Autoimmune disease re-examined in light of metagenomic concepts

Amy D. Proal

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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.

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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 co-morbidity 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.

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Abbreviations

1,25-D	1,25-dihydroxyvitamin-D
16S rRNA	16S ribosomal RNA
^{1}H NMR	proton NMR
25-D	25-hydroxyvitamin-D
ACE	angiotensin-converting enzyme
AMPs	antimicrobial peptides
ANA	antinuclear antibody
anti-dsDNA	anti- double-stranded deoxyribonucleic acid
anti-EBNA-1	anti-EBV nuclear antigen-1
anti-TTG	anti-tissue transglutaminase
AR	androgen receptor
ARA	American Rheumatism Association
ASCA	anti-Saccharomyces cerevisiae antibodies
B. anthracis	*Bacillus anthracis*
B. burgdorferi	*Borrelia burgdorferi*
B. cereus	*Bacillus cereus*
B. fragilis	*Bacteroides fragilis*
BAK1	BRI1-associated receptor kinase 1
BUN	blood urea nitrogen
C. elegans	*Caenorhabditis elegans*
CD	Crohn's disease
CDC	Centers for Disease Control
CFS	chronic fatigue syndrome
CHD	coronary heart disease
ChIP-seq	ChIP-Sequencing
CK	creatinine kinase
CMV	cytomegalovirus
CYP24	cytochrome P450C24
CYP24A1	cytochrome P450C24A1
CYP27A1	cytochrome P450 27A1
CYP27B1	25-Hydroxyvitamin D3 1-alpha-Hydroxylase
DDD	degenerative disc disease
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
EBV	Epstein–Barr virus
eGFR	estimated glomerular filtration rate
ERB,	estrogen receptor beta
ESR	erythrocyte sedimentation rate
GCR	glucocorticoid receptor
GFR	glomerular filtration rate
GWAS	genome-wide association study
H. hepaticus	*Helicobacter hepaticus*

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
Mtb	*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
PBPs	penicillin-binding proteins
PKA	protein kinase A
pmol/L	picomoles per Liter

PTN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
PXR	pregnane X receptor
RA	rheumatoid arthritis
RCTs	randomized controlled trials
RF	rheumatoid factor
RNA	ribonucleic acid
S. aureus	*Staphylococcus aureus*
SARS	severe acute respiratory syndrome
SLE	systemic lupus erythematosus
SNPs	single nucleotide polymorphisms
T3	triiodothyronine
T4	thyroxine
TACO	transfusion-associated circulatory overload
TBC	tonsil B cells
TLR	toll-like receptor
TLR2	toll-like-receptor 2
TNF-alpha	tumor necrosis factor-alpha
VDR	vitamin D receptor
µm	micrometer

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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

Proal AD, Albert PJ, Blaney GP, Lindseth IA, Benediktsson C, Marshall TG. Immunostimulation in the era of the metagenome. *Cellular & molecular immunology* 2011;8:213-25.

Proal AD, Albert PJ, Marshall TG. Autoimmune disease and the human metagenome. In: Nelson KE, ed. *Metagenomics of the Human Body*: Springer; 2010:231-75.

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Blaney GP, Albert PJ, Proal AD. Vitamin D metabolites as clinical markers in autoimmune and chronic disease. *Ann N Y Acad Sci* 2009;1173:384-90.

Albert PJ, Proal AD, Marshall TG. Vitamin D: the alternative hypothesis. *Autoimmun Rev* 2009;8:639-44.

Proal AD, Albert PJ, Marshall TG. 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;1173:252-9.

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.[1] 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

pathogens. 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.[2] 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."[3] 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

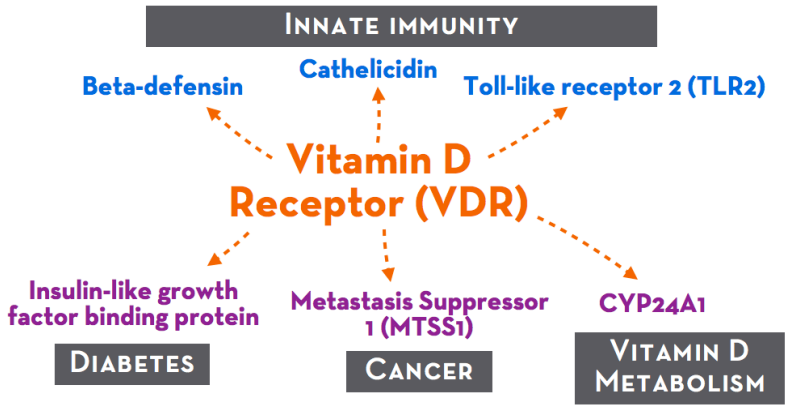

Figure 1. Select proteins transcribed by the Vitamin D

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.

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