious agents. In addition to its antibacterial activity, alpha-defensin human neutrophil peptide-1 inhibits HIV and influenza virus entry into target cells. It diminishes HIV replication and can inactivate cytomegalovirus, herpes simplex virus, vesicular stomatitis virus and adenovirus. In addition to killing both gram positive and gram-negative bacteria, human beta-defensins HBD-1, HDB-2, and HBD-3 have also been shown to kill the opportunistic yeast species Candida albicans. Cathelicidin also possesses antiviral and antifungal activity.

In 2005, Wang et al. demonstrated that the VDR expresses at least 913 genes, many connected to autoimmune conditions and cancers. Last year a UK-based team used chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq) to identify 2776 binding sites for the VDR along the length of the human genome. Significantly, the binding sites were unusually concentrated near a number of genes associated with susceptibility to autoimmune conditions. Such genes include IRF8 (multiple sclerosis) and PTPN2 (Crohn’s disease and type 1 diabetes).

In 2007, Marshall used in silico emulation to show that the sulfonolipid capnine, which is created by biofilm bacterial species in the genera Cytophaga, Capnocytophaga, Sphingobacterium, and Flexibacter, could bind to and slow the activity of the VDR. This work suggested that microbes may be able to directly alter VDR ligand binding pocket occupancy, and subsequently VDR expression, in order to gain a survival advantage.

Xu et al. used an early cDNA microarray to study cellular gene expression altered by Mycobacterium tuberculosis infection. VDR expression was downregulated. This was not unexpected as the VDR expresses TACO, a protein critical to intraphagocytic survival of M. tuberculosis. Barrelia burgdorferi, another obligate pathogen, also reduces expression of VDR. HIV uses the VDR to recognize its own long terminal repeat promoter region in order to transcribe its own genome.

In addition, EBV, which has been associated with many autoimmune diseases, very effectively downregulates expression of VDR in immature lymphblastoid cell lines. This is an especially elegant persistence mechanism.

Key metabolic changes within the nucleated cells

When microbial ligands dysregulate the VDR, the receptor fails not only to transcribe key antimicrobials but also CYP24A1, a well-studied enzyme which breaks down excess 1,25-dihydroxyvitamin D (1,25-D) into 25-hydroxyvitamin D. Thus, when activity of the receptor is thwarted, 1,25-D levels rise.

Indeed, Bell has pointed out that a number of infectious diseases—tuberculosis, AIDS with Pneumocystis carinii pneumonia, and AIDS with cytomegalovirus infection, disseminated candidiasis—have high levels of 1,25-D leaking into the bloodstream.

A cross-sectional analysis of 100 patients with autoimmune disease showed that a similar dynamic seems to occur in autoimmune disease. Confirmation of this observation has been demonstrated in Crohn’s disease and rheumatoid arthritis, with Kavathia et al. tying higher levels of 1,25-D to greater disease severity in sarcoidosis patients, and Mawer et al. finding that 1,25-D levels were particularly elevated in the synovial fluid surrounding the joints of subjects with rheumatoid arthritis.

We have previously predicted, based on molecular in silico emulation, that at higher concentrations, 1,25-D interferes with expression of several of the body’s other key nuclear receptors, including the glucocorticoid receptor, the androgen receptor and the thyroid receptor. These receptors also express various families of AmPs—20, 17 and 15 families, respectively, out of the 22 analyzed by Brahmachary. As the concentration of 1,25-D accumulates within the nucleated cells, our model predicts that it would increasingly occupy the ligand-binding pockets of these receptors, displacing their endogenous ligands. For example, in the case of alpha-thyroid, the agonist T3 would have to compete with the antagonist 1,25-D for access to the receptor ligand-binding pockets. As the levels of 1,25-D continue to rise, expression of the AmPs by alpha-thyroid would be downregulated. Glucocorticoid receptor and androgen receptors would be similarly affected, leading to a profound suppression of the innate immune system’s ability to respond to the intracellular attack.

Thus, dysregulation of the VDR by pathogenic components of the microbiota could cause flow-on effects that effectively disable the bulk of the body’s AmPs, leaving the host increasingly immunocompromised. The same phenomenon could explain, at least in part, why many autoimmune diseases are characterized by dysregulated hormonal expression—a symptom that often becomes exacerbated as the disease progresses.

The complete set of mechanisms by which persistent intracellular microbes slow innate immune activity has yet to be definitively determined. However, it likely occurs on a much broader scale than previously supposed, as most of the human microbiome is still understudied. Each pathogen that decreases VDR expression makes it easier for other pathogens themselves to slow immune activity even further, creating a snowball effect.

Successful infection

We refer to this dynamic state, in which the host microbiome shifts further and further away from a natural homeostatic state, as successive infection. Human genes are up- or downregulated by acquired components of the microbiota, and infected cells progressively struggle to correctly produce human metabolites in the presence of the numerous proteins, enzymes and metabolites generated by the pathogenic genomes. Indeed, Kanchwala et al. showed that patients with sarcoidosis expressed the antimicrobial peptide cathelicidin less than healthy subjects, and that the sickest sarcoidosis patients expressed it least of all. In patients with Crohn’s disease, Wang et al. also demonstrated a decline in levels of cathelicidin, while Wilken et al. showed reduced TLR2 mRNA expression in patients with Lofgren’s syndrome.

After a certain level of dysbiosis has occurred, people may well reach the point where they can be diagnosed with an autoimmune/inflammatory condition. Many, however, incrementally present with aches and pains often attributed to ‘normal aging’. For example, mice lacking the cathelicidin gene, which is robustly transcribed by the VDR, have longer periods of wound healing than their wild-type counterparts. The absence of this key AmP in a murine model might be
compared with impaired wound healing among the elderly.\textsuperscript{54} The term ‘inflammaging’ has been coined to explain ‘the now widely accepted phenomenon that aging is accompanied by a low-grade chronic, systemic upregulation of the inflammatory response, and that the underlying inflammatory changes are common to most age-associated diseases’.\textsuperscript{55}

Further support for successive infection comes from the recent metagenomic studies that show that there does not appear to be a core microbiome across people.\textsuperscript{56} Even among relatively homogeneous populations of fewer than 100 individuals, only a ‘tiny fraction’ of the microbial species inhabiting the gut are shared by other community members.\textsuperscript{56,57} Similar variability has also been identified in the skin.\textsuperscript{7} These unanticipated discrepancies in microbial inhabitants parallel the variability in presentation of chronic inflammatory disease.

Over 100 years ago, Theobald Smith commented ‘it is what bacteria do rather than what they are that commands attention, since our interest centers in the host rather than in the parasite’.\textsuperscript{58} That many of the pathogens driving the autoimmune disease state may survive by gradually slowing the immune response adds additional weight to the contention that immunostimulation rather than immunosuppression is more likely to facilitate reversal of these chronic conditions.

**IMMUNOPATHOLOGY**

Unfortunately, immunostimulation in infectious disease is complicated by a cascade of reactions, including inflammation, which occur as part of the immune response to microbial death.\textsuperscript{59} As others have done, we use the term ‘immunopathology’ to refer to a systemic inflammatory response consistent with elevated immune activation.\textsuperscript{60,61}

It is well understood that the symptoms of the flu, or any other acute microbial illness, stem from an inevitable battle between man and microbe, a clash that ensues as the immune system releases a host of cytokines and chemokines in an attempt to eradicate offending infectious agents.\textsuperscript{62} Additionally, the dead microbial debris must be cleaned up, placing an additional load on the immune system.\textsuperscript{63}

Thus, pathogens driving an infectious disease state cannot be killed without, at the very least, a temporary increase in patients’ symptoms, inflammatory markers or both. While patients may be administered with some palliative medications, they must endure the burden of inflammation if the host immune system is to prevail.

This is consistent with the autoimmune disease process being driven largely by the presence of chronic pathogens. Autoimmune diseases are characterized by a relatively continuous inflammatory process. This suggests that the uninterrupted effort by the immune system to secrete cytokines and chemokines is an attempt to keep pathogenic load under control.\textsuperscript{64} Microbial death leads to the release of toxins and debris into the bloodstream. The death of intracellular pathogens is particularly difficult for the host to manage, as the body must deal with both the by-products of entire human cells undergoing phagocytosis and apoptosis, as well as the microbes that once inhabited them. In addition, innate immune activity is signaled to the adaptive immune system, initiating the generation of antibodies from the scraps of both cellular and pathogenic debris.

In chronic inflammatory disease, the conflict between man and microbe rarely ends. Perhaps, because chronic microbes appear so effective at progressively and cumulatively slowing the innate immune response, the body ultimately seems unable to reverse the disease state. What results is a stalemate, where the immune system strives to target the persistent microbes but never fully succeeds, and the initial low-grade inflammation becomes continuous. As far back as 1929, Boas and Michelson commented ‘[w]hen the battle waged between the invading organism and the body’s resistive forces becomes a stalemate, chronicity results’.\textsuperscript{65}

Therefore, once a patient with autoimmune disease has accumulated a high enough microbial load, periods of relief may paradoxically correspond to times when the immune system is most compromised, unable to mount an effective immune response against pathogens. Autoimmune diseases are often characterized by patterns of relapse punctuated by periods of remission. Indeed, remission may actually signal a kind of exhaustion on the part of the immune system. On the other hand, relapse, which is often accompanied by a new infection or stress, may represent the immune system’s best effort at a response.

This suggests that, if efforts are made to restore the immune response in these immunocompromised patients, any subsequent renewed attack against pathogens will lead to symptom exacerbation.

The literature offers a number of examples of therapies that deliberately stimulate the immune response in an effort to target chronic pathogens, and, in the process, generate an increase in symptoms as part of a microbial die-off response. This reaction was first described by Jarisch and Herxheimer during therapy of secondary syphilis using mercury, and became known as the Jarisch–Herxheimer reaction.\textsuperscript{66} In the 100 years since Jarisch and Herxheimer, researchers have noted this reaction in a broad spectrum of infectious diseases such as relapsing fever, Lyme disease, leptospirosis, brucellosis, tuberculosis, Vincent’s angina and African trypanosomiasis.\textsuperscript{1,60,66} Symptom exacerbation varies depending on the nature of the pathogens targeted, but is generally accompanied by a complex clinical reaction including reports of abrupt onset of fever, chills, myalgias, headache, tachycardia, hyperventilation and hypotension.

**Immune reconstitution inflammatory syndrome (IRIS)**

More recently, a type of immunopathology has been observed in HIV/AIDS patients. During IRIS, HIV/AIDS patients experience the worsening or onset of systemic inflammatory clinical signs and symptoms following treatment with highly active antiretroviral therapy (HAART). This syndrome results when HAART allows for partial recovery of the immune response. This causes renewed and exuberant host immunological responses towards opportunistic infectious agents, agents that the host accumulated during prior periods of immunosuppression.\textsuperscript{67}

A number of well-known readily cultured pathogens have been conclusively linked to IRIS: the herpes viruses, cytomegalovirus, hepatitis B and C, M. tuberculosis, Mycobacterium avium complex and Cryptococcus neoformans.\textsuperscript{68} However, many more microbes likely contribute to the reaction since AIDS clinicians do not yet have access to the metagenomic tools. Certainly, the existence of IRIS in culture-negative HAART patients suggests that more microbes may be present than the few that have already been isolated.\textsuperscript{69}

Interestingly, patients experiencing IRIS often ‘develop’ autoimmune conditions as a manifestation of immune restoration. These include sarcoidosis and other granulomatous reactions,\textsuperscript{70,71} diabetes mellitus, rheumatoid arthritis,\textsuperscript{72} systemic lupus erythematosus,\textsuperscript{73} Guillain–Barre syndrome,\textsuperscript{74} Graves disease\textsuperscript{75} and autoimmune thyroid disease.\textsuperscript{68,76} This suggests that these patients accumulated microbes that are directly involved in the pathogenesis of these disease states.

**Our therapeutic approach**

Over the past 8 years we have developed a therapy for autoimmune disease that appears to strongly activate the innate immune response. Treatment is based on the use of a putative VDR agonist, olmesartan.
medoxomil, which, by re-activating the receptor, appears to gradually restore expression of the numerous AmPs, Toll-like receptors (TLRs) and other antimicrobials expressed by the VDR.

Olmesartan medoxomil was developed as a mild hypotensive, an angiotensin II type 1 receptor antagonist. Typically it is dosed 20–40 mg once a day. However, this drug has a unique affinity for the VDR nuclear receptor, for which it is most probably a partial agonist.\(^\text{38,77}\) In order to be effective in this targeting, the dosing has to be more frequent as the VDR’s half-life is only 4–6 h before it is broken apart by caspase-3, and protease activity.\(^\text{79}\) Thus, when dosed at 4- to 8-h intervals, VDR stimulation persists between doses.

Olmesartan has at least two identified effects on the immune system. By inhibiting angiotensin II binding to its receptor, which occurs under most dosing regimes, the expression of nuclear factor-kappaB is reduced.\(^\text{79}\) This lowers the cell’s production of inflammatory cytokines. We have found that as the dosing interval shortens, immune activation becomes noticeable above about 20 mg every 8 h, achieving saturation at about 40 mg every 6 h. Patients have reported a further palliative effect at even higher doses, but the exact mechanism for this has not yet been validated.

It should be noted that olmesartan is considered a very safe drug\(^\text{80–83}\) for which the US FDA has not dictated any unsafe dosing level. However, there are definite sequelae that some might consider to be ‘adverse events’—autimmune patients initiating this therapy appear to experience immunopathology, sometimes severe immunopathology. They generally report consistent increases in overall malaise, particularly those related to the specific symptoms of their disease. At the same time, markers of inflammation rise. It should be noted that healthy people administered with the same medications experience no such reaction.\(^\text{80}\)

After months, or sometimes years of dealing with these symptomatic flares, the very symptoms that wax and wane in accordance with administration of olmesartan begin to disappear, resulting in reports of symptomatic improvement in, and in some cases, eventual resolution of the symptoms. Inflammatory markers generally return to their normal range.

For example, LZ is a 58-year-old female diagnosed with rheumatoid arthritis in 1996. In the 5 years that followed, she was administered with high-dose antibiotics along with frequent cortisone injections. Despite treatment, her disease progressed and she had joint damage in hands and feet. In 2001, LZ began 2000–5000 IU of vitamin D daily, dehydroepiandrosterone, armor thyroid, hydrocortisone and bio-identical hormone supplementation. In August 2004, LZ’s measured levels of ANA were 1:160. Following the test, patient stopped vitamin D and was administered with 40 mg of olmesartan four times daily. Over the course of several years, she was prescribed rotating combinations of certain subinhibitory antibiotics including minocycline, clindamycin and azithromycin. His erythrocyte sedimentation rate (ESR) was 25 mm/h, his C-reactive protein (CRP) was 17.1 mg/l and his bath ankylosing spondylitis disease activity index (BASDAI) was 8.8 (Figure 2). After 26 months (April 2007), the two markers and index rose. ESR went up to 25 mm/h, CRP climbed to 21.6 mg/l and his BASDAI was 9.2. Three years later in April 2010, a total of more than 5 years since starting the therapy, ESR declined to 4 mm/h, CRP fell to 6.7 mg/l and BASDAI descended to 5.3. In addition to improvement in markers of ankylosing spondylitis, LZ reported a decline in ankylosing spondylitis symptoms, as well as less depression and improved irritable bowel syndrome.

It may seem unrealistic that a VDR agonist could cause what appears to be immunopathology, let alone eventual improvement in patients with so widely differing disease states. Yet, we have been collecting many reports of improvement of patients with a wide range of autoimmune and inflammatory conditions.

The antimicrobial peptides activated by the therapy are able to target vastly different pathogens under very different circumstances. Some even have activity against certain species of antibiotic-resistant bacteria.\(^\text{84}\) Zasloff concludes ‘Acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable’.\(^\text{85}\)

Because it can be differentially spliced, the cathelicidin protein itself can respond to a range of very different microbial challenges. In humans, the cathelicidin antimicrobial peptide gene encodes an inactive precursor protein (hCAP18) that is processed to release a 37-amino-acid peptide (LL-37) from the C-terminus. LL-37 is susceptible to proteolitic processing by a variety of enzymes, generating many different cathelicidin-derived peptides, each of which has specific targets. For example, LL-37 is generated in response to Staphylococcus aureus, yet LL-37 represents <20% of the cathelicidin-derived peptides, with the smaller peptides being much more abundant and able to target even more diverse microbial forms.\(^\text{29}\)

Beta-defensin expression is modulated in response to bacteria-derived molecules and/or cytokines and chemokines produced by the immune system and damaged cells. For example, in immune cells,
its production is upregulated following exposure to bacteria, lipopolysaccharides, interferon-gamma and interleukin-beta among others. So again, the beta-defensin response will differ depending on the presence and abundance of these and other factors that are, in turn, determined by the unique nature of every individual’s microbiota.

**Olmesartan appears to potentiate pulsed subinhibitory antibiotics**

Antibiotics may be generally ineffective at generating immunopathology if a patient is immunocompromised. Under these conditions, the immune system may not be able to potentiate the actions of the antibiotics in a manner that would allow them to generate significant microbial die-off. The following case history illustrates how, when certain subinhibitory antibiotics are taken in conjunction with the immunostimulant olmesartan, patients generally become much more sensitive to these antibiotics.

BG is a 56-year-old male who was first diagnosed with rheumatoid arthritis in June 2002. He also complained of fatigue and depression. In February 2004, BG was administered with 200 mg of minocycline every other day, 200 mg of Celebrex daily and Advil as needed. BG reported improvement in all major symptoms within weeks. In April 2005, Celebrex was lowered to 100 mg every day. At this point, BG reported being ‘unaware’ of rheumatoid arthritis symptoms. On a scale of 1–10, with 10 being the most severe, he rated his overall well-being as a 1. In September 2005, he was administered with 40 mg of olmesartan four times daily. His symptom levels remained constant. After 2 weeks, 25 mg of minocycline every other day was introduced. Within 48 h, BG reported exquisite photosensitivity, complaining that daylight ‘hurt his eyes’ and ‘made him feel ill’. Over the course of several weeks, his symptoms increased greatly to the point where he rated his overall well-being as an 8.5. After 5 weeks, BG discontinued olmesartan and resumed 200 mg of minocycline every other day. He reported immediate relief. In September 2005, BG resumed olmesartan four times daily and 25 mg of minocycline on alternate days. He experienced a spike in symptoms once more. Over a few months, immunopathology gradually decreased on this dose. At present, BG has been on the treatment for over 4 years. In September 2010, BG reported overall well-being at a 2.

**Neurological comorbidities**

Since our therapy was originally developed, an increasing number of mental diseases have been tied to microbes. In a seminal 2010 study, a team of Harvard researchers showed that amyloid beta can act as an antimicrobial peptide, having antimicrobial activity against eight common microorganisms including *Streptococcus, S. aureus* and *Listeria*. This led study author Rudolph E. Tanzi to conclude that amyloid-beta is ‘the brain’s protector’. A large subset of autism spectrum disorder patients show evidence of bacterial and/or viral infections with Nicholson’s group showing unique urinary metabolites associated with the disorder. Thus, it is not implausible that an immunostimulatory treatment could cause mental in addition to physical immunopathology.

For example, AW is a 59-year-old male who was diagnosed with severe depression in 2000. In 2004, he went on total disability due to severe depression. AW also suffered from several comorbidities including chronic fatigue syndrome (CFS). CFS/myalgic encephalomyelitis is now believed by many to be an autoimmune illness. Between 2000 and 2007, AW was administered with the psychotropic medications Celexa and Ritalin with limited clinical improvement. He was weaned from Celexa and Ritalin in January 2007. The patient began 40 mg of olmesartan four times daily in October 2007. In the following month, AW noted increased symptoms associated with his CFS. In the following years, symptoms of depression and fatigue were further exacerbated upon administration of clindamycin and azithromycin. AW temporarily discontinued treatment starting in November 2009 through June 2010. In June 2010, he started olmesartan again, 40 mg four times daily. AW’s CFS symptoms persisted but symptoms of depression improved. The patient’s supervising psychologist reported in June 2010 that AW’s ‘functioning and emotional adjustment has improved considerably’ which the psychologist ‘attributed to treatment of underlying medical issues’. In September 2010, AW remains on the treatment and disability due to his CFS but no longer complains of depression.

When our treatment was first administered in 2001, we were surprised to receive reports of significant neurological immunopathology. However, we have now grown accustomed to receiving frequent reports in which not just depression, but obsessive compulsive disorder, anxiety, dyslexia, cognitive dysfunction and mania all fluctuate
in the same manner as physical symptoms upon administration of olmesartan and, in some cases, pulsed subinhibitory antibiotics.

Subclinical infection
Clinicians have long reported a phenomenon known as ‘minocycline-induced lupus’ in which certain patients administered with minocycline appear to develop the autoimmune condition.99 In fact, there is no plausible mechanism that explains how minocycline can cause lupus—or any other disease.99 A more logical explanation may be that certain patients harbor persistent bacterial species that predispose for subclinical 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. As Krawitt has argued, the same is likely true for ‘minocycline-induced hepatitis’.91 Many of the patients on our immunostimulatory therapy have also reported the temporary development of new symptoms, suggesting that the unmasked subclinical infections may be more common than currently supposed.

JM is a 54-year-old female diagnosed with endometriosis (diagnosed in 1986), chronic fatigue syndrome (2000) and a number of comorbidities. In January 2006, JM was administered with 40 mg of olmesartan four times daily. In April, she was also administered with 25 mg of minocycline every other day. JM reported increases in symptoms including but not limited to the following: body pain, fatigue, lightheadedness, insomnia, photosensitivity, anxiety and depression. In August 2009, she developed acute shingles with distribution of the left greater occipital nerve branch. Shingles were managed with oral and topical Valtrex. By November 2009, JM’s symptoms were stable and tolerable, although she reported an increase in fatigue after beginning 125 mg of Bactrim DS every other day. In March 2010, JM discontinued taking all antibiotics but remained on 40 mg of olmesartan four times daily. By April 2010, JM reported global improvement.

Most of the symptoms that JM found exacerbated upon olmesartan and subinhibitory antibiotic administration were symptoms that she had previously experienced before starting therapy. However, JM had never reported any history of shingles infections. It is likely that her activated immune response unmasked a previously subclinical infection. This same phenomenon, including treatment-induced appearance of shingles, has also been reported in IRIS.92

Potential severity of immunopathology
We have found very strong immunopathology to be quite common, particularly among patients who have been ill for decades and/or have taken immunosuppressants for extended periods of time. Physicians must be aware that helping severely ill patients manage immunopathology may present a significant clinical challenge.

PF is a 65-year-old female diagnosed with metabolic syndrome (diagnosed in 1995), osteopenia (2004), fibromyalgia (2002) and undiagnosed gastrointestinal symptoms. By 2004, she was taking 1200 IU of vitamin D a day for several years, which she discontinued in March 2005. PF was administered with olmesartan four times daily starting in March 2005. In June 2005, she was also administered with 50 mg of clindamycin every other day. In November 2008, PF experienced acute and severe diarrhea and dehydration, which required hospitalization for several weeks. Tests were negative for acute infections including Clostridium difficile. Due to these severe bowel symptoms, olmesartan was discontinued. PF was finally stabilized on ramipril and losartan after 2 weeks. After taking 20 mg of olmesartan, she experienced nausea, vomiting and diarrhea within 2 h.

This patient was not able to stay on our immunostimulatory treatment despite the fact that she was able to tolerate the immunopathology associated with her diabetes and CFS. Instead, her physician was forced to terminate therapy because of her severe bowel immunopathology. PF is an example of a patient who was simply too ill to tolerate immunopathology that at a lower level might otherwise have allowed for eventual improvement or recovery. Even so, over the course of the treatment, PF did experience significant improvement in bone density. Between June 2004 and the June 2010, the patient’s total hip bone mineral density increased 4.8%, while anterior/posterior spine (L1–L4) decreased 1.3% since baseline.

Cases like that of PF emphasize the importance of actively managing immunopathology with the goal that a patient’s symptoms remain in a tolerable range. Patients would almost certainly die from sepsis if it were somehow possible that their entire pathogenic microbiota could be targeted at once. Indeed the literature has several reports of fatal reactions among patients with syphilis in which too strong a Jarisch–Herxheimer reaction was induced.93–95 Our treatment protocol encourages physician and patient to work together to adjust levels of olmesartan and antibiotics in order to continually achieve a tolerable level of immunopathology.

Many patients experience an inflammatory reaction for several years before reporting significant improvement. While we expected immunopathology as a result of olmesartan administration to occur for at least several months, we did not anticipate how profound and prolonged the reaction could be. In our experience, patients with severe illness often manage immunopathology for 4–7 years before presenting with objective markers indicating significant improvement or disease resolution.

The length of time it takes seriously ill patients to report symptom remission on this therapy has some medical precedent. The preferred regimen for the treatment of latent tuberculosis is 9 months of isoniazid—which is also intended to kill intracellular pathogens.96 Notably, both treatments may involve an immunopathological-style reaction.97,98 However, isoniazid treatment is aimed at killing only one major pathogen whereas patients with autoimmune disease may be targeting multiple phyla of pathogenic microbes. By contrast, treatments like HAART in HIV patients target polymicrobial communities of opportunistic infections including viruses, fungi and bacteria. Beatty has noted that symptoms of IRIS among these patients can occur as long as 3 years after initiating treatment.99

The long periods of immunopathology experienced by some patients on our treatment could speak to the possible inadequacies associated with the therapy. However, it may also reflect the sheer number and virulence of the pathogenic microbes present in autoimmune disease.

Recently diagnosed patients
Conversely, patients who start our treatment early after disease onset and have not previously been administered with immunosuppressants often find that their immunopathology is much easier to tolerate and experience faster symptom improvement.

At the age of 34 years, in January 2007, AC was diagnosed with her first inflammatory condition, mixed connective tissue disease. Several months later, she had ANA of 1:2520 and RF of 12. Several other diagnoses followed in the next 2 years including neuropathy, Sjögren’s and Raynaud’s syndromes, muscle fasciculation, vulvodynia, dermatitis and cervical dysplasia. AC began 40 mg of olmesartan four times daily in September 2009, less than 3 years after her initial diagnosis. Prior to beginning treatment with olmesartan, AC reported...
that on a scale of 1–10, with 10 being the most severe, high levels of muscle (7), joint (8) and vulva (10) pain. Six months into treatment, those scores declined: muscle (3), joint (3) and vulva (3). In September 2010, 1 year after beginning treatment, she rates those symptoms, respectively, as 1, 1 and 2. In February 2010, the patient’s bloodwork was negative for ANA. RF has not been retested since. AC has reported similar 12-month improvements in dry mouth (5–0), dry eye (6–3), burning tongue (8–0) and noise intolerance (5–0). Only fissured tongue (9–8) has remained unchanged at this point in therapy.

Cases like AC strongly suggest that the sooner an immunostimulatory treatment is started, the less immunopathology a patient may have to manage, and the quicker the potential recovery. This underscores the importance of immunostimulatory therapies being researched in further depth so that they might become increasingly used as first-line treatment options for autoimmune disease.

**SURROGATE OUTCOMES FOR DISEASE MUST BE CAREFULLY CHOSEN**

Since microbes seem to play a significant role in the autoimmune disease process, we must necessarily reconsider the role of the various metabolites associated with the markers we use to assess those conditions. Many inflammatory disease states are marked by both metabolic fluctuations and physical presentations that would not be considered ‘normal’. We observe shifts in white blood cell count, cholesterol levels, blood pressure and measures of kidney function (such as blood urea nitrogen (BUN) and creatinine) to be common.

In responding to elevated or depressed markers associated with inflammatory disease, physicians have a broad range of available therapeutic strategies. Statins, diuretics, hypoglycemics, tumor necrosis factor-α antagonists, vitamin D and thyroid hormones each target a particular surrogate outcome associated with disease. Six of the world’s 10 top-selling drugs in 2010 are marketed at targeting outcomes that are surrogate outcomes.

Some physicians argue that autoimmune diseases should be treated until laboratory abnormalities resolve. However, while surrogate outcomes have some utility in signaling the presence of inflammatory disease, it has become increasingly less clear the extent to which changes in a particular marker associated with disease alters the course of the disease itself; ‘few surrogate markers’ have been shown to ‘capture the effect of a treatment’.

More and more researchers and physicians routinely deprecate evidence devoid of outcomes that matter most to patients and their caregivers (i.e., patient-important outcomes). This evolution in approach is borne out of experience. According to Grimes and Schulz, ‘thousands of useless and misleading reports on surrogate endpoints litter the medical literature’.

While a number of drugs are highly effective at altering measurable metabolites, they barely affect the progression of disease. For example, in low-risk individuals with high cholesterol, statins have a marginal, if not absent, effect in protecting against cardiovascular disease.

There are two possibilities in metabolite and disease interaction, cause and effect. It is critical that we do not try to intervene to drive purely associative metabolites back into range, as that may disrupt systemic homeostasis and possibly delay disease resolution.

**Markers of anemia**

The low levels of blood cells characteristic of anemia of chronic disease (ACD) are relatively common among autoimmune conditions and obesity. A related hallmark of ACD is increased uptake and retention of iron within cells. In their *New England Journal of Medicine* review, Weiss and Goodnough write that despite treatment guidelines, ‘anemia of chronic disease remains underrecognized and undertreated’. Anemia should be actively managed, they put forth, because the condition ‘has been associated with a relatively poor prognosis’ and is associated with suboptimal oxygen delivery.

However, Zarychanski and Houston state that ACD is fundamentally an adaptive physiologic response which benefits the patient during times of infection with Baker and Ohi offering a similar argument. As a nutrient that is essential for the survival of many microbes, increased iron availability promotes microbial growth. In fact, the ability of a particular species of bacteria to glean iron from its host is often a good indicator of its virulence. It is logical then that the body sequesters iron in response to an infection. Kemna *et al.* showed that injecting human volunteers with lipopolysaccharides, a component of the cell walls of gram-negative bacteria, leads to a significant decrease in serum iron. In iron-deficient conditions, blood plasma is moderately effective at inhibiting bacterial growth.

It has been our experience that both white and red blood cell counts surge and wane during therapy, tending to normalize as the inflammation resolves. Therefore, while it might otherwise seem reasonable for a clinician to directly manage ACD using iron supplements, doing so may actually lead to microbial proliferation.

**25-hydroxyvitamin D (25-D)**

Lower than normal levels of the metabolite 25-D, which is widely believed to serve as a marker of vitamin D status, have been independently associated with all-cause mortality and increased prevalence of at least 40 different chronic inflammatory diseases. Over the last decade, low levels of 25-D have generated interest among physicians, with many concerned that failing to supplement puts the patient at greater risk for further disease. Proponents of heavy supplementation have stated that adult humans can take extraordinary levels of vitamin D for prolonged periods of time without risk of adverse effects.

However, the issue of what serum level of the secosteroid is optimal for health may be less conclusive than some have suggested. As previously discussed, microbes including *M. tuberculosis*, *Borrelia* and EBV have been shown to downregulate the activity of the VDR. As expression of CYP24A1 diminishes, 1,25-D levels rise. When the hormone/secosteroid rises above a normal range, it may downregulate, via the nuclear receptor pregnane X receptor, the amount of pre-vitamin D converted into 25-D. The result is that 25-D levels drop.

Thus, in patients suffering from VDR dysregulation, there is a strong possibility that a low 25-D level is a result rather than a cause of the disease process. A similar pattern has been demonstrated in VDR knockout mice. While there are clear biological differences between humans and rodents, VDR knockout mice also show a marked increase, by a factor of 10, in serum 1,25-D and a clear reduction in serum 25-D to almost undetectable levels.

This challenges the entire concept of vitamin D ‘deficiency’ and helps explain why many patients with inflammatory disease present with low levels of 25-D even when they are consuming large amounts of the secosteroid or are exposed to abundant sunlight.

Supplemental vitamin D has been widely lauded for conferring immunosuppressive effects: Arnson *et al.* write ‘[v]itamin D affects the immune system at many levels and by a number of mechanisms... Vitamin D has multiple immunosuppressant properties... On the whole, vitamin D confers an immunosuppressive effect’. Indeed, in a 2010 study of pregnancy-associated breast cancer, higher levels of 25-D were positively correlated with serum antibodies to EBV,
suggesting that EBV is able to better proliferate in patients who take vitamin D. Further evidence for vitamin D's activity as an immunosuppressant comes in the range of reports of short-term symptom resolution in autoimmune patients taking vitamin D. In animal models, administration of vitamin D has been shown to effectively inhibit signs of autoimmunity, even when animals had 'sufficient' vitamin D.

Vitamin D may be a case where a substance has inadvertently become popular in autoimmune disease because of its immunosuppressive properties and subsequent ability to lower inflammation and immunopathology. Ingestion of an immunosuppressant would counteract an immunostimulatory therapy, leading to treatment failure even while a patient experiences modest symptomatic improvement in the short-term.

**Blood pressure**

Low blood pressure is often taken to be a cause of disease, but in many circumstances, hypotension is one of the net results of microbial death. Hudgins found that injecting human volunteers with a small amount of endotoxin—which typically enters the blood stream when gram-negative bacteria are destroyed—not only produces fever and activates coagulatory and inflammatory processes, but leads to a drop in blood pressure. One lab worker ingested very large amounts of Salmonella endotoxin and found his blood pressure drop to 42/20 mmHg.

Unfortunately, artificially raising a patient’s blood pressure back to the range normally correlated with health does not alter the bioavailability of endotoxins or the underlying disease state. In many cases, intervention to raise or lower the blood pressure is unnecessary, especially because additional medications may affect immune homeostasis. We find that blood pressure surges and wanes during treatment, but usually settles into the normal range as the inflammation wanes.

An illustrative case is that of AY, a 54-year-old female who began treatment with a diagnosis of chronic fatigue syndrome in 1993 and a number of comorbidities. In November 2004, prior to treatment, AY's blood pressure was measured as 75/45 mmHg. That month, she began four times daily dosing of 40 mg of olmesartan. Initially, symptoms of CFS and photosensitivity increased. Over the subsequent year, AY's blood pressure ranged between 65/45 and 75/50 mmHg. In January 2005, the patient was administered with 25 mg of minocycline every other day. The dose was slowly increased until she reached a maximum of 100 mg every other day. Symptoms intensified including several episodes of syncope. Starting in March 2005, the patient continued rotating combinations of minocycline, clindamycin and azithromycin, all taken in pulsed subinhibitory doses, which resulted in an increase in nearly all disease symptoms. However, in January 2006, A.Y.'s blood pressure rose to 90/60 mmHg. In August 2006, she reported increased tolerance to light and noise, less insomnia, increased exercise tolerance but still limited functionality. In March 2008, AY became able to travel and reported greatly decreased photosensitivity, increased exercise tolerance but still limited functionality. In March 2009, the patient’s blood pressure read 110/65 mmHg. In April 2010, AY discontinued all antibiotics, remaining on 40 mg of olmesartan taken three times daily. AY reported being able to exercise vigorously. The latest blood pressure was taken at this time and read 120/75 mmHg.

It should be noted that AY was taking the same dose of olmesartan when her blood pressure was 75/45 mmHg as when it later stabilized to 120/75 mmHg, further suggesting that her original low blood pressure was not directly related to treatment medications.

**Blood urea nitrogen and creatinine**

Several studies have pointed to elevated markers of kidney function as a risk factor for disease. In the absence of further context, a physician administering an immunostimulatory therapy might be tempted to withdraw treatment in the face of such measures. However, increases in nitrogenous waste may also reflect host-defensive measures.

During acute infections, proinflammatory cytokines and interferon-gamma stimulate the production of nitric oxide as do bacterial lipopolysaccharides. Nitric oxide acts as a highly potent microbicidal and tumoricidal agent, and has immunomodulatory functions.

Research has also shown that nitric oxide acts as an effector of the innate immune system targeting adenoviruses and other similar viruses. As a result, during acute infections we see a sudden and dramatic increase in excretion of urinary nitrite, a stable metabolite of nitric oxide. Fever, the body’s evolutionarily conserved response to infection, is also accompanied by urinary excretion of creatinine, urea and ammonia.

A 2003 study found that among 117 hemodialysis patients, those who had high serum values of BUN were less likely to have the acute infection, Helicobacter pylori. This clinical work suggests that temporary markers of kidney stress may correlate with a robust and successful immune response. In certain cases, elevated kidney metabolites are associated with improved clinical outcomes. A recent Japanese 4-year follow-up study of 877 men found that lower serum creatinine was significantly associated with an increased risk of type 2 diabetes.

Studies of immunopathology indicate that the kidneys and liver must deal with the burden of toxins released after microbial death. This, in addition to the factors described above, may lead to a decline in markers of kidney function. While the kidneys are under a certain amount of stress, bringing such metabolites back into range would require interfering with the immunopathological reaction. This defeats the purpose of administering the treatment in the first place. Therefore, to a certain extent, physicians may want to consider allowing BUN, creatinine, or other kidney markers to remain out of range provided that these metabolites are carefully monitored and immunopathology is tolerable. As with blood pressure, we typically see that, if left alone in this fashion, kidney metabolites will come back into range as immunopathology eventually subsides.

EJ is a 53-year-old female diagnosed with CFS. She also complains of musculoskeletal pain. EJ began 40 mg of olmesartan four times daily in May 2007. After starting subinhibitory doses of minocycline and azithromycin, EJ reported increases in pain and fatigue while, around the same time, her epidermal growth factor receptor (EGFR) plummeted from 86 to 38 ml/min. After only normal readings, EJ’s creatinine was first measured out of range 7 months into therapy at 1.53 mg/dl. However, in the course of the next year, her eGFR climbed to 53 and eventually 80.3. During the same period of time, EJ’s creatinine dropped to 0.76. As of September 2010, EJ reports her musculoskeletal pain as 0, but still complains of fatigue and cognitive problems. Her latest kidney readings are a creatinine of 0.79 and eGFR of 75.9.

Although EJ’s creatinine and eGFR fluctuated substantially out of the normal range during some of the time on the treatment, the metabolites stabilized without intervention. As expected, her most pronounced drop in eGFR corresponded to a period in which she experienced very high levels of immunopathology.

A more extreme example is that of BB, a 56-year-old male with a diagnosis of sarcoidosis. He began 40 mg of olmesartan four times
daily in December 2005 and began rotating combinations of subinhibitory minocycline, azithromycin and clindamycin shortly thereafter. As seen in Figure 3, several months after starting treatment, measures of renal function initially declined sharply, reaching out-of-range levels in BUN (18 of 21 measures), creatinine (19 of 22) and GFR (14 previous tests). However, he is now 95% free of his previous symptoms and no longer takes oral or inhaled steroids.

This case is interesting in that typically a patient with kidney metabolites such as those of BB would likely be preparing for dialysis or experiencing symptoms of kidney failure. Instead, BB is relatively symptom-free. Again, his test results fluctuate in a way that suggests they are tied to immunopathology. This implies that in BB’s case, factors other than progressive kidney deterioration may be affecting his BUN, creatinine and GFR. It is important to note that even though we have received many case histories in which patients’ kidney metabolites fluctuate out of range, we have had no reports of patients needing dialysis.

IMMUNOSTIMULATIVE THERAPIES NEED FURTHER STUDY
In studying therapeutic approaches designed to induce immunopathology, we must grapple with several ethical issues that have received minimal attention thus far.

Accepting discomfort
Physicians and researchers, especially in the context of clinical studies, feel an acute imperative to relieve pain and discomfort whenever possible. Anything less, many have argued, would be unethical or immoral. Pullman writes ‘[t]he duties to relieve pain and suffering are clearly matters of moral obligation’.

Lohman writes that access to pain treatment is a ‘human right’, while Rollin states that necessary suffering is any suffering which is ‘impossible to alleviate’. However, medical providers have limited reservations with administering painful treatments or conducting uncomfortable procedures that lead to well-characterized positive outcomes: sentinel node biopsy in suspected cancer, major shoulder surgery, certain dental procedures, etc. Nor are most clinicians categorically opposed to using medicines that have a range of serious side effects, chemotherapy being an obvious example. Given sufficient reason for causing near-term discomfort, many patients and physicians are perfectly willing to seriously consider these types of trade-offs.

Therapies are typically thought of as having two categories of effects: therapeutic effects and adverse effects. The former is intentional and makes a patient feel better, and the latter is unintended and makes a patient feel worse. Yet, certain therapies that induce immunopathology have the potential to be a hybrid of the two: by necessity, the treatment is intended to make a patient feel worse. In other words, in the context of an immunopathology-inducing therapy, an adverse effect is not so much a collateral effect of the treatment, but the treatment itself.

As we have discussed, our immunopathology-inducing protocol can cause a sustained exacerbation in symptoms over at least several years. However, in spite of the treatment’s length, we have found many patients are more than willing, considering the gravity of the prognoses they face, to commit themselves to such therapy.

It should be noted that the immune system may become healthier over the course of treatment. As microbes dysregulating the nuclear receptors are increasingly eliminated, and infected cells are replaced by their healthy counterparts, the immune response gains substantial strength. Thus, in some cases, immunopathology may actually become more severe several years into treatment. Physicians and patients should be aware that symptomatic improvement on an immunostimulatory treatment is rarely linear.

Blinding, randomization and study design
The randomized controlled trial is regarded as the gold standard of measuring the efficacy of a therapy. Blinding the intervention to participants and physicians further reduces the effect of treatment bias. However, in the case of an immunopathology-inducing therapy, the severity of the symptom response would invariably make blinding impossible—patient and physician would know in short order the treatment arm to which a participant had been assigned. Given that therapies that generate immunopathology seem to be required at least several years before sicker patients report substantial improvement, randomization also poses a challenge. To be sure, subjects could be
assigned to the immunopathology-inducing group, but the potential for early withdrawal is clearly much higher for multiyear treatments that necessitate symptom exacerbation, even if only in the short term.

The number of patients who decline to participate and withdraw early may make an intention to treat analysis untenable. In any case, any study design for a therapy which makes patients feel significantly uncomfortable has the potential to incur self-selection bias: people who commit themselves to a challenging multiyear therapy are qualitatively different from people who decline the treatment.

Another key consideration for study design is the length of the trial. Trials that assess overall patient outcomes, such as improvement in activities of daily living, would need to last several years. Alternatively, trials of efficacy (i.e., those looking at microbial death) could be concluded in a month or 2. It has been reported that HIV/AIDS patients on antiretroviral drugs experience IRIS within weeks of beginning therapy. This is consistent with our experience when using immunostimulation in autoimmune disease.

An essential feature of the design of any clinical trial is informed consent, and this is especially important in therapies that generate immunopathology. Patients need to expect to experience temporary well-defined increases in symptoms, markers of inflammation, and disease state. They must also appreciate why such increases are necessary, and how they would work with their physician to manage their symptoms.

SUMMARY
Over the past decade, molecular sequencing technology and metagenomic analyses have revolutionized the field of microbiology. The human body, once deemed largely sterile, is now known to harbor thousands of genomes—many of which have still to be named and characterized. These genomes continually interact with the human genome in both health and disease. Not just single pathogens, but entire communities of microbes differ substantially between healthy individuals and those with autoimmune disease. The body is likely not creating antibodies against self, but instead in response to these pathogenic genomes. Indeed, many ‘autoantibodies’ have already been linked to the presence of pathogens.

Those pathogens that can persist intracellularly have access to DNA translation and repair mechanisms, and can also affect gene expression. This activity mandates a fundamental reconsideration of how chronic diseases are treated. Rather than immunosuppression, immunostimulation may be the true key to reversal of these conditions.

The symptoms of an acute infection result from a battle between man and microbe. A similar battle, in which the immune system releases cytokines and microbes form endotoxins or debris, would occur if pathogens were killed in chronic disease. Thus, if chronic microbes are successfully targeted, patients would necessarily be expected to experience symptom exacerbation. In effect, patients recovering from autoimmune disease may need to feel worse before they feel better.

Interestingly, these responses have been described in the literature, generally as being paradoxical. Yet symptom exacerbation resulting from an immunostimulative approach towards pathogens is paradoxical only because it defies previously held intuition. The immunostimulatory approach we have studied uses a putative VDR agonist to reactivate the innate immune response. Case histories suggest that patients with a variety of autoimmune diagnoses experience prolonged immunopathology. Many subjects additionally experienced neurological immunopathology, often for several years before improvement. This emphasizes the systemic nature of autoimmune disease processes and the significant number of pathogenic responses that seem to be involved in the development of these disease states.

A broad array of medications and supplements are effective at reducing discomfort of patients with chronic disease. Our reports suggest that many of these drugs, whose actions at the molecular level are not fully understood, may generate feelings of clinical and subjective improvement precisely because they suppress and slow activity of the immune system.

In addition, accumulating evidence from metagenomic studies is pushing physicians and researchers to reflect upon the wisdom of using interventions in an attempt to alter the body’s metabolites. The more prominently microbes figure in our understanding of the pathogenesis of chronic disease, the more likely it is that therapies should be tailored to support the body as it responds to infection. In many cases, out-of-range markers seem to be a necessary part of the healing process.

Nevertheless, there remains a need to identify microbe-specific markers. Metabolomics is the emerging field which studies microbial metabolites. As metabolomics and metagenomics evolve, we should expect the emergence of technologies capable of more optimally defining a treatment regime based on examination of specimens from blood, urine and swabs.

There needs to be more collaboration between researchers and clinicians in order to more tightly define the immunopathology we have observed.


68 Schaffner A. Fever—useful or noxious symptom that should be treated? Ther Umsch 2006; 63: 185–188.


Summary and link to General Discussion

In this chapter “Immunostimulation in the era of the metagenome”, we discussed an immunostimulative therapy that attempts to increase innate immune activity via a drug aimed at re-activating the VDR. The putative VDR agonist olmesartan may correct VDR dysregulation and prime the immune system to kill the intracellular pathogens driving the autoimmune disease process. In parallel, pulsed low-dose bacteriostatic antibiotics would assist in bacterial eradication. We have presented ten case reports of patients whose physicians had used this immune therapy in order to illustrate the paradigm shifts when immunostimulative medications are used in the place of immunosuppressants. We examined the effects of the treatment in a variety of autoimmune diagnoses, most showing improvement and/or reversal of disease symptoms.

The most important observation made was that when the innate immune system was invigorated to target chronic pathogens, the resulting storm of cytokines and chemokines led to a temporary rise in disease inflammation and symptoms. We refer to this process as immunopathology. In this chapter, we have described the different forms of immunopathology observed in treated patients. We also discussed some challenges that physicians and scientists will need to face as they attempt to optimize potential curative treatments for autoimmune disease in the future.
General Discussion

This thesis presents a working hypothesis describing the pathogenesis of many serious idiopathic diseases. To the best of my knowledge, this thesis is the first body of original work to propose a model for autoimmune disease informed by metagenomics. We synthesize data and concepts from metagenomic biological research, in silico emulations, and data from clinical studies of autoimmune disease. If the pathogenic description described in this thesis is valid, it will significantly change both the description and understanding of autoimmune disease.

Key Points

1. The human body is not a sterile compartment but harbors a prolific microbiota. The number of microbial cells in the body exceeds human cells by a factor of at least ten. While the human genome consists of 23,000 genes, the number of unique genes in the microbiome is known to be at least 100 times greater.

2. The human microbiome exists not only in the gut and other mucosal surfaces but also in tissues, blood, and inside the cells. Key to disease is that the RNA and proteins from intracellular microbes directly interfere with transcription, translation, and cellular repair mechanisms.

3. Koch’s postulates stipulate that a given infectious disease must be caused by a given species of microbe. Yet it is now more plausible that autoimmune disease results from dysbiosis of whole communities of microbes - microbes that interact so extensively that the line between species is unclear.
4. In order to survive in the face of the human immune response, persistent components of the microbiota must necessarily employ survival mechanisms. Those mechanisms, which weaken the host innate immune system, including expression by the human VDR of cathelicidin and TLR2, provide a significant survival advantage.

5. The exact composition of a given person’s microbiota varies according to several factors including familial exposure, site and duration of exposure to infections, blood transfusions, and use of antibiotics or immunosuppressants. The sum of a person’s infectious history determines the level and type of interference between the host and parasitic proteomes and metagenomes. This drives the uniqueness of each disease presentation, no two of which are exactly alike. Embracing the co-morbidities allow us to focus on the shared mechanisms by which microbes disrupt the body’s pathways.

6. In order to restore microbial homeostasis, the body’s immune response must be reinvigorated to recognize persistent pathogens. Therefore, immunosuppression is counterproductive if one wishes to target the underlying causes of autoimmune disease. At best, it can lead to short term symptomatic relief with relapse in the longer term.

7. When the immune system is killing pathogens inside a cell, the likelihood of killing the cell through apoptosis or autophagy is very high. Additionally, the storm of inflammatory and toxic byproducts resulting from microbial death is significant. The resulting surge in symptoms (immunopathology) causes patients to initially feel worse before they start to feel better. It follows that feel-good substances therefore cannot be targeting the causal microbes and are most likely acting through immunosuppressive pathways. Any claimed long-term efficacy of such substances must be very suspect. The substances in question include the secosteroid transcriptional activator called vitamin D.
8. Hormonal differences between women and men cause the sexes to express different antimicrobial defenses. In addition, the VDR is expressed in the cycling endometrium, potentially causing women to suffer more from VDR dysregulation than their male counterparts. These differences suggest that over time, women may be more susceptible to certain microbes than men and may acquire them more easily. This would partially account for the higher prevalence of autoimmune disease in women.

9. The body sets up a different metabolic homeostasis in disease than in health. Thus, low levels of 25-D in patients with inflammatory disease are likely a result rather than a cause of the disease process. This disturbance of homeostasis is observed with other metabolites such as cortisol and cholesterol. This means that a physician may have to allow these metabolites, and others, to remain out of range during periods of immunopathology in order for the body to attempt to return to a state of homeostasis.

10. Autoantibodies are notoriously polyspecific. Increasingly the evidence is showing that they are created in response to the disease-causing microbiota. Thus, autoimmune activity may be collateral damage from the immune system's fight against chronic infection rather than the result of a hyperactive adaptive immune response. Ultimately, Ehrlich's instinct was at least partially correct: there is no such thing as autoimmunity.

11. The human genome can no longer be studied in isolation. Rather, it must be analyzed in the context of or how it interacts with the metagenome and with the metatranscriptome.

**Translating the science into practice**
In 2010, J. Craig Venter, who first sequenced the human genome, commented, “If anything, we don't really know how to read the genome and it can't tell us very much right now... We couldn't even be certain from my genome what my eye color was. Isn't that sad? Everyone was looking for miracle 'yes/no' answers in the genome. ‘Yes, you'll have cancer.’ Or ‘No, you won't have cancer.’ But that's just not the way it is.... [The Human Genome Project has had] close to zero [medical benefit]. We have, in truth, learned nothing from the genome other than probabilities. How does a 1 or 3 percent increased risk for something translate into the clinic? It is useless information.”

Venter's genome is “useless” because it has not yet been interpreted in concert with the microbial genomes that also persist in his body. Mainstream medicine has yet to embrace the clinical utility of the metatranscriptome, which impacts nearly all areas of medicine including how we react to vaccines, medications, and environmental toxins. In the same vein, the human superorganism is often viewed as more of a fascinating factoid than a paradigm on which to base or reinterpret research. While it is widely assumed that inflammatory disease is caused by a combination of genetic and environmental factors, researchers studying the human genome often have little contact with those studying the microbiome. Much of the lack of utility described by Venter is due to this disconnect.

As the importance of the metatranscriptome becomes more fully appreciated, researchers attempting to set a “correct” level for vitamin D intake will need to interpret their findings in the context of how VDR expression is continually altered by a number of major pathogens. Moreover, the traditional concept that intake of probiotics is beneficial must be re-examined now that microbes such as *Bifidobacteria* and *Lactobacillus* are known to directly impact pathways associated with autoimmune disease.¹ To reduce mistakes in characterization of
the human genome, it is important to make sure that tools do not mistakenly amplify RNA from intracellular microbial genomes.

**Moving away from reductionist approaches**

Recently Schutzer *et al.* used mass spectroscopy proteomics to analyze the cerebrospinal fluid of patients with Chronic Fatigue Syndrome (CFS), Post Treatment Lyme Disease (nPTLS) and so-called healthy subjects.² The group detected more than 2,600 proteins in each group with 692 proteins unique to the Lyme patients and 738 unique to the CFS group. CFS and nPTLS groups shared significantly more proteins (305) than either group shared with healthy controls.

In discussing the study the team wrote, “As with most technologic methods we expect multiple replicate analyses of the highly fractionated samples would result in a reduction of the number of seemingly unique proteins identified for each disease group.” However, if the results were interpreted in the context of our model, our team would seek to do the exact opposite. We would hope that future studies would investigate even more proteins associated with each disease state, as this would expand our understanding of the microbes capable of contributing to each condition. We would also be more interested in the proteins associated with both nPTLS and CFS, as such overlap would support our hypothesis that any one microbe can contribute to a variety of disease states. This approach would require us to accept the complexity inherent to each disease state and, as we have tried to do with our current work, focus our efforts on identifying new ways to describe and analyze the system.”
Some metagenomic researchers have suggested that studies with larger sample sizes and more in-depth analyses of specific genomes might better inform our understanding of the metagenome in health and disease. This resembles the approach currently being employed by many GWAS (genome-wide association studies) investigators. These researchers believe that GWAS with staggering sample sizes and an increased focus on a growing number of SNPs may finally lead to an understanding of the “missing heritability.” Unfortunately these GWAS have only bogged down the field of genomics in a morass of complexity. An increasing number of SNPs are identified but few show more than a minimal association with any specific diagnosis.

It would be a shame if future metagenomic researchers decide to adhere exclusively to a similar path in which reams of new data are generated absent context of how this might transfer to the clinic. The reason that our immunostimulative therapy has the potential to help patients with inflammatory disease today is that we have chosen not to fixate on the complex interplay between the microbes in any given disease state. Instead, we identified a common pathway that, if stimulated, enables the innate immune system to decide which species should be eliminated in order to restore health.

**Other considerations**

1. **Health and disease are not discrete states.**

The medical community traditionally divides people into two categories: healthy or sick. However, a person’s microbiota continually changes over time as new microbes are incorporated into the superorganism. As described in Chapter 6, in some people, the accumulation leads to chronic symptoms at an early or middle age, while others will not suffer until "aging" in their later years. Thus, health and disease are not discrete states; there
is a continuum between the two. For example, most studies that look for microbes in chronically ill individuals find that members of the control group harbor them as well, albeit usually with differing incidence. This should not be a surprise but an expected outcome. It follows that people who are not yet overtly ill may also need to take measures to maintain the homeostasis and healthy trajectory of their microbiotas. This would provide an entirely new avenue for preventative medicine.

2. We must study antibiotics more critically.

Antibiotics have saved countless lives over the past century. However, this may have also engendered a false sense of confidence that we fully understand the manner in which they work and should be used. For example, as described in Chapter 5, one of the most important, yet virtually ignored properties of the beta-lactam antibiotics, is that while they effectively target acute bacterial forms, they foster the development of chronic variants without cell walls. This alone could account for much of the increase in chronic disease over the last decades, but is not commonly used to inform clinical practice.

In the same vein, high-dose antibiotics have been shown to have profound effects on immune function in addition to their actions on any microbes. This means that, paradoxically, patients taking high doses of antibiotics frequently exhibit symptoms characteristic of immunosuppression. They generally feel better while the high dose antibiotic is being administered but relapse after it is discontinued. At least one study has shown that intravenous cephalosporin is purely palliative.³

Other studies are just beginning to reveal that commonly used antibiotics have many more actions at the molecular level than previously realized. For example, the bacteriostatic antibiotics minocycline and clindamycin may be a direct agonist of the PXR nuclear
Concerns about antibiotic resistance continue to grow, but perhaps a better understanding of the full spectrum of an antibiotic's actions at the molecular level could help us better dose and design these drugs. If used in a more informed and careful fashion, they would form a more powerful part of our arsenal against bacterial pathogens.

3. Use of probiotics may have unintended effects.

In the 1930's cane beetles in Australia were found to be destroying much of the country's sugar cane crop. It was decided that cane toads (*Bufo marinus*) should be bred in Hawaii and subsequently transferred to Australia in an effort to control the beetles. Hundreds of toads were released into the Australian tropics in 1935. Unfortunately, it soon became apparent that the toads could not jump high enough to eat the beetles, which persisted on the upper stalks of the cane plants. Furthermore, the toad population quickly burgeoned and began to outcompete and decimate many native species. The cane toads were also toxic to would-be predators such as native snakes.

The gut harbors a microbial ecosystem. The equivalent of the toads in the above example of biological pest control could be certain strains of probiotic bacteria. It is seductive yet simplistic to assume that dumping large amounts of so called beneficial microbes into the gut will simply kill "bad" microbes. Instead, introducing "beneficial" bacteria into a sick patient may actually cause such species to swap genes with pathogenic microbes. This could alter the interactions between the microbial genomes and the human genome, creating a different microbiota that the immune system may have difficulty managing. As described in Chapter 5, because of the size of their genomes, "good" microbes may well have undesirable properties as well.
Patients often do report feeling better after taking probiotics. However, it may be that adding new microbes into the gut simply diverts the immune system away from dealing with other pathogenic species. This would temporarily decrease immunopathology and disease symptoms but not target the root cause of the problem.

4. Physicians must be on the alert for neurological immunopathology.

It is clear from our case reports that immunopathology does not just occur in the body but also in the brain. It is now understood that microbes can cross the blood-brain barrier transcellularly, paracellularly, and intracellularly. Microbes have already been implicated in diseases including Alzheimer's, autism spectrum disorder, and schizophrenia. A very high number of patients with inflammatory disease exhibit mental co-morbidities. Furthermore, it appears that microbes in other parts of the body can impact the brain. For example, the composition of bacteria in the gut has been linked to depression and anxiety.

This means that patients on immunostimulatory therapy will almost certainly have to contend with changes in the way they think and feel. We have found that physicians must continually monitor their patients' mental as well as physical health. Patients who undertake immunostimulative therapy must have a support system in place, which would help them manage immunopathology and become educated about the wide range of symptoms they may encounter due to immunopathology.

5. Chronic components of the microbiome affect susceptibility to acute infection.

It is well known that the immune system weakens with age and in chronic disease. Our model of successive infection offers stronger explanatory power of this phenomenon at the
molecular level. Since the health of the microbiome is continually altered over a lifetime, its composition at any point in time significantly impacts how a person will respond to an acute infection. If the proteome and innate immune response are already dysregulated by the persistent microbiota, risk of morbidity and mortality by acute pathogens also becomes much higher.

During the recent outbreak *E. coli O104:H4* strain in Germany, the preponderance of cases occurred in women. As described in Chapter 1, women have an extra site of VDR expression in the cycling endometrium. This could account for why several pregnant women also developed the more serious forms of the illness.

Many immunologists predict that it is only a matter of time before more acute pathogens mutate into forms that might lead to serious, worldwide epidemics. Already thousands have died from SARS, HIV, tuberculosis and other “super bugs.” If preventative measures were available to maintain the health and homeostasis of an individual's chronic microbiota, priming the body to best deal with these acute invaders, then control of these epidemics might well become easier. At the very least, a move away from the regular use of immunosuppressive medications towards immunostimulative medications could prove very useful in the control of acute pathogens.

6. We must learn to explore more alternative hypotheses.

This thesis repeatedly invokes the alternative hypothesis. Our model opens up numerous avenues of inquiry that will be valuable in allowing other research teams to also examine their data in a new light. For example, studies which draw the conclusion that subjects with a particular inflammatory condition have low 25-D levels and are thus suffering from "vitamin D
deficiency“ also fit our alternative model. The 25-D levels may actually be a result of the disease process.

On their website, the supplementation advocacy group Vitamin D Council asked, “One of the great mysteries in human biology is the fact that most human breast milk is deficient in vitamin D. How could Nature overlook such an important nutrient in the ‘perfect food’?” Many studies have shown that breast-fed infants tend to perform better on standardized tests and display higher overall levels of intelligence than their formula-fed counterparts. This is almost invariably interpreted as a sign that breast milk contains a substance that baby formula lacks. Instead, the converse may be true – the intelligence discrepancy could be due to the fact that formula contains an extra ingredient with potential immunosuppressive properties: the secosteroid vitamin D.

**Continued support**

To be truly useful our model needs to be continually tested against data accumulating from new studies. Since the 2011 publication of “Immunostimulation in the era of the metagenome,” dysbiosis of microbial communities has been further reported in chronic inflammatory diseases including type 1 diabetes, autism, and COPD. We continue to receive physician case reports that are consistent with our model.

In 2009, the US Institute of Medicine (IOM) commissioned a report on vitamin D research by the Tufts Medical Center Evidence-based Practice Center. According to the report’s abstract: “The majority of the findings concerning vitamin D, calcium, or a combination of both nutrients on the different health outcomes were inconsistent.” For a variety of diseases, the report repeatedly finds few or no controlled studies showing an association between vitamin
D intake and disease.” Based on this assessment and speeches from several vitamin D researchers (including myself), IOM opted not to raise the daily recommended intake towards the high levels recommended by many top “experts” in the field. Instead, as we have, they called for a shift away from epidemiological observation towards more comprehensive studies and long-term results.

**Weaknesses**

Several aspects of this model require further validation. This includes data derived from *in silico* emulations, such as those that suggest that elevated 1,25-D can alter expression of thyroid beta and other nuclear receptors. To some extent, case studies of patients on our immunostimulative therapy offer validation of our model. Several reports demonstrate that thyroid, adrenal, and sex hormone levels return to range after 1,25-D levels drop. Yet these reports are not being collected in a controlled setting. Nor can we assume that these metabolite changes are due to the same mechanisms suggested by our model.

Another challenge lies in more accurately characterizing immunopathology. Physician reports indicate that the reaction is often extremely profound and sustained. Yet at the moment, what appears to be a dramatic change in inflammation and cell death is poorly detected by standard laboratory tests. While physicians have reported changes in markers such as CRP, white blood cell count, and “autoantibody” levels, such changes vary among patients and not in a reliable manner. With such inconsistencies in signs and symptoms of immunopathology, it is no wonder that the phenomenon has previously gone essentially unrecognized. Indeed the best biomarker that we have identified as an indicator of the dysregulated microbiota is higher than normal levels of 1,25-dihydroxyvitamin-D.
It is critical then that researchers identify new, unique biomarkers that would allow physicians to specifically track immunopathology. We anticipate that new immunopathology-specific markers might be identified in the metabolome or the proteome since the microbiota and the immune response both alter the body’s protein expression and microbial composition. Hopefully this would only require analysis of blood or urine, rather than the cerebrospinal fluid as in the Schutzer study\(^2\).

Assuming that immunopathology is indeed an inevitable response to immunostimulation, it is very important that we develop new ways to manage the reaction. At present, there are very few drugs that can temper immunopathology since the vast majority of common palliatives impair the immune response. If used too often, palliatives call into question the rationale for stimulating the innate immune response in the first place. That said, our case studies suggest that the best way to prevent severe immunopathology is to start patients on an immunostimulative treatment at an early stage in the disease process – before the microbiota becomes dominant.

**Challenges in testing**

Testing our pathogenesis/model is not as straightforward as it might seem. Testing and improving our model has presented us with many challenges. As explored in the section “Men are not large mice without tails”, in Chapter 5, there are a host of difficulties in trying to use an animal model to recreate a system in which our model might be accurately tested. The most obvious problem is that many pathways, including the VDR system, are different in mice and man (and even between higher primates and man). Thus, the manner in which the murine microbiota has evolved to persist in its host is undoubtedly different from the manner in which the human microbiota has evolved to persist. Also, to date, it has not been possible
to re-create the process of successive infection in mice in order to induce a human chronic disease. This means that although the symptoms of an “autoimmune” condition can sometimes be re-created in a mouse model, the pathogenesis or reason for the appearance of those symptoms is almost certainly different from the reason similar symptoms appear in *Homo sapiens*. However, despite these issues, some regulatory agencies will not give permission for *in vivo* studies to proceed in the absence of evidence generated from experimental animal models. This represents a serious barrier to the pace of scientific change.

*In vitro* research also presents certain difficulties. Researchers are accustomed to testing the effects of drugs on ready-made cell lines. However such cell lines do not account for the effects of an intracellular microbiota. Given that the microbiota varies between people, among people, and over time, it may be impossible to duplicate the microbiota, and its interaction with the human genome, in an *in vitro* setting. For example, we can try to test the effects of various compounds on VDR expression. However, *in vivo* the VDR is likely compromised by the microbiota leading towards inflammatory disease. If we are unable to duplicate these variables in the laboratory, is such an experiment even worth conducting?

In order to perform a successful *in vitro* study it is also essential that we understand the mark we plan to measure. There are currently 51 identified nuclear receptors and the function of most of them is still unknown. The VDR plays a critical role in the expression of numerous important genes, and yet mice that are VDR-null still survive. Clearly there are a great number of redundant functions in a mammal. There will remain a huge gap in our ability to understand the underlying mechanisms of clinical phenomena until more nuclear transcriptomes become available.
The optimal way to test our model is in an *in vivo* environment, yet working with human subjects presents its own set of obstacles. As mentioned in Chapter 6, we have found institutional review boards (IRBs) are often uncomfortable allowing patients to feel worse as a result of immunopathology. Several years ago we attempted to begin a clinical trial at West China Hospital (WCH) in Chengdu, China in an effort to test our immunostimulative model in the therapy of patients with ankylosing spondylitis. Although our clinical collaborators understood the need for patients to experience immunopathology, they were still generally uncomfortable. West China Hospital’s review board took the issue further, stating that patients on any treatment should not feel worse. We were told that every symptom of immunopathology would have to be palliated with a medicine that attempted to reverse the symptom. Furthermore, if any measure of the patient's blood work went out of range, even temporarily, the patient would be given a medication to bring the metabolite back into range, or possibly be excluded from the study.

We agree that the symptoms of immunopathology are very difficult to manage and that palliation will be necessary in some cases. However, treating the patient in this manner would result in the inevitable prescription of many medications with numerous confounding side effects. Our ability to observe the immune response to olmesartan most likely would have been greatly reduced. This intervention would then negate the utility and outcome of the study. Consequently we did not proceed with the WCH study. However, until the clinical community is ready to work within a new paradigm that does not require immunosuppression, pursuing such a study may be quite difficult.

In the meantime, we continue to gather case reports and series from our clinical collaborators. While some have argued that such anecdotal reports are of no value, we have
learned a great deal from them. For example, it is clear that the immunopathological reaction appears to be consistent across a wide variety of inflammatory diagnoses.

**Rethinking assumptions about the human microbiota**

Most physicians have little, if any, training in metagenomics. This makes our work very difficult, as physicians ultimately have to interpret the manner in which to optimally move our metagenomic model from bench to bedside. The Faculty of 1000 rated my 2009 paper "Autoimmune disease in the era of the metagenome" as a "must read":

*The clear message of this article is that molecular genetic studies of diseases must include analysis of resident bacterial genomes and not focus solely on the human genome. The observations presented in this review are thought-provoking and offer new ways of thinking about the origin of diseases and possible novel treatments.*

*The essence of this eye-opening article is that 90% of the cells in the human body are bacterial, that bacterial genomes are expressed in humans and that they have profound positive or negative influences on health. Compelling evidence is presented that humans are 'superorganisms' whose molecular functions result from the sum of interactions between the human genome and a multitude of microbial genomes. The advantages of bacterial colonization and its profound influence on the diversity of the human metagenome are emphasized. The human genome is composed of about 30,000 genes, while the endogenous microbial flora contribute millions of genes. In addition, transcription of the human genome is altered by the presence or absence of bacterial products. Differences in bacterial colonization account for individual variations*
in gene expression. Lastly, the potential pivotal role of endogenous microbial genomes on the induction and persistence of autoimmune disease is emphasized.

Even so, the clinical infectious disease expert, Mark Crislip, M.D., is on record as saying that the publication exaggerates the number of potential disease-causing pathogens capable of causing disease stating, “Of our thousand bacterial species, I only have to worry about a couple dozen.”

At the same time, researchers in the metagenomics community are not making a compelling case to clinicians explaining why their work should be taken seriously. Metagenomic findings that offer compelling new connections to disease progression and proliferation are overshadowed by assertions that the microbiota is largely helpful and innocuous. For example, in 2010 Gordon et al. decoded viral DNA in stool samples provided by four identical twin pairs and their mothers. They sequenced the DNA from stool samples collected at three different times over a one-year period. Amazingly, more than 80% of the viral genetic sequences found, which included sequences characteristic of both animal and bacterial viruses, had never been previously reported. When asked about the findings Gordon commented, "This is a largely unexplored world." He did not rule out the possibility that the viruses could, at least under certain conditions, contribute to disease processes. However the message the team communicated in the press release, and subsequently to the public, was very different. Despite the fact that the majority of these viruses had never been studied, they were repeatedly described as harmless and even beneficial. Yet Edward DeLong of MIT commented that, in oceans, the modality of viruses has tended to be predatory. In his opinion, Gordon’s work was interesting because the “faecal microbiota seems to be driven by prophages, which tend to basically integrate their genetic material into the host genome and hide there — it's a much more stable situation."
Our model allows us to interpret prophage action a little differently. The integration of viral DNA into a host genome offers significant potential for some pathogenicity and genomic instability, albeit this is more likely over the long term. It is certainly premature to assume stability when the study was only of one-year duration. Yet apparently, because subjects were not acutely ill at the time of the study, it was decided that their fecal viral loads would best be described as innocuous.

In our experience, researchers who stray from this tone may be accused of unnecessarily scaring the public. To some extent, metagenomic researchers are wise in avoiding the pitfalls of their colleagues who jumped to premature conclusions in studying the human genome. However, there is no need to swing to the other extreme and soft pedal the serious implications of many metagenomic discoveries.

**Final thoughts**

In the United States, the number of people with chronic diseases is projected to increase steadily for the next 30 years. Partnership for Solutions National Program Office estimates that it will reach 157 million by 2010. In 2004, almost half of all Americans, or 133 million people, lived with a chronic condition. At that time, people with chronic conditions accounted for 83 percent of health care spending, and those with five or more chronic conditions had an average of almost fifteen physician visits and filled over 50 prescriptions each year.\(^\text{12}\)

According to the World Health Organization, deaths from “noncommunicable” diseases are projected to increase by 15% globally between 2010 and 2020.\(^\text{13}\)
The rate of chronic health conditions among children in the United States increased from 12.8% in 1994 to 26.6% in 2006, particularly for asthma, obesity, and behavior and learning problems, according to results of a new prospective study published in the 2010 paper in *Journal of the American Medical Association*. One team concluded in a recent meta-analysis that if Americans keep gaining weight at the current rate, 75 percent of U.S. adults will be overweight and 41 percent obese by the year 2015.

Chronic disease prevalence is increasing at the same time that the theory of "autoimmunity" still guides our clinical approaches. Meanwhile, the majority of autoimmune researchers continue to look for answers to disease in the human genome alone. Rates of autoimmune disease are escalating as the metagenomic and autoimmune research communities continue to operate in virtual isolation. These trends persist as substances such as the secosteroid vitamin D are added to an increasing number of foods and supplements.

Consequently, there is an urgent need for the novel paradigms, including those in our model, to be further explored and tested. Ultimately, as we factor the microbiota and the metatranscriptome into our understanding of chronic disease we must make a choice. We can continue to use immunosuppressive substances that offer short-term palliation at the cost of long-term relapse. Or, we can move towards immunostimulatory approaches. While difficult in the short-term, these measures may improve long-term preventative and curative outcomes.

**References**


