DNA METHYLATION PATTERNS AS A BIOMARKER OF DISEASE RELAPSE AND REMISSION IN PATIENTS WITH ANCA-ASSOCIATED VASCULITIS

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ABSTRACT

Britta Elyse Jones: DNA Methylation Patterns as a Biomarker of Disease Relapse and Remission in Patients with ANCA-Associated Vasculitis
(Under the direction of Ronald J. Falk)

This dissertation is focused on the role of DNA methylation in anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV), an autoimmune condition characterized by vascular inflammation and organ damage. Pharmacologically induced remission is complicated by relapses. Potential triggers of relapse are immunological challenges and environmental insults, both of which are associated with changes in epigenetic silencing modifications. Alterations in histone modifications implicated in gene silencing are associated with aberrant autoantigen expression.

In Chapter 1 I establish a link between DNA methylation, a model epigenetic gene silencing modification, and autoantigen gene expression and disease status in AAV, by measuring gene-specific DNA methylation of the autoantigen genes, myeloperoxidase (*MPO*) and proteinase 3 (*PRTN3*), in leukocytes of AAV patients followed longitudinally (n=82) and healthy controls (n=32). Patients with active disease demonstrated hypomethylation of *MPO* and *PRTN3* and increased expression of the autoantigens; in remission DNA methylation generally increased. Longitudinal analysis divided AAV patients into two groups based on whether DNA methylation increased or decreased from active disease to remission. In patients with increased DNA methylation, *MPO* and *PRTN3* expression correlated with DNA methylation. Kaplan-

Meier estimate of relapse revealed patients who increased DNA methylation at the *PRTN3* promoter had a significantly greater probability of a relapse-free period, independent of ANCA serotype. Patients with decreased DNA methylation at the *PRTN3* promoter were more likely to relapse with a hazard ratio of 4.55. Changes in the DNA methylation status of the *PRTN3* promoter predict likelihood of stable remission and may explain autoantigen gene regulation.

Chapter 2 focuses on gene-specific DNA methylation patterns of purified neutrophils and CD14+ monocytes isolated from patients with AAV. I measured DNA methylation at *MPO* and *PRTN3* along with the mRNA expression of those genes and found evidence that DNA methylation in monocytes may be contributing to the altered methylation patterns seen in Chapter 1 from total leukocytes while altered expression of autoantigen genes in neutrophils may, instead, be impacted by histone modifications. Efforts to isolate T cells uncovered a CD3- CD4- fraction of cells in our CD4-enriched isolation from active patients exhibiting high autoantigen gene expression which may further contribute to the disease etiology of AAV.

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Ralph Waldo Emerson is quoted as saying “nothing great was ever achieved without enthusiasm”. It is an understatement to say that everything I have accomplished in graduate school came through the grace of my own enthusiasm and the enthusiasm others had in teaching, and guiding me. It took more than a village to get me to the point of writing this document; indeed, it took more than one department in the School of Medicine. I would be remiss if I didn’t acknowledge the scientists, athletes and people who have made this journey a triumph of quality over quantity.

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My life has been charmed by the opportunity to grow up close to three of my grandparents--they have, collectively, taught me the joys of dry ice and liquid nitrogen, the importance of using my voice to advocate for myself and others, and the value of good posture and a nice pair of shoes. My brothers are possibly the only people who know what traveling around the world, twice, as a member of the Jones clan feels like. I am eternally grateful to be in a family where normal looks so boring and even more grateful to have their support as a young

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While it is entirely possible that the first year I competed in the local science fair I made my life and the lives of those around me beyond miserable, I am grateful that my parents did not abandon me in the pick-up lane of my middle school one afternoon as I often feared. Indeed, they stuck with me and my budding love of all things weather-related and fostered in me a deep appreciation for how things work and how much in the world around us is a puzzle waiting for someone to sit down and piece together. My father often speaks of the 'wall of science' and how each of us contributes our findings, brick by brick as we work to fill in the gaps. I hope that this dissertation is one of many bricks I have the opportunity to contribute—not only by sheer mass but also by scientific value.

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James Watson once said, "the major problem, I think, is chromatin…you can inherit something beyond the DNA sequence. That's where the real excitement of genetics is now".

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AAV	anti-neutrophil cytoplasmic autoantibody associated vasculitis
AP-1	activated protein 1, transcription factor
ANA	antinuclear antibodies
ANCA	anti-neutrophil cytoplasmic autoantibody
BPI	bactericidal/permeability-increasing protein
BVAS	Birmingham vasculitis activity score
c-ANCA	cytoplasmic-anti-neutrophil cytoplasmic autoantibody
CF	cystic fibrosis
CGI	CpG island
CS	corticosteroid
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
EGPA	eosinophilic granulomatosis with polyangiitis
ELANE	elastase
ELISA	enzyme-linked immuno-absorbent assay

EWAS	epigenome-wide association studies
Foxp3	transcription factor forkhead box P3
FCR	fragment, crystallizable receptor
G-CSF	granulocyte colony-stimulating factor
GPA	granulomatosis with polyangiitis
H3K27me3	histone 3 lysine 27 trimethylation
HC	healthy control
HLA-DR	human leukocyte antigen, antigen D related
HMR	hypomethylated region
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
IL-8	interleukin 8
Lim	renal limited necrotizing and crescentic glomerulonephritis
LTF	lactotransferrin
MALDI-TOF	matrix assisted laser desorption/ionization time of flight
MHC	major histocompatibility complex
MPA	microscopic polyangiitis

MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NF-AT	transcription factor, nuclear factor of activated T cells
p-ANCA	perinuclear-anti-neutrophil cytoplasmic autoantibody
PBMC	peripheral blood mononuclear cell
PMN	polymorphonuclear cell
PR3	proteinase 3
RA	rheumatoid arthritis
RT-PCR	real time polymerase chain reaction
SLE	systemic lupus erythematosus
TCR	T cell receptor
TET	ten-eleven translocation family enzymes
Treg	regulatory T cell
USF	upstream stimulatory factor
WBC	white blood cell

PROLOGUE: DNA METHYLATION IN AUTOIMMUNE DISEASE

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a systemic autoimmune condition characterized by vascular inflammation and organ damage. Observations from *in vitro* and experimental animal models indicate a pathogenic role for ANCA, which can activate neutrophils and monocytes, causing destructive necrotizing vascular inflammation (*1*). The necrotizing vasculidities associated with ANCA include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA) and renal limited necrotizing and crescentic glomerulonephritis (*2*). The etiology and pathogenesis of AAV are influenced by genetic factors, environmental exposures, infections and characteristics of the innate and adaptive immune system. ANCA target antigens in the cytoplasm of neutrophils and monocytes. Myeloperoxidase (MPO) and proteinase 3 (PR3) are two prominent autoantigen targets of ANCA; it is the interaction between ANCA and target autoantigens that induces AAV.

AAV is characterized by therapy-induced disease remission, which may be punctuated by periods of disease relapse (*3*-*7*). Relapses can be triggered by immunological challenges and environmental insults, both of which are associated with changes in epigenetic silencing modifications. Specific factors that promote remission or permit disease relapse are unknown; however, because expression of autoantigen genes is elevated during active disease, clues to disease states emerge from understanding mechanisms regulating autoantigen expression (*8*). Recent research on transcriptional dysregulation from our laboratory found increased autoantigen

mRNA that coincided with *de novo* protein synthesis of MPO and PR3 in neutrophils (*9*). Our group previously proposed a model wherein these normally silenced genes are upregulated as a consequence of reduction in the histone modification histone H3 lysine 27 trimethylation (H3K27) (*10*). This modification is associated with DNA methylation, raising the possibility of combinatorial mechanisms responsible for autoantigen gene silencing. Thus, there is reason to believe that altered epigenetic modifications have the ability to impact the pathogenesis of AAV.

When Conrad Waddington first postulated that environmental signals might have the ability to modify genes and determine the fate of a single cell, he thought an additional layer of information must exist around or above the genome—the epigenome (*11*). Genes had yet to be visualized at this point in the early 20th century, thus this postulation and the accompanying concept of epigenetics were cast aside in favor of more testable hypotheses in a field soon to become developmental biology. It was not until 1975 that DNA methylation was explored as a potential epigenetic regulator (*12, 13*). By the late 20th century, epigenetics came to describe modifications of the DNA or associated proteins that carry information during cell division, exclusive of DNA sequence variation (*14*). Today, the methylation of the fifth position of cytosine is one of the most mechanistically understood epigenetic modifications (*15*). DNA methylation patterns are established and modified in response to environmental factors by three main DNA methyltransferases (DNMTs); the loss of any one of these enzymes is lethal in mice (*16*).

The field of epigenetics, which includes the study of DNA methylation, holds the potential to explain mechanisms involved in aging, human development, cancer, heart disease mental illness and autoimmunity. Epigenetics is now considered to be at the "epicenter of modern medicine" by some investigators who believe the field may ultimately play a greater role

in disease than genetics (*17*). Just as Conrad Waddington first theorized, scientists continue to study epigenetics in an effort to better explain the relationship between the genome and the environment. Inclusion of epigenetics in studies of the etiology of human diseases is thought to uncover modifications useful in disease prevention and therapy. Already, DNA methyltransferase inhibitors are being used in the treatment of hematologic cancers; demonstrating a combination of immune-checkpoint-inhibitor agents and epigenetic modulators (*18, 19*). Unlike sequence mutations, epigenetic changes are, by definition, reversible. Thus, the greatest promise of epigenetics in the field of medicine lies in the possibility of new therapies.

Chromatin modifications

Chromatin is made up of DNA and proteins that form chromosomes in the nucleus of the cell. There are two types of chromatin modifications: histone modifications and ATP-dependent chromatin remodeling complexes. A nucleosome is comprised of about 200 base pairs of DNA wrapped around a core of histone proteins, which can be chemically modified. Covalent modifications by specific enzymes to the histone proteins allow transcriptional regulatory proteins access to condensed genomic DNA to alter gene expression. Proteins work with transcription factors in activating or silencing genes by acetylation or deacetylation of histones, respectively (*17*). ATP-dependent chromatin remodeling complexes regulate gene expression by moving or restructuring nucleosomes (*20*). Additionally, the density of nucleosome packing along DNA can also influence epigenetic changes and impact gene expression. The mechanisms of maintaining chromatin modifications during cell division are more complicated than for maintaining DNA methylation (*21, 22*).

In response to environmental factors, DNA methylation patterns are established and modified by a complex interplay of DNMT1, 3A and 3B that catalyze the transfer of methyl groups to DNA. DNA methyltransferases can be divided into maintenance methyltransferases (DNMT1) and *de novo* methyltransferases (DNMT3A and 3B). During DNA replication, DNMT1 propagates symmetrically methylated CpGs through recognition of the nascent strand opposite a previously methylated position. DNMT1 functions throughout the life of an organism to maintain the methylation pattern established by the *de novo* methyltransferases. DNMT1 can accumulate errors over successive rounds of cell division which can lead to aberrant expression of previously silenced genes (*23*).

De novo methyltransferases, DNMT3A and DNMT3B, add methyl groups to previously unmethylated CpGs and are known to be active during germ cell development and early embryogenesis, when DNA marks are re-established after phases of genome demethylation (*24*). Thus, both DNMT3A and 3B are capable of mediating methylation-independent gene repression. With regard to disease etiology, the replication of DNA methylation patterns during mitosis is sensitive to the environment.

DNA methylation

Epigenetic changes have profound effects on gene expression by modifying the accessibility of DNA to transcription factors (*25*). In mammals, cytosine methylation is restricted to the symmetrical CpG context (*26, 27*). Compared to other epigenetic modifications, CpG methylation is thought to be relatively stable; but it can be affected by environmental changes as well as genetic mutations, leading to epigenetic instability and disease. Three conserved enzymes: DNMT1, 3A and 3B are responsible for the deposition and maintenance of methyl

groups that are essential for normal development (*28, 29*). Of the 28 million CpGs in the mammalian genome, 60-80% are methylated. Clusters of CpGs averaging 1,000 base pairs long and characterized by elevated cytosine and guanine base composition are referred to as CpG islands (CGIs). CGIs comprise fewer than 1% of total genomic DNA (*30*), yet these CGIs are present at transcription start sites of more than half of all human genes (*31, 32*). Despite growing interest in the study of changes in DNA methylation, the vast majority of genomic methylation patterns are relatively static across tissues and throughout life; exceptions include the germ line and pre-implantation development.

In contrast to the rest of the genome, where CpG dinucleotides are heavily methylated and rapidly lost through deamination, CpG sites within CGI promoters are normally free from DNA methylation and do not have an elevated mutation rate (*32, 33*). These genes show differences in their patterns of transcription initiation and are reported to have higher levels of activation-associated chromatin modifications. CGI promoters are not usually repressed by DNA methylation; instead they are silenced by histone (H3K27) methylation (*34, 35*). Genes with CGI promoters have a characteristic transcription-associated chromatin organization. Active genes with CGI promoters have a distinct step-like series of modified nucleosomes after the transcription start site (*36*). The maintenance of an unmethylated state at a promoter overlapping with a CGI requires DNMTs to be excluded; a maintenance that is heavily influenced by transcription factor binding. CGIs can accrue heritable methylation if they are truncated or depleted of known transcription factor binding sites (*37, 38*). Transfer of a specificity protein 1 binding site into an endogenously methylated locus induces local demethylation, confirming dominance of transcription factor binding over DNA methylation (*37*). Histone modifications, variants and nucleosome positioning work alongside DNA methylation to regulate gene

expression by modifying the accessibility of promoter regions to transcription machinery (*39, 40*).

Forty-five percent of all human gene promoters do not contain a sufficiently dense population of CpGs to constitute CGIs, yet the role of DNA methylation in the control of non-CGI promoters in normal and pathological processes is not fully understood (*41*). The regulation of non-CGI promoters by DNA methylation plays an important role in the establishment and maintenance of cell identity (*42*). Despite their low CpG density, genes with non-CGI promoters share many epigenetic features also associated with CGI promoter genes. Active non-CGI promoters display a nucleosome-depleted region immediately upstream of the transcription start site. The epigenetic signatures comprising DNA methylation, histone marks and nucleosome occupancy of non-CGI promoters are almost identical to CGI promoters. This suggests that aberrant methylation patterns of non-CGI promoters may also impact altered gene expression, contributing to disease processes such as tumorigenesis and autoimmunity (*14, 23, 43*).

Both of the autoantigen genes, *MPO* and *PRTN3*, are considered to have non-CGI promoters. Proteinase 3 contains enough CpGs in the promoter to allow for quantification of DNA methylation in the promoter as well as in the three CGIs across the gene body. Myeloperoxidase contains two CGIs in the gene body and fewer than 6 CpGs in the promoter. Previous studies measuring DNA methylation at CGIs that are remote from annotated transcription start site showed evidence of promoter function, indicating strong correlation between these CGIs and transcription initiation (*44, 45*). The CGIs at intragenic regions are more frequently methylated during development and may contribute more nuanced regulatory functions (*46*).

Quantification of DNA methylation is crucial for understanding the roles gene expression and silencing play in the development of autoimmunity. This quantification was made more accessible with the development of a technique wherein genomic DNA is treated with sodium bisulfite, which converts unmethylated cytosines to uracil and leaves methylated cytosines unchanged (*47*). The bisulfite conversion method is the basis of many platforms used to measure the amount of CpG methylation from genome-wide to gene-specific interrogation (*48*).

Tools for measuring DNA methylation can be divided into genome-wide platforms and targeted or gene-specific platforms. Genome-wide methylation studies are increasingly being referred to as epigenome-wide association studies (EWAS) and include a number of different microarray-based methods. Following bisulfite conversion of genomic DNA, the Illumina Infinium methylation assay uses two site-specific probes for the methylated and unmethylated loci followed by single-base extension of the probes to incorporate a fluorescently-labeled ddNTP; the ratio of fluorescent signals from methylated and unmethylated sites determines the level of DNA methylation at close to 500,000 CpG sites (*49*). High-throughput sequencing now enables complete methylomes to be elucidated in a variety of cells and tissues (*50*). One of the most commonly used platforms for targeted quantification of DNA methylation is bisulfite sequencing in which primers are designed around regions of interest containing multiple CpG dinucleotides (*51*). EWAS techniques are not always the most cost-effective for local DNA methylation studies. In this body of work I utilized the EpiTYPER® DNA methylation analysis technology (Agena Bioscience) which allows for the design of amplicons up to 600 base pairs in length and interrogating ten to hundreds of CpGs. After bisulfite-conversion of genomic DNA, primers are used to amplify the target regions (amplicons). Treatment with reverse primers containing the T7 promoter tag allows for *in vitro* RNA transcription followed by base-specific RNA cleavage.

Cleavage products derived from a template with a methylated cytosine will differ from products derived from a template with an unmethylated cytosine by 32 Daltons. This difference in cleavage products is detected using matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, and the ratio of fragments determines the percent methylation.

DNA methylation in autoimmune diseases

In essence, autoimmunity is the loss of self tolerance and is associated with a number of risk factors. It is thought to develop when genetically predisposed individuals encounter environmental agents that trigger a disease. Despite the many years since Dr. Waddington's initial hypothesis relating the environment to gene modifications, researchers continue to believe that interactions between the genetic elements and epigenetic changes caused by environmental agents may be responsible for inducing autoimmune disease. The list of genetic loci with the ability to predispose an individual to certain autoimmune conditions continues to grow; yet alongside that growing list is the evidence that genetic sequence mutations do not explain the entirety of autoimmune disease development. Autoimmune disease concordance in identical twins is often incomplete, indicating a requirement for additional factors, presumably from the environment (*52, 53*). Environmentally-induced epigenetic changes, particularly DNA methylation, have already been shown to contribute to the environment-host interaction in some autoimmune diseases (*54*).

Systemic lupus erythematosus (SLE) is one of the most thoroughly researched autoimmune diseases; some form of lupus erythematosus is estimated to affect five million people worldwide. There is evidence that impaired T cell methylation occurs in SLE patients

along with the knowledge that DNA demethylating drugs can cause a lupus-like disease. Specifically, procainamide and hydralazine inhibit DNA methylation and are known to cause antinuclear antibodies (ANAs) in most people and a lupus-like disease in a genetically pre-disposed subset (*55, 56*). Hydralazine can also cause systemic vasculitis and for this reason, patients treated with hydralazine have been excluded from these studies (*57*).

DNA demethylation has been shown to predict changes in T cell gene expression, contributing to the pathogenesis of lupus. T cells from SLE patients with active disease have hypomethylated DNA due to decreased DNMT1 (*58-60*). The demethylation of CD4+ T cells in SLE patients can cause increased expression of CD11a and CD70 (*61, 62*). CD11a expression leads to T cell autoreactivity while CD70 expression stimulates B cells to produce autoantibodies. Additionally, defects acquired in the T cell extracellular signal-regulated kinases (ERK) pathway are sufficient to cause lupus-like autoimmunity, likely through the down regulation of DNMTs in T cells (*63*). Together, these and other studies demonstrate that demethylation in CD4+ T cells is sufficient to cause autoimmunity (*23*).

There is altered *DNMT1* mRNA expression in SLE, rheumatoid arthritis (RA) and multiple sclerosis (MS) (*64*). While significantly less is understood about the role of epigenetics in RA and MS, there is evidence that altered DNA methylation occurs in both of these autoimmune diseases as well as AAV. Rheumatoid arthritis is believed to be driven by a T cell response to an environmental trigger and studies have shown aberrant gene expression in synovial fibroblasts that lack genetic mutations, suggesting epigenetic mechanisms may play a role in the disease etiology (*65*). Additionally, synovial fibroblasts isolated from RA patients have demethylated DNA along with increased expression of repetitive DNA elements normally

silenced by methylation (*66*); this demethylation may lead to the generation of auto-reactive T and B cell clones, similar to those seen in lupus (*58*).

Multiple sclerosis is a chronic neurodegenerative autoimmune disease caused by a combination of genetic and environmental factors. Studies of individuals with comparable genetic backgrounds but living in different geographic regions revealed significant differences in disease prevalence, suggesting a role for epigenetics in MS (*67*). The inflammation and demyelination in relapsing-remitting MS may be related to the increased differentiation of T cells toward a T-helper 17 phenotype, an epigenetically regulated pathophysiological mechanism. There may also be a role for increased histone acetylation in the exacerbation of progressive MS (*68*).

Lupus, RA and MS are all autoimmune diseases with both a genetic and environmental component; while our understanding of the genetic mutations that impact disease prevalence is rapidly growing, our understanding of how the environment contributes to disease development is largely unknown. Added to this list of autoimmune diseases is ANCA-associated vasculitis, a systemic autoimmune disease characterized by episodes of destructive vascular and extravascular inflammation (*69-73*). Aberrantly elevated autoantigen expression suggests a critical factor in AAV is the dysregulation of autoantigens, possibly due to epigenetic modifications at *MPO* and *PRTN3* (the gene that encodes PR3) (*8, 74-76*). Thus, both ANCAs and autoantigen expression are important for the development of AAV.

In normal mature neutrophils, the autoantigens are stored in cytoplasmic granules and their expression is dramatically reduced or completely silenced. Two well-known epigenetic modifications capable of inducing gene silencing are histone 3 lysine 27 trimethylation (H3K27me3) and DNA methylation (*77-79*). The relationship between H3K27me3, Polycomb

Repressive Complex 2, the H3K27 methyltransferase, and DNA methylation is complex including evidence in stem cells and cancer for combinatorial associations between H3K27me3 and DNA methylation (*34, 80, 81*). The dysregulation of *MPO* and *PRTN3* in patients with active AAV has been linked to reduced H3K27me3 (*10*). Whether DNA methylation regulates *MPO* and *PRTN3* expression has not been investigated in the context of AAV.

Immune cell types in AAV

MPO and PR3 are the two most prominent target antigens for ANCA in AAV and are found in the granules of neutrophils and the lysosomes of monocytes. The genes encoding the autoantigens, *MPO* and *PRTN3,* are silenced in normal mature neutrophils and monocytes, but aberrantly expressed in patients with AAV. Clinical evidence along with both *in vitro* and *in vivo* studies suggest that neutrophils are an important effector cell in the pathogenesis of AAV (*2, 82*). Activated neutrophils have been identified in kidney glomeruli taken from renal biopsies of AAV patients. Additionally, the number of activated intraglomerular neutrophils correlates with the severity of renal injury, as inferred from serum creatinine levels (*83*). *In vitro* studies have shown ANCA can activate cytokine-primed neutrophils and cause degranulation, the release of inflammatory cytokines and damage to the surrounding endothelial cells (*69, 84-86*). Neutrophils comprise a majority (45-65%) of total leukocytes in the peripheral blood of healthy individuals. Infection, disease and pharmaceutical therapy can impact the percentage of neutrophils in the peripheral blood. Patients with AAV can have fluctuating neutrophil counts in their peripheral blood due, in part, to the use of therapies, like corticosteroids, which can impact neutrophil count (*87*).

Primed neutrophils activated by ANCA can initiate an inflammatory amplification loop and cause destructive necrotizing lesions. Acute injury then initiates a response to the injury by monocytes that transform to macrophages and recruit T cells, which are key to creating granulomatous inflammation (*88*). Monocytes can also be activated by ANCA, leading to the production of proinflammatory cytokines that can further attract and activate neutrophils, amplifying tissue injury (*89-91*). Murine studies have found that monocytes alone are not sufficient to cause acute necrotizing lesions (*92*).

T cells are another major cell type involved in the pathogenesis of AAV; the dysregulation of T cells can permit B cells and plasma cells to produce autoantibodies. T cells are critically involved in the genesis of the ANCA autoimmune response by inducing both a pathogenic autoantibody response via B cells as well as through ineffective suppression of the autoimmune ANCA response by regulatory T cells (*93*). Our research group has recently observed disruption of the suppressive regulatory T cell network in patients with AAV along with an increase in the frequency of a distinct proinflammatory effector T cell subset (*94*). Murine studies have shown neutrophils activated by ANCA can deposit MPO in glomeruli, thereby involving autoreactive anti-MPO CD4+ T cells in the induction of glomerular lesions (*95*).

In general, lymphoid cells depend on DNA methylation to be able to proliferate and respond to extracellular signals. Conditional Dnmt1-knockout in naïve B and T cells hinders their proliferative capacity (*96-98*). DNMT1 is likely more important in immune cell regulation than during erythropoiesis. Dnmt1-null naïve CD4+ T cells upregulate cytokines normally methylated and silenced, while Dnmt1-null CD8+ T cells upregulate CD4+ T cell cytokines (*98, 99*).

There is growing interest in characterizing the role of immune cell subsets involved in the pathogenesis of autoimmune disorders such as SLE, RA, MS and AAV (*100*). Although, the dynamic cell heterogeneity present in this patient population suggests purified cell populations would be a more accurate basis for understanding disease pathogenesis and etiology, there is likely much to be learned from examining DNA methylation in total leukocytes, especially since a careful study has not been reported in AAV.

Central hypothesis

The role of epigenetic modifications in the pathogenesis of autoimmune diseases is poorly understood compared to genetics; however, a number of studies in the past decade have further characterized aberrations in DNA methylation and histone modifications in autoimmune diseases including SLE, RA, MS and AAV. The central hypothesis of this body of work is that altered DNA methylation profiles exist in patients with AAV and contribute to the pathogenesis of this disease. This body of work incorporates two main sub-hypotheses: at loci within *MPO* and *PRTN3* i) changes in leukocyte DNA methylation exist between healthy controls and patients with AAV and correlate with *MPO* and *PRTN3* mRNA expression ii) DNA methylation patterns in purified neutrophils and CD14+ monocytes differ between healthy individuals and patients with AAV.

The central hypothesis and sub-hypotheses will be addressed in the following two chapters. In Chapter 1, I establish a link between DNA methylation, autoantigen gene expression and disease status in AAV by measuring gene-specific DNA methylation in leukocytes of the autoantigen genes, *MPO* and *PRTN3*. Epigenome-wide DNA methylation is measured in both AAV patients and healthy controls; differential methylation is found to be specific to a small

fraction of the genes interrogated. I find differential expression of DNMT1 in patients with AAV. I also find gene-specific DNA methylation associates with *MPO* and *PRTN3* mRNA expression in this patient cohort. In Chapter 2, I focus on which cell populations are responsible for *MPO* and *PRTN3* mRNA expression. I isolated neutrophils and CD14+ monocytes from patients with AAV and found differential DNA methylation and mRNA expression at loci within *MPO* and *PRTN3*. In an effort to purify CD4+ T cells from AAV patients, I discovered a sub-population of cells within the CD4-enriched isolation that are capable of expressing both autoantigen genes in patients with active disease. The search for the specific cell type responsible for the expression of *MPO* and *PRTN3* is ongoing but I have confirmed it is not a progenitor cell and that it may contain either or both CD45 and HLA-DR on the surface.

Chapter 1

CHANGES IN GENE-SPECIFIC DNA METHYLATION PREDICT STABLE REMISSION IN PATIENTS WITH ANCA-ASSOCIATED VASCULITIS[1]

Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCAs) target antigens in the cytoplasm of neutrophils and monocytes. Two prominent ANCA autoantigens are myeloperoxidase (MPO) and proteinase 3 (PR3). The interaction between ANCA and target autoantigens induces ANCA-associated vasculitis (AAV), a systemic autoimmune disease characterized by episodes of destructive vascular and extravascular inflammation (*69-71*). AAV is associated with aberrantly elevated expression of target autoantigens (*8, 74-76*). In normal mature neutrophils, the autoantigens are stored in cytoplasmic granules and their expression is dramatically reduced or completely silenced. The elevated autoantigen expression observed specifically in patients with AAV suggests that a critical factor in the disease is the dysregulation of autoantigens. Thus, both ANCAs and autoantigen expression are important for the development of AAV.

Another prominent feature of AAV is that therapy-induced disease remission may be punctuated by periods of disease relapse (*3-7*). It remains to be understood what factors promote remission or permit disease relapse; however, the elevated expression of autoantigens during active disease suggests mechanisms regulating autoantigen expression may be important in

[1]This chapter consists of material from a manuscript submitted to the *Journal of the American Society of Nephrology* May 2016.

understanding disease states (*8*). At the transcriptional level, the dysregulation of *MPO* and *PRTN3* (the gene that encodes PR3) has been linked to alterations in histone modifications that are associated with gene silencing (*10*). The role of DNA methylation, a model epigenetic modification capable of inducing stable gene silencing, has not been investigated in the context of AAV (*77, 78*). Hence, the role of gene-specific DNA methylation in the regulation of *MPO* and *PRTN3* expression during the natural history of AAV is not known.

I do not presently know whether DNA methylation changes during the course of disease or remains static, yet different from healthy individuals. The presence of static hypomethylation in AAV patients would be consistent with the concept that an epigenetic state predisposes individuals to disease. Alternatively, dynamic alterations in the DNA methylation profile that occur with changes in disease status may indicate a role for epigenetic modifications in disease status. Characterization of DNA methylation in AAV patients would bolster the understanding of the molecular mechanisms involved in disease pathogenesis and inform disease prognosis.

In this study I investigated DNA methylation associated with AAV-related autoantigen genes, *MPO* and *PRTN3,* in patients with AAV. A longitudinal analysis showed that (i) *MPO* and *PRTN3* DNA methylation was reduced in patients with active disease and associated with mRNA expression of these genes and (ii) *MPO* and *PRTN3* DNA methylation increased during disease remission and associated with reduced transcription of these genes. The dynamics of DNA methylation at the *PRTN3* promoter revealed AAV patients with increased DNA methylation during disease remission at the *PRTN3* promoter had an increased probability of a relapse-free period ($p<0.0001$). Patients with decreased DNA methylation at the *PRTN3* promoter were 4.55 times more likely to relapse ($p=0.0001$); this suggests changes in DNA methylation at the *PRTN3* promoter can predict long-term prognosis for AAV patients.

Materials and Methods

Study Design

The objective of this study was to characterize the DNA methylation patterns in paired patients with AAV through states of disease activity and remission. This was an observational study of DNA methylation changes, both globally and at specific loci. AAV patients were enrolled at UNC–Chapel Hill clinics and followed in the Glomerular Disease Collaborative Network (*101, 102*). Patients and healthy volunteers were recruited, according to the guidelines of the Institutional Review Board (IRB study #97-0523) by the University of North Carolina Office of Human Research Ethics. Study subjects gave informed, written consent and participated according to UNC IRB guidelines. I stopped collecting patient samples once I achieved a statistically significant difference between active patients and healthy controls and a statistically significant hazard ratio for patients with decreased DNA methylation at the *PRTN3* promoter. I used a power analysis to calculate the sample size (n=100) necessary to achieve a reliable measurement of DNA methylation changes in patients with AAV. Preliminary data from a smaller sample size was used to recalculate our power analysis and change our sample size to 80 unique patients. De-identified patient and healthy control samples were assigned randomly to plates for DNA methylation analysis and run in duplicate on separate plates. Paired samples from the same patient were rarely run on the same plate or in the same batch. Generally, samples were processed in the order that they were retrieved from the freezer or the order in which the patients presented at clinic. Investigators who quantified the results were blinded with regard to the type of patient or control being analyzed.

Patient Cohort

Patients were diagnosed according to the Chapel Hill Consensus Conference (*103, 104*). ANCA serotypes were determined by indirect immunofluorescence and antigen-specific PR3 and MPO enzyme-linked immune-absorbent assays (ELISA) (*105*). Disease activity was determined by the 2003 Birmingham Vasculitis Activity Score (BVAS) in conjunction with clinical signs of activity. In this study, patients with a BVAS of 0 and no clinical or laboratory evidence of active disease were considered to be in remission. Active disease was defined as a BVAS >0 with clinical and/or laboratory evidence of disease (*106*). A total of 82 patients with AAV and 32 healthy controls were chosen for this study based on the availability of paired active/remitting disease samples, clinical data and laboratory data (Table 1.1). Patients with suspected or confirmed drug-induced forms of AAV, that were ANCA negative by ELISA, or had overlapping disease were excluded. Patients taking known epigenetic modifiers were also excluded from this study. Patient demographics were similar between healthy controls and AAV patients with regard to age, gender and race.

AAV patients were selected for this study based on the availability of total leukocyte DNA and RNA collected at a point of clear disease activity or disease remission (on or off therapy). In addition to the presence of DNA and RNA samples for each patient, I also ensured there was adequate clinical information including BVAS and a list of immunosuppressant therapies the patient was taking at the time of each sample collection. For longitudinal studies I selected patients for whom samples were available at a time of disease activity and remission and were, on average, 18 months apart. These criteria were established prospectively. No outliers have been excluded from this study.

Sample	Age	Sex	Race	mRNA Expression

				PRTN3	*MPO*	*DNMT1*
1	47	M	W	NA	NA	NA
2	57	M	W	16.05	105.72	1.22
3	52	M	W	NA	NA	NA
4	66	F	A	NA	NA	NA
5	52	F	W	NA	NA	NA
6	22	F	W	2.09	33.12	1.84
7	56	F	W	28.29	66.92	0.75
8	51	F	W	NA	NA	NA
9	59	F	W	NA	NA	NA
10	69	M	W	19.56	66.98	0.84
11	67	M	W	1.85	38.93	1.21
12	65	M	W	51.72	102.3	0.81
13	51	F	W	28.64	29.89	1.18
14	59	F	B	9.69	33.14	0.71
15	58	F	W	NA	NA	NA
16	57	F	W	5.56	38.14	0.97
17	57	F	W	14.79	77.65	0.9
18	54	M	W	45.4	118.73	1.49
19	22	M	A	NA	NA	NA
20	22	M	W	8.07	25.14	0.79
21	58	F	W	11.29	42.73	0.87
22	26	M	B	2.46	25.03	1.03
23	22	M	A	NA	NA	NA
24	58	F	W	26.67	24.77	0.7
25	56	F	W	1.81	28.46	0.86
26	77	M	W	15.06	34.61	0.84
27	69	M	W	13.74	58.12	0.56
28	56	M	W	10.71	35.89	NA
29	64	F	W	NA	NA	NA
30	56	M	W	33.21	175.38	0.76
31	47	M	W	50.53	156.82	0.92
32	57	F	W	NA	NA	0.85

Table 1.1. Healthy individual demographics. Age, gender, race and mRNA expression of *PRTN3*, *MPO* and *DNMT1* for each of 32 healthy controls.

RNA and DNA isolation from total leukocytes

Total circulating leukocyte RNA was obtained from EDTA-treated whole blood using RNA STAT-60 (Tel-Test "B", Friendswood, TX, USA). Qiagen reagents (Chatsworth, CA)

including the RNeasy Mini Kit and RNase-Free DNase Set, were used to isolate RNA from total leukocytes. Sodium citrate-treated whole blood was used to isolate DNA from total leukocytes. For DNA isolation I used Cell Lysis Solution, Protein Precipitation Solution, DNA Hydration Solution (all Puregene Accessories, available through Qiagen) and RNase A from bovine pancreas (Sigma-Aldrich, St. Louis, MO).

DNA methylation studies

Total leukocyte DNA was bisulfite-converted in duplicate using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Bisulfite-treated DNA samples were used in three separate platforms for measuring DNA methylation: EpiTyper MassARRAY (Agena, La Jolla, CA), bisulfite sequencing (Zymo Research) and Illumina Infinium HumanMethylation450 BeadChip (Illumina, Inc.).

Targeted MALDI-TOF mass spectrometry (EpiTYPER®, Agena Bioscience) was carried out at seven amplicons within *MPO*, *PRTN3*, *LTF* and *ELANE* (Figure 1.1B,C; Figure 1.2). Primer pairs were designed using EpiDesigner software (www.epidesigner.com) (Table 1.2). A cohort of 82 AAV patients and 32 healthy individuals were run on this platform, in duplicate. In accordance with the standard protocol and following amplification of 650ng of bisulfite-converted DNA, in duplicate, the PCR products underwent the SAP treatment and T-cleavage reaction in preparation for quantitative analysis of DNA methylation. Mean DNA methylation was measured by averaging the CpGs in each individual amplicon.

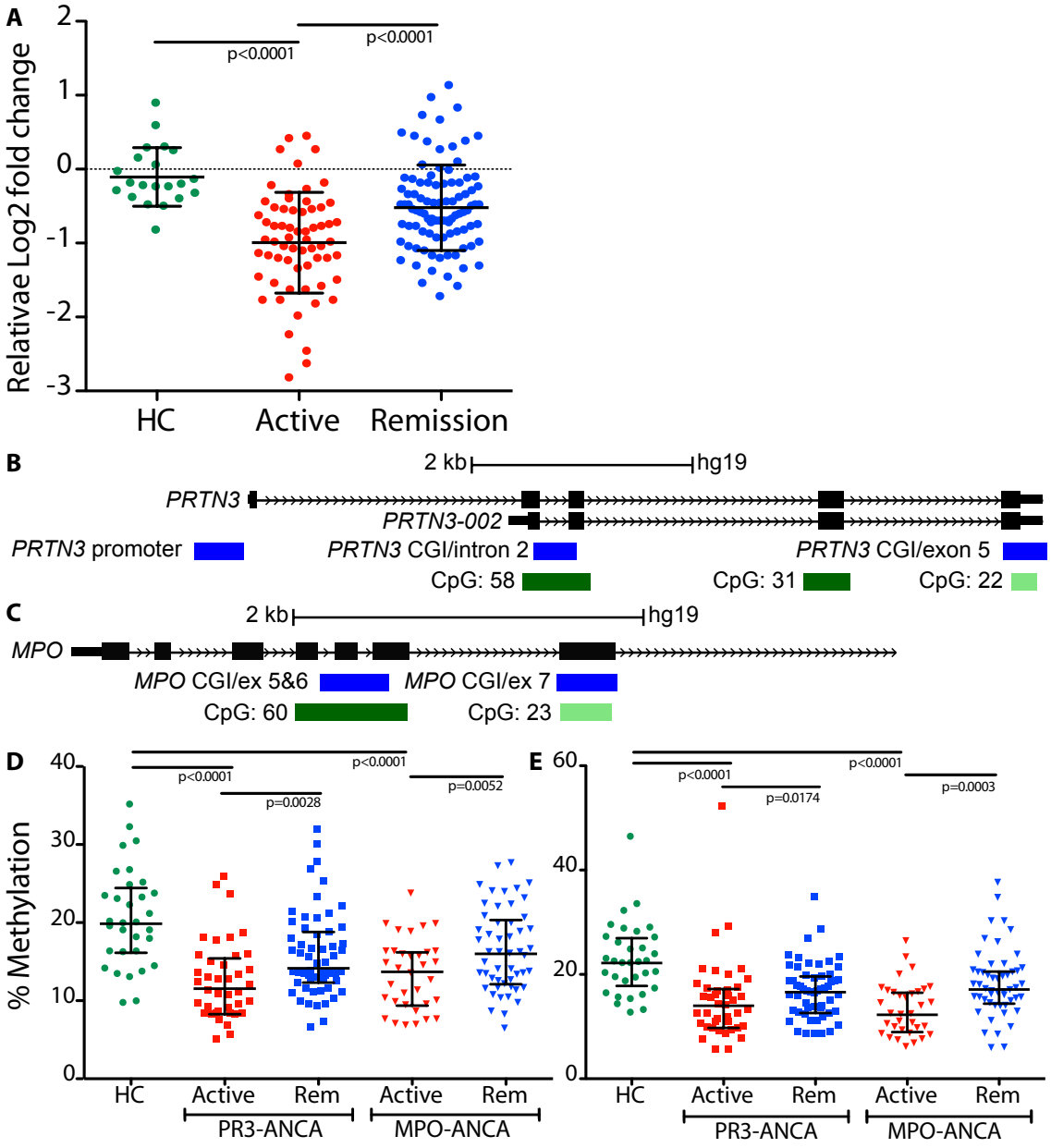

Figure 1.1. Relative *DNMT1* expression and DNA methylation at *PRTN3* and *MPO* loci comparing active to remission states. (**A**) Two-fold decrease in mean *DNMT1* expression in active patients (red) compared to healthy controls (green); mean expression in remitting patients (blue) was 1.5-fold higher than active patients. Bars shown are mean and standard deviation; $p<0.025$ is considered significant after accounting for multiple testing. (**B**) Three *PRTN3* amplicons covering: the promoter, a CGI and intron 2, a CGI and exon 5. (**C**) Two *MPO*

amplicons covering: a CGI and exon 7; a CGI and exon 5-6. Gene is shown in black, amplicons in blue, CGIs in green. Amplicon-wide cross-sectional DNA methylation patterns at the (**D**) *PRTN3* promoter and (**E**) *MPO* CGI/exon 5-6. Green circles are healthy controls; squares are PR3-ANCA patients; triangles are MPO-ANCA patients; active patients are red; patients in remission are blue. Bars shown are median with interquartile range; $p<0.0125$ is considered significant after accounting for multiple testing.

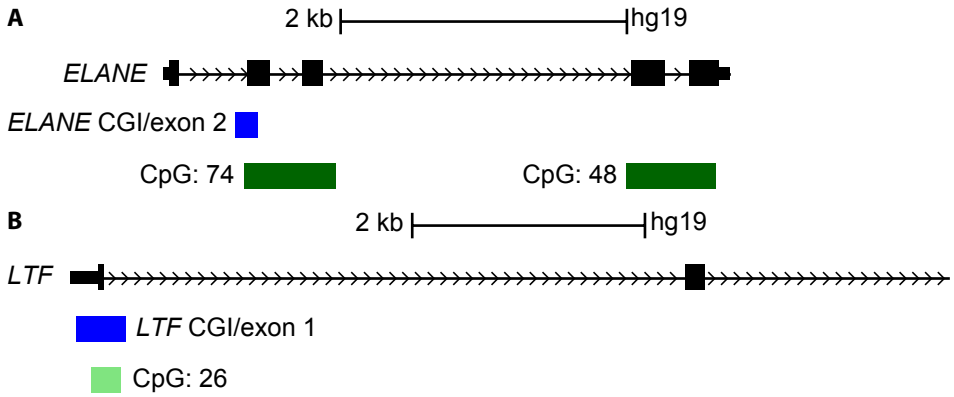

Figure 1.2. Amplicon locations within *ELANE* and *LTF*. (**A**) Amplicon shown covering part of a CGI and exon 2 within the *ELANE*. (**B**) Amplicon shown covering a CGI and exon 1 within the *LTF*. Gene is shown in black, amplicon in blue, CGIs in green.

Gene	Primer
PRTN3 (Taqman)	Forward 5'-TGT CAC CGT GGT CAC CTT CTT-3' Reverse 5'-CCC CAG ATC ACG AAG GAG TCT AT-3' Probe FAM-TTG CAC TTT CGT CCC TCG CCG-TAMRA
MPO (Taqman)	Forward 5'-CCA GGA AGC CCG GAA GAT-3' Reverse 5'-CGG AAG GCA TTG GTG AAG A-3' Probe FAM-TGC CCA CGT ACC GTT CCT ACA ATG ACT C-TAMRA
DNMT1 (Taqman)	Thermo Fisher cat. #4331182
PRTN3 (amplicon 5, methylation)	Forward 5'-aggaagagagTTAAAGGGGGAAGAAAATTTTTAGA-3' Reverse 5'-cagtaatacgactcactatagggagaaggct ATTACCCAATACCCAAACTAAATCC-3'
PRTN3 (amplicon 20, methylation)	Forward 5'-aggaagagagAAGTGTTGTTGGGTGGGTTTTT-3' Reverse 5'-cagtaatacgactcactatagggagaaggct AACACCTTAATCCACCCCAACTT-3'
PRTN3 (amplicon 22, methylation)	Forward 5'-aggaagagagTTGTGATGGTATTATTTAAGGAATAGATTT-3' Reverse 5'-cagtaatacgactcactatagggagaaggct CCTCCCCTCCCTATATAAAAAAAAC-3'
MPO (amplicon 11, methylation)	Forward 5'- aggaagagagGTTGGGGGTGGTTGTAGGAAT -3' Reverse 5'-cagtaatacgactcactatagggagaaggct CAACTAACCCCATTACATAAACATAAA-3'
MPO (Amplicon 12, methylation)	Forward 5'-aggaagagagAAGTTGATTTGTTAGGAAGTAGGGG-3' Reverse 5'- cagtaatacgactcactatagggagaaggct AACCTCTCTCTATACCTCAAATCCC-3'
LTF (amplicon 3, methylation)	Forward 5'-GGA GTT TTG TTT TGT TTT AGG GTT T-3' Reverse 5'-CTC CTA TTC CTC CCC ATA TAA AAA A-3'
ELANE (amplicon 5, methylation	Forward 5'-TGG TGG GGG ATT TAG AGG TT-3' Reverse 5'-AAC TAC AAA AAC ACC ATA AAA AAC CA-3'

Table 1.2. Primers used for Taqman mRNA expression studies and DNA methylation studies.

Targeted bisulfite sequencing for DNA methylation analysis was done on a replication cohort of 77 patient samples and 19 samples from healthy individuals (96 samples) at six of the same loci studied interrogated using the Agena platform. Sixteen primers were designed, synthesized (Integrated DNA Technologies) and validated by Zymo Research (Table 1.3). Targeted amplification of these samples was performed according to manufacturer's protocol (Zymo Research).

Gene_Amplicon	Chromosome	Browser position	
PRTN3_005	19	840498	840945
PRTN3_022	19	847822	848215
PRTN3_020	19	843571	843964
MPO_11	17	56356480	56356874
MPO_12	17	56355180	56355526
LTF_003	3	46506126	46506547
ELA_005	19	852792	852954

Table 1.3. Gene coordinates for each of the seven loci studied.

Illumina Infinium HumanMethylation450 BeadChips were used to analyze DNA methylation on a genome-wide scale in ten longitudinally paired AAV patients and four age-matched healthy individuals (24 samples). This platform allows for interrogation of >485,000 methylation sites per sample, covering 99% of RefSeq genes. After bisulfite treatment, the Mammalian Genotyping Core at UNC-Chapel Hill performed the remaining assay steps following the specifications and using the reagents supplied by the manufacturer (*107*).

Taqman mRNA expression studies

Quantitative detection of *DNMT1* mRNA levels from patient samples was determined as relative to three healthy control samples run on each plate. Quantitative detection of *MPO* and *PRTN3* mRNA levels from patient samples was determined using a standard curve. The standard

curve for *MPO* mRNA levels was generated using HL60 cells, a cell positive for *MPO* mRNA, diluted with Jurkat cells, a cell line negative for *MPO* mRNA. The standard curve for *PRTN3* mRNA levels was generated using THP-1 cells, a cell positive for *PTRN3* mRNA, diluted with Jurkat cells, a cell line negative for *PRTN3* mRNA. *MPO* and *PRTN3* mRNA levels for patients and healthy donor samples were determined by $2^{-\Delta\Delta Ct}$ calculations and expressed relative to standard curves. Primers and probes for *MPO* and *PRTN3* can be found in Table 1.2. Cytochrome c oxidase (*COX5B*) was used as mRNA internal control (*8*). Primers and probes were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA) and Integrated DNA Technologies, Inc. (Coralville, IA). Quantitative RT-PCR assays were performed on an ABI PRISM 7900HT sequence detection system using the TaqMan EZ RT-PCR kit (Applied Biosystems) (Yang, et al., manuscript submitted). Elevated expression of these autoantigen genes is considered 2 standard deviations above the mean expression of healthy individuals; for *PRTN3* high expression is >90, for *MPO* high expression is >180.

Genome-wide DNA methylation analysis

Analysis of the Illuminia Infinium HumanMetylation450 BeadChip array was performed in R (*108*) using the minfi package (*109*) and the UCSC hg19 knownGene genome annotation (*110*). The red and green intensities were converted to methylation data using background correction and SWAN normalization (*111*). Probes with single nucleotide polymorphisms at the CpG interrogation site or at the single base extension were omitted from analysis. Beta values were logit transformed to draw boxplots and compare DNA methylation at specific sites. These boxplots where made using the default R settings for the boxplot function.

Statistical Analysis

Comparisons between two independent groups were done using Wilcoxon rank sum test. Bonferroni corrections were used in situations with multiple comparisons between groups. Mean DNA methylation was measured by averaging the CpGs in each individual amplicon. Methylation at individual CpGs was found to be either static or dynamic in a pattern mirroring that shown in the mean DNA methylation. Log transformed correlation for DNA methylation and the expression of autoantigen genes was done by Spearman correlation coefficients. Kaplan-Meier curves with log rank tests were used to display and compare relapse-free survival times. These curves were used to evaluate the proportional hazards assumption, and then proportional hazards models were used to model the effect of DNA methylation (*PRTN3* promoter, *PRTN3* promoter CpG13, or *MPO* CGI/exon 5-6) on time to relapse. Potential confounders were modeled controlling for DNA methylation with one additional variable at a time. Univariate predictors of flare are reported as hazards ratios and 95% confidence intervals with a two-sided *P*-value of 0.05 or less considered statistically significant. DNA methylation change between the active and remission groups was analyzed with signed-rank test on all available longitudinal samples within patients (n=65) and then limited to the first pair collected chronologically for individuals (n=60). Results were almost exactly the same, thus results displayed include the full number of pairs. All analyses were done by R and SAS 9.4 (SAS Institute, Cary, NC, USA).

Results

Decreased *DNMT1* expression in AAV patients

Our investigation of DNA methylation in patients with AAV began by exploring alterations in the expression of the DNA methyltransferase 1 gene (*DNMT1*). Previous gene expression studies demonstrated that expression of *DNMT1* was decreased in patients with AAV compared to healthy individuals (Yang, et al., manuscript submitted). I confirmed differential *DNMT1* expression by quantitative real-time PCR in leukocytes collected from a cohort of AAV patients during disease activity and remission (Table 1.4). The relative mean *DNMT1* expression among AAV patients during disease activity was two-fold less than the mean expression among healthy individuals ($p<0.0001$) (Figure 1.1A). The mean *DNMT1* expression of AAV patients in disease remission was 1.5-fold higher than patients with active disease.

Genome-wide studies show differential DNA methylation is restricted to specific loci

I then carried out genome-wide DNA methylation studies to determine if reduced *DNMT1* expression in AAV patients with active disease resulted in genome-wide hypomethylation. Genome-wide DNA methylation was measured with the Illumina Infinium HumanMethylation450 BeadChip. The M value median and range among patients with active disease and healthy individuals were nearly identical, indicating no obvious differences in genome-wide DNA methylation despite differences in *DNMT1* expression (Figure 1.3A).

Variables		Healthy individuals (32)	PR3-ANCA patients (42)	MPO-ANCA patients (40)
Age	N	32		
	Mean ± SD	52.78±14.61		
	Median(IQR)	56.50(51.00, 59.00)		
Sex	Female	16(50.00%)	17(40.48%)	19(47.50%)
	Male	16(50.00%)	25(59.52%)	21(52.50%)
Race	Caucasian	27(84.38%)	35(83.33%)	32(80.00%)
	Other	5(15.63%)	7(16.67%)	8(20.00%)
Diagnosis	EGPA		0(0.00%)	2(5.00%)
	GPA		27(64.29%)	8(20.00%)
	LIM		0(0.00%)	12(30.00%)
	MPA		15(35.71%)	18(45.00%)
Sample number			98	86
	Active Samples		42	35
	Active samples taking CS		18	16
	Remitting samples		56	51
	Active samples taking CS		14	9
Active, new onset			10(10.20%)	11(12.79%)
Long-term remission, off therapy			12(12.24%)	18(20.93%)
Age of active sample	N		42	35
	Mean ± SD		56.21±14.53	53.51±20.22
	Median(IQR)		57.00(50.00, 67.00)	57.00(33.00,69.00)
Age of remission sample	N		56	51
	Mean ± SD		57.57±16.75	52.47±18.99
	Median(IQR)		59(46.50, 71.00)	54.00(38.00,66.00)
Active sample BVAS	N		42	35
	Mean ± SD		8.07±6.91	9.49±6.42
	Median(IQR)		6.00(2.00,13.00)	8.00(6.00,12.00)
Remission sample BVAS	N		56	51
	Mean ± SD		0±0	0.11±0.43
	Median(IQR)		0.00(0.00,0.00)	0.00(0.00,0.00)

PR3, proteinase 3; MPO, myeloperoxidase; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; Lim, renal limited vasculitis; BVAS; Birmingham Vasculitis Activity Score; CS, corticosteroids.

Table 1.4. Demographics summary for AAV patient cohort. Gender, race and diagnosis information for each patient group alongside BVAS and corticosteroid use for active and remitting samples.

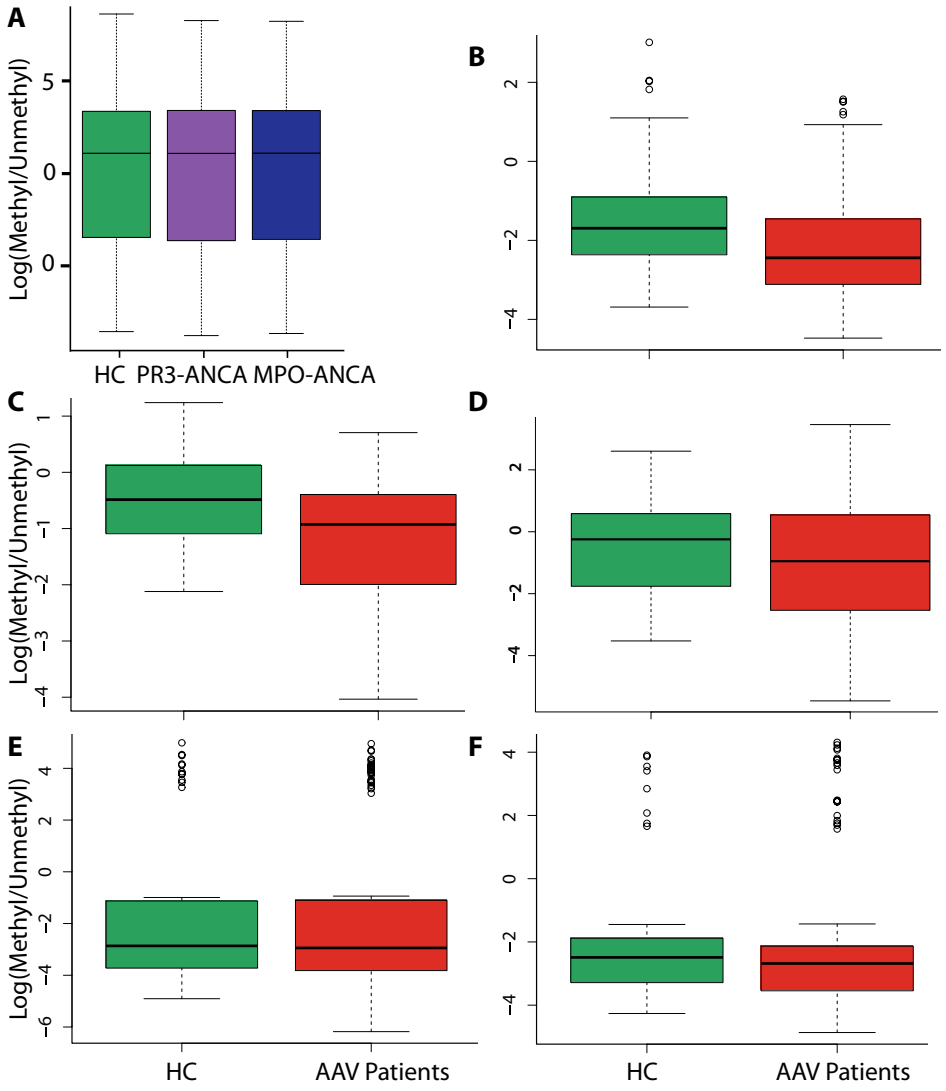

Figure 1.3. Epigenome-wide DNA methylation in patients with AAV. (**A**) Mean M value (log(methylated signal/unmethylated signal)) at 485,512 CpGs for 4 healthy controls (left, green) 6 PR3-ANCA active patients (middle, purple) and 4 MPO-ANCA active patients (right, blue). Median methylation at all CpG dinucleotides in healthy controls (left, green) compared to AAV patients (right, red) at (**B**) *PRTN3*, (**C**) *MPO*, (**D**) *ELANE*, (**E**) *LTF* and (**F**) *BPI*. For each graph, the line represents the median, the box the first and third quartiles, and the whiskers represent the maximum and minimum (A, B, D) values or 1.5 interquartile range (C, E, F).

Differential DNA methylation at autoantigen genes

DNA methylation at most loci, genome-wide, was unchanged between patients and healthy controls; however, gene-specific differences in DNA methylation status were detected at *MPO* and *PRTN3*. The median DNA methylation at all CpG dinucleotides in *MPO* and *PRTN3* was less in AAV patients with active disease than healthy controls (Figure 1.3B,C). I then determined how many other genes contained differential DNA methylation comparable to *MPO* and *PRTN3*. To do this, I identified the CpGs with the largest decrease in mean M value in active patients compared to healthy controls within *MPO* and *PRTN3*. The mean M values at these CpGs were compared to the decrease in M value between active patients and healthy controls at CpGs of all other genes. Of 19,654 unique genes, a greater decrease in DNA methylation in active samples was found for only 849 genes and 1,050 genes compared to *MPO* and *PRTN3*, respectively (Table 1.5). Approximately 96% of genes exhibited a smaller decrease in DNA methylation in active patients compared to healthy controls. These results strongly suggest that DNA methylation changes in AAV patients are restricted to specific loci, including *MPO* and *PRTN3*.

	Largest mean M-value difference (Active v HC)	**Genes with larger mean M value difference**	**Unique genes**	**% Genes hypomethyl v autoantigen genes**
MPO	-1.67	849	19654	3.8%
PRTN3	-1.42	1050		4.1%

Table 1.5. Genome-wide DNA methylation supports differential methylation at *MPO* and *PRTN3.*

To test if the changes in DNA methylation at *MPO* and *PRTN3* were a feature of other neutrophil granule genes, methylation was measured locally at CpG islands (CGIs) in neutrophil elastase (*ELANE*), lactotransferrin (*LTF*) and bactericidal/permeability-increasing protein (*BPI),* three neutrophil granulocyte genes with altered mRNA expression in patients with AAV and are known target antigens of ANCA in patients with inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (*8, 112, 113*). I found smaller differences in DNA methylation between AAV patients and healthy controls in the neutrophil granulocyte gene *ELANE* than those found at *MPO* and *PRTN3* (Figure 1.3D). The genome-wide DNA methylation studies found no difference between patients and healthy controls at *BPI* or *LTF* (Figure 1.3E,F).

AAV patient-derived leukocytes exhibit hypomethylation of loci within *MPO* and *PRTN3*

Based on the data from the genome-wide DNA methylation study, I screened our larger cohort of paired samples from patients during disease activity and remission for methylation changes at *MPO* and *PRTN3*. DNA methylation was measured on bisulfite-converted DNA from total leukocytes using the MassARRAY method which profiles methylation of multiple CpGs at specific loci (*114*). Three loci in *PRTN3* and two loci in *MPO* were analyzed (Figure 1.1B,C). While the *PRTN3* promoter does not contain a typical CGI, it does contain 15 CpGs that overlap binding sites for transcription factors that regulate myeloid gene expression. The other two analyzed regions in *PRTN3* included CGIs: one is contained in exon 2 and the alternative promoter which I reported was active in AAV patients with active disease (*115*), the other is contained within exon 5 and the 3'UTR. DNA methylation at the 3' boundary of genes encoding the 3'UTR can regulate transcriptional activity (*116, 117*). In *MPO,* a CGI spanning exons 5 and 6 was chosen as a region of interest based on our previous report of DNA methylation at this

region (*10*). The second region screened in *MPO* contains a CGI that spans exon 7. Data from ENCODE indicates both regions are DNase I sensitive and enriched in transcription factor binding sites, suggesting transcriptional regulatory properties. These amplicons cover CpGs that were also interrogated using the genome-wide DNA methylation platform (Illumina Infinium HumanMethylation450 BeadChip). The *MPO* promoter was not probed because the promoter is CpG-poor with only seven CpGs within 500 base pairs of the transcription start site; of those CpGs, six are in a repetitive sequence that prevented designing a reliable amplicon for the MassARRAY platform.

Consistent with the genome-wide DNA methylation study, I found that MPO- and PR3-ANCA patients with active disease were hypomethylated compared to healthy individuals at four loci within *MPO* and *PRTN3* ($p<0.0001$) (Figure 1.1D,E; Figure 1.4A,B). However, DNA methylation at these sites rebounded when patients were sampled during disease remission. I then looked at DNA methylation at *ELANE*, *LTF* and a CGI overlapping the alternative promoter of *PRTN3.* Overall, I found low DNA methylation, which remained static with no discernable differences between patient serotype, healthy controls or disease status (Figure 1.5). To determine if the platform used to measure DNA methylation influenced the observed changes, I validated a subset of the samples analyzed by MassARRAY with bisulfite sequencing. These two platforms for measuring DNA methylation produced comparable methylation patterns between healthy controls, active and remitting patient samples at identical CpGs (Figure 1.6). Thus, DNA methylation at *MPO* and *PRTN3* is reduced in patients with active AAV compared to patients in remission and healthy individuals, and the changes in DNA methylation occurred at specific loci within *MPO* and *PRTN3*.

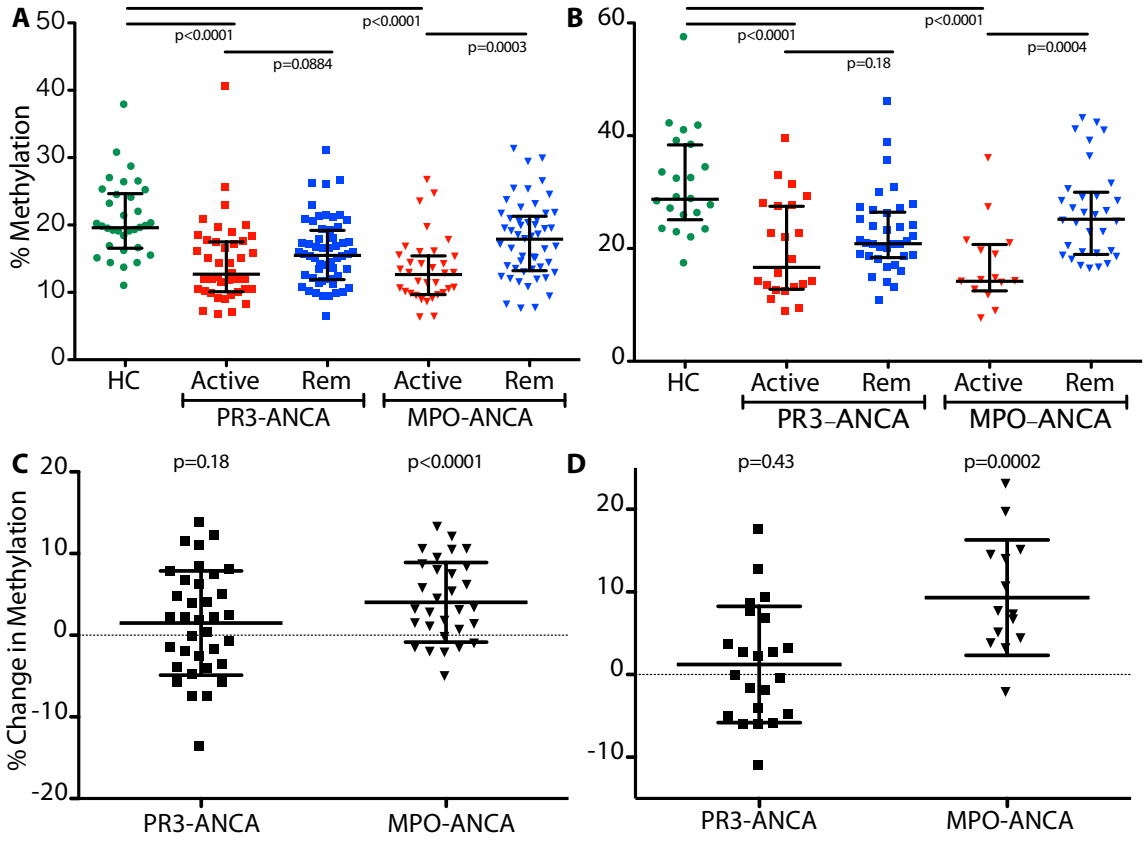

Figure 1.4. DNA methylation at additional loci within *PRTN3* and *MPO*. Cross-sectional methylation at (**A**) *PRTN3* CGI/exon 5 and (**B**) *MPO* CGI/exon 7. Healthy controls (green circles); PR3-ANCA patients (squares); MPO-ANCA patients (triangles); active patients are red and patients in remission are blue; bars shown are median with interquartile range; p<0.0125 is considered significant after accounting for multiple testing. Mean longitudinal methylation change from disease activity to remission at (**C**) *PRTN3* CGI/exon 5: PR3-ANCA patients 1.49%, MPO-ANCA patients 4.36%; and (**D**) *MPO* CGI/exon 7: PR3-ANCA patients 1.22%, MPO-ANCA patients 9.31%. Error bars are standard deviation; p-values shown are as different from zero, where p<0.025 is significant after accounting for multiple testing.

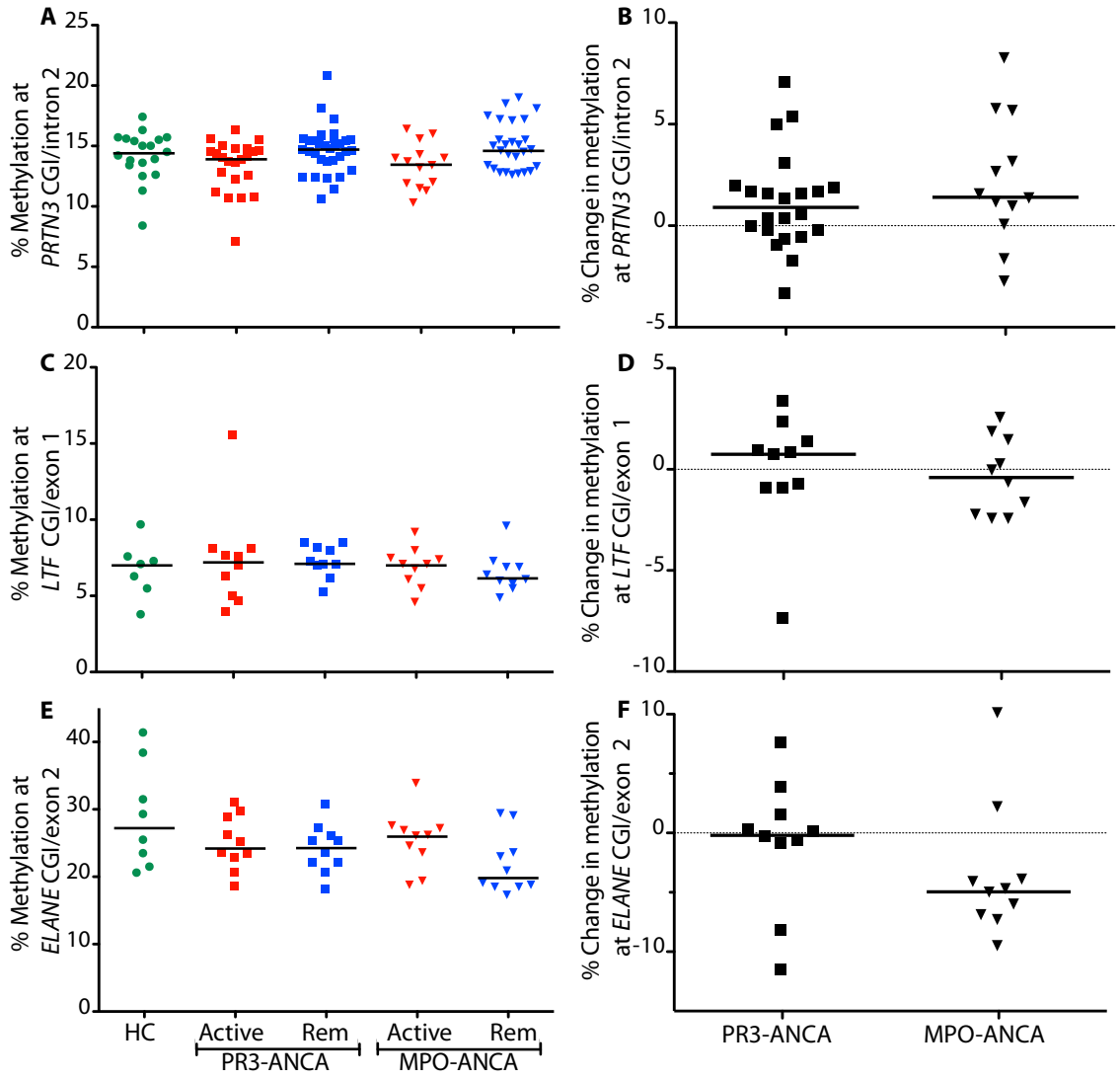

Figure 1.5. DNA methylation at loci within additional granulocyte genes. Green circles are healthy controls; squares are PR3-ANCA patients; triangles are MPO-ANCA patients; active patients are red and patients in remission are blue. (**A**) Cross-sectional and (**B**) longitudinal methylation at CGI/intron 2 in *PRTN3*. (**C**) Cross-sectional and (**D**) longitudinal methylation at a CGI/exon 1 in lactotransferrin (*LTF*). (**E**) Cross-sectional and (**F**) longitudinal methylation at a CGI and exon 2 in elastase (*ELANE*). Bars are median methylation (**A,C,E**) or median longitudinal methylation change from disease activity to remission (**B,D,F**).

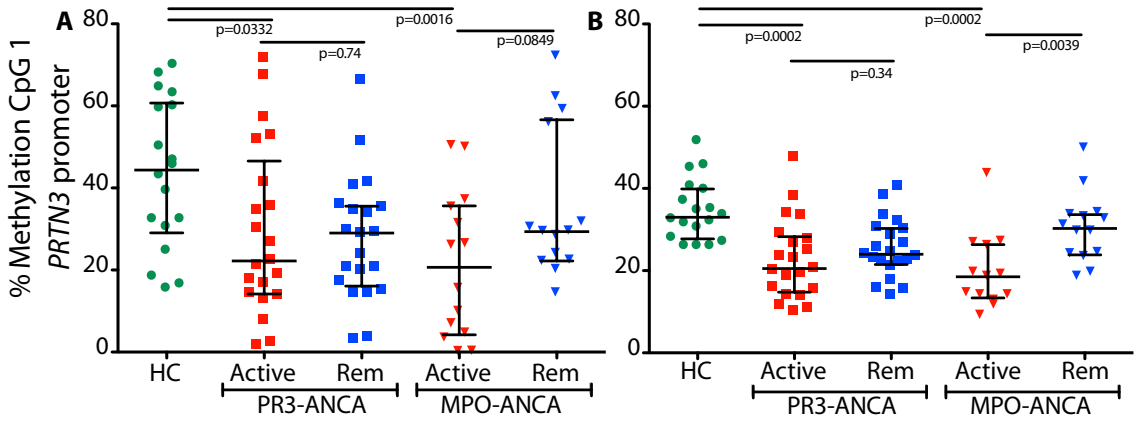

Figure 1.6. DNA methylation platform comparison. Bisulfite sequencing compared to MassARRAY. (**A**) Cross-sectional methylation at CpG 1 *PRTN3* promoter for (**A**) bisulfite sequencing and (**B**) MassARRAY. Green circles are healthy controls; squares are PR3-ANCA patients; triangles are MPO-ANCA patients; active patients are red and patients in remission are blue. Bars are median and interquartile range for all three graphs; $p<0.0125$ is considered significant, accounting for multiple testing.

Correlation of mRNA expression with DNA methylation

I compared the level of *DNMT1* expression to DNA methylation measured at *MPO* and *PRTN3* to confirm the association found in the genome-wide DNA methylation data. *DNMT1* mRNA expression correlated positively with DNA methylation at all four loci ($r= 0.4858$ at *PRTN3* promoter; $r= 0.5464$ at *MPO* CGI/exon 5-6; $p<0.0001$) (Figure 1.7). *MPO* and *PRTN3* mRNA expression were measured to determine if expression of autoantigen genes were regulated by DNA methylation. There is a significant correlation between DNA methylation within *MPO* and *PRTN3* and mRNA expression of these respective genes (*PRTN3* promoter $r= -0.2828$; *MPO* CGI/exon 5-6 $r= -0.3155$; $p<0.0001$) (Figure 1.8). The correlation of cross-

sectional measurements suggests that DNA methylation in total leukocytes has a minor influence on expression of these autoantigen genes; however, following patients longitudinally may be more valuable than measuring expression and methylation at a single point in time.

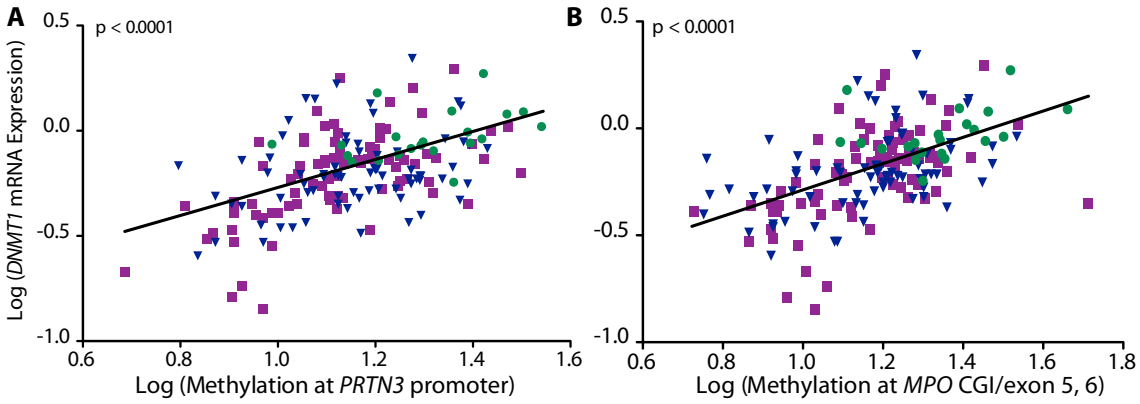

Figure 1.7. Correlation of DNA methylation and mRNA expression at *DNMT1*. Healthy controls (green circles); MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares). Log transformed correlation between methylation at the (**A**) *PRTN3* promoter and *DNMT1* expression (n=179; r= 0.4858) and (**B**) correlation between methylation at *MPO* CGI/exon 5-6 and *DNMT1* expression (n=177; r= 0.5464).

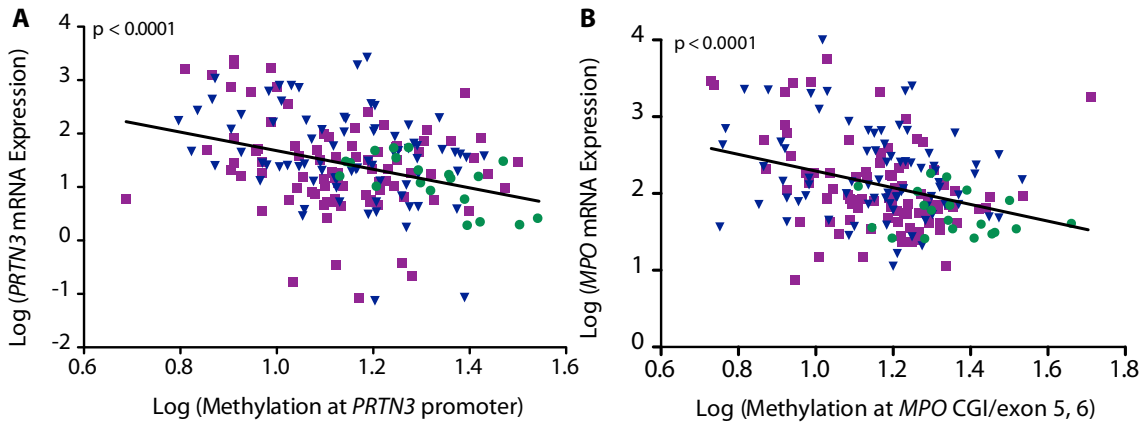

Figure 1.8. Correlation of DNA methylation and mRNA expression at *PRTN3* and *MPO*. Total leukocytes from healthy controls (green circles); MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares). Log transformed correlation between DNA methylation at the (**A**) *PRTN3* promoter and *PRTN3* expression (n=187; r= -0.2828) and (**B**) DNA methylation at *MPO* CGI/exon 5-6 and *MPO* expression (n=186; r= -0.3155).

Longitudinal DNA methylation studies uncover two distinct patterns in AAV patients

I hypothesized that methylation changes relate to disease status in patients with AAV; to this end, I measured DNA methylation in paired patient samples collected an average of 18 months apart and quantified the difference in methylation (remission minus active methylation) at loci within *MPO* and *PRTN3*. At the *PRTN3* promoter, the mean change in DNA methylation is comparable between PR3-ANCA patients (3.62%) and MPO-ANCA patients (3.99%) and significantly different from zero (p=0.0003 and 0.0007, respectively) (Figure 1.9A). These data indicate that most patients exhibit increased DNA methylation at the *PRTN3* promoter upon disease remission, regardless of serotype. At other loci, changes in DNA methylation status depended on serotype. At the *MPO* CGI/exon 5-6, the mean DNA methylation change in MPO-

ANCA patients is similar to that at the *PRTN3* promoter (4.36%) but not in PR3-ANCA patients (1.85%) (p=0.0008 and 0.12, respectively) (Figure 1.9B). Similarly, changes in DNA methylation at both *PRTN3* CGI/exon 5 and *MPO* CGI/exon 7 were significantly different from zero in MPO-ANCA patients, but not different from PR3-ANCA patients (Figure 1.4C,D).

Thus far, all DNA methylation values shown are averaged values of the 13-39 CpGs within each locus studied. Closer analysis shows that individual CpGs within each locus are characterized by either static or dynamic DNA methylation patterns that are similar to those seen in the averaged methylation data. The DNA methylation patterns at individual dynamic CpGs within the *PRTN3* promoter (Figure 1.10A,C) and *MPO* CGI/exon 5-6 (Figure 1.10B,D) mirror the patterns seen when averaging the CpGs across an amplicon in both cross-sectional and longitudinal analyses. The longitudinal analysis of DNA methylation revealed that although patients were more likely to have increased DNA methylation when in remission, there were patients with decreased DNA methylation during remission. Importantly, this segregates patients into two groups where the role of DNA methylation was examined separately.

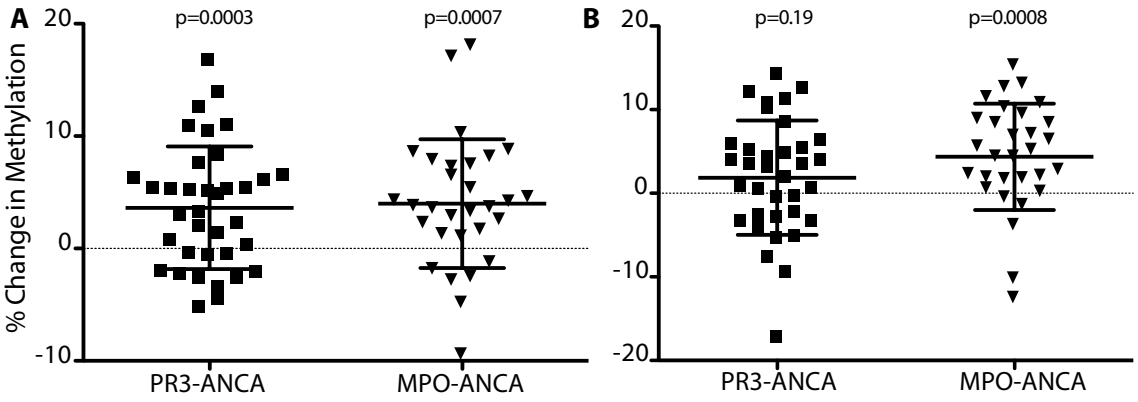

Figure 1.9. Longitudinal change in DNA methylation from disease activity to remission. Mean and standard deviation shown; p-values are as different from zero, where $p<0.025$ is significant after accounting for multiple testing. PR3-ANCA patients are squares and MPO-ANCA patients are triangles. Mean DNA methylation change at the (**A**) *PRTN3* promoter: PR3-ANCA patients 4.03%, MPO-ANCA patients 3.69% and the (**B**) *MPO* CGI/exon 5-6: PR3-ANCA patients 3.06%, MPO-ANCA patients 4.93%.

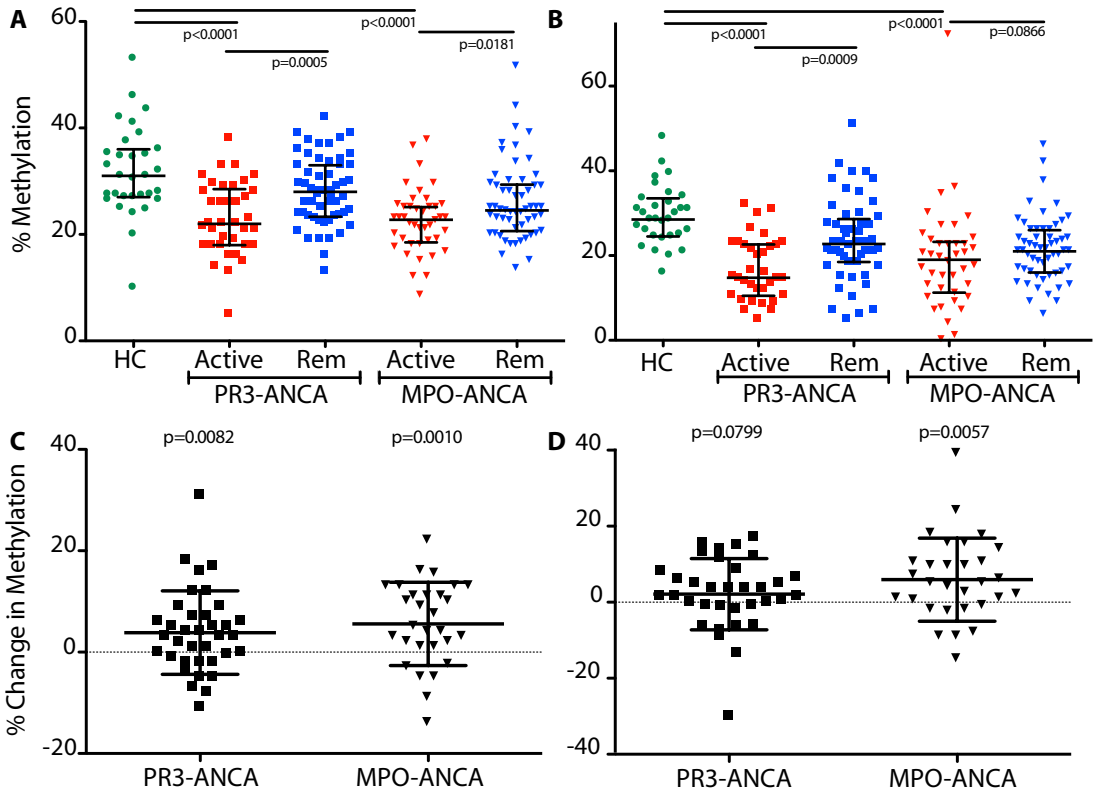

Figure 1.10. DNA methylation changes at individual CpGs within the *PRTN3* promoter and *MPO* CGI/exons 5-6. Green circles are healthy controls; squares are PR3-ANCA patients; triangles are MPO-ANCA patients; active patients are red and patients in remission are blue. Bars shown are median with interquartile range; p<0.0125 is significant after accounting for multiple testing. Cross-sectional methylation at (**A**) CpG 7, 8 *PRTN3* promoter and (**B**) CpG 38 *MPO* CGI/exons 5-6. Mean longitudinal methylation change from disease activity to remission at (**C**) CpG 7, 8 *PRTN3* promoter: PR3-ANCA patients 3.85%, MPO-ANCA patients 5.57% and (**D**) CpG 38 *MPO* CGI/exons 5-6: PR3-ANCA patients 2.11%, MPO-ANCA patients 5.95%. For longitudinal graphs, p-values shown are as different from zero where p<0.025 is significant after accounting for multiple testing, bars are mean and standard deviation.

Autoantigen gene expression correlates with gene-specific DNA methylation in AAV patients

I investigated the relationship between autoantigen mRNA expression and DNA methylation in patients with increased gene-specific DNA methylation and patients with decreased gene-specific DNA methylation. Stratifying paired patient data by DNA methylation increase (Figure 1.11A,E) or decrease (Figure 1.11B,F) revealed two levels of association between autoantigen mRNA expression and DNA methylation. The correlation between *PRTN3* mRNA expression in patients with increasing DNA methylation at the *PRTN3* promoter is much stronger (r= -0.3390, p=0.0013) (Figure 1.11C) than the patients with decreasing methylation (r= -0.08322, p=0.63) (Figure 1.11D); this correlation is also stronger than that seen in the entire cohort (Figure 1.8A). The same trend is found at *MPO* CGI/exon 5-6: patients with increasing DNA methylation correlate with *MPO* mRNA expression (r= -0.3735, p=0.0004) (Figure 1.11G) while patients with decreasing methylation do not correlate (r= -0.08508, p=0.64) (Figure 1.11H). These data suggest DNA methylation plays a stronger role in regulating expression in patients who exhibit increased gene-specific DNA methylation during disease remission.

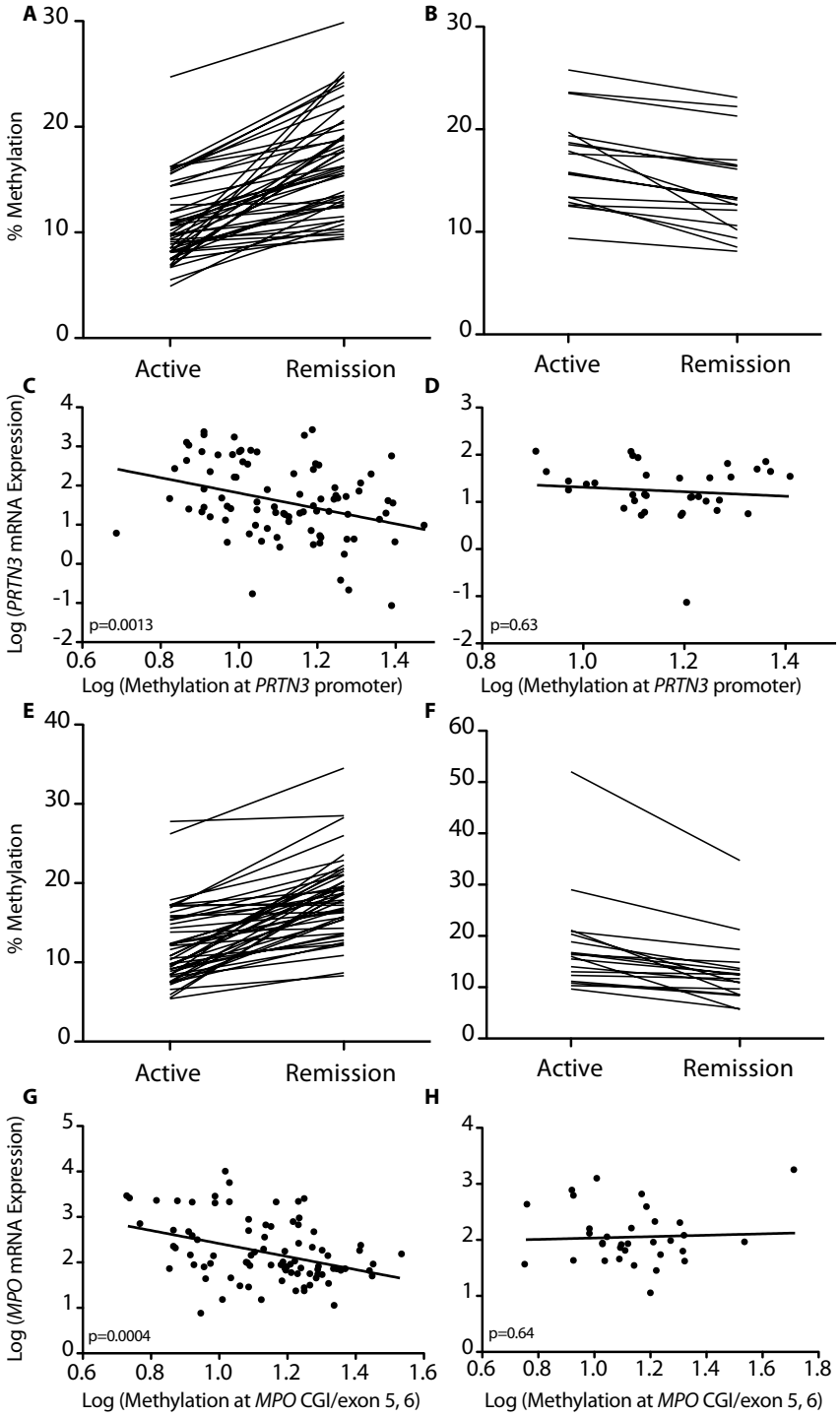

Figure 1.11. AAV patients stratified by DNA methylation increase or decrease. Paired patients with (**A**) increased DNA methylation and (**B**) decreased DNA methylation in disease remission at the *PRTN3* promoter. (**C**) DNA methylation at the *PRTN3* promoter compared to expression

of *PRNT3* in patients with increased methylation in disease remission (panel A) (r= -0.3390). (**D**) DNA methylation at the *PRTN3* promoter compared to expression of *PRNT3* in the samples with decreased methylation in disease remission (panel B) (r= -0.08322). Paired patients with (**E**) increased methylation and (**F**) decreased methylation in disease remission at *MPO* CGI/exon 5-6. (**G**) DNA methylation at *MPO* CGI/exon 5-6 compared to expression of *MPO* in patients with increased methylation in disease remission (panel E) (r= -0.3735). (**H**) DNA methylation at *MPO* CGI/exon 5-6 compared to expression of *MPO* in patients with decreased methylation in disease remission (panel F) (r= -0.08508). P-values of <0.05 are considered significant.

Change in DNA methylation at the *PRTN3* promoter is an indicator of relapse in AAV

I then questioned if the change in DNA methylation could inform disease prognosis. Within our longitudinal cohort, 65 patients continued to be followed in our clinic. Thirty-four of these patients remain in stable disease remission with a mean clinical follow-up time of 27 months since entering disease remission. Of the paired patients who relapsed, the average time to relapse was 31 months (SEM ±4.9) for those exhibiting increased gene-specific DNA methylation and 16 months (SEM ±2.7) for those exhibiting decreased gene-specific DNA methylation. I compared time to relapse in patients with increased DNA methylation to those with decreased methylation at each locus using Kaplan-Meier survival curves. The analysis using the change in DNA methylation at the *PRTN3* promoter revealed that the relapse-free probability was higher among patients (n=50) with increased DNA methylation upon disease remission than among patients (n=15) with decreased DNA methylation ($p<0.0001$) (Figure 1.12A). At *MPO* CGI/exon 5-6, the relapse-free probability was slightly higher among patients with increased DNA methylation than among patients with decreased methylation, yet the probability of relapse was not significantly different between the two groups ($p=0.41$) (Figure 1.12B).

I further subdivided the patients with increased or decreased DNA methylation by serotype to determine if ANCA specificity contributed to relapse-free probability. At the *PRTN3* promoter, the relapse-free probability partitioned patients based on change in DNA methylation rather than ANCA serotype. At *MPO* CGI/exon 5-6, the relapse-free probability was not significantly different after dividing patients by ANCA serotype (p=0.60) (Figure 1.12C,D). Stratifying patients based solely on MPO-ANCA or PR3-ANCA serotype showed similar relapse-free probability that was not statistically significant (p=0.81) (Figure 1.12E). This suggests an increase in DNA methylation in disease remission at the *PRTN3* promoter indicates a better disease prognosis than a decrease in DNA methylation at this locus.

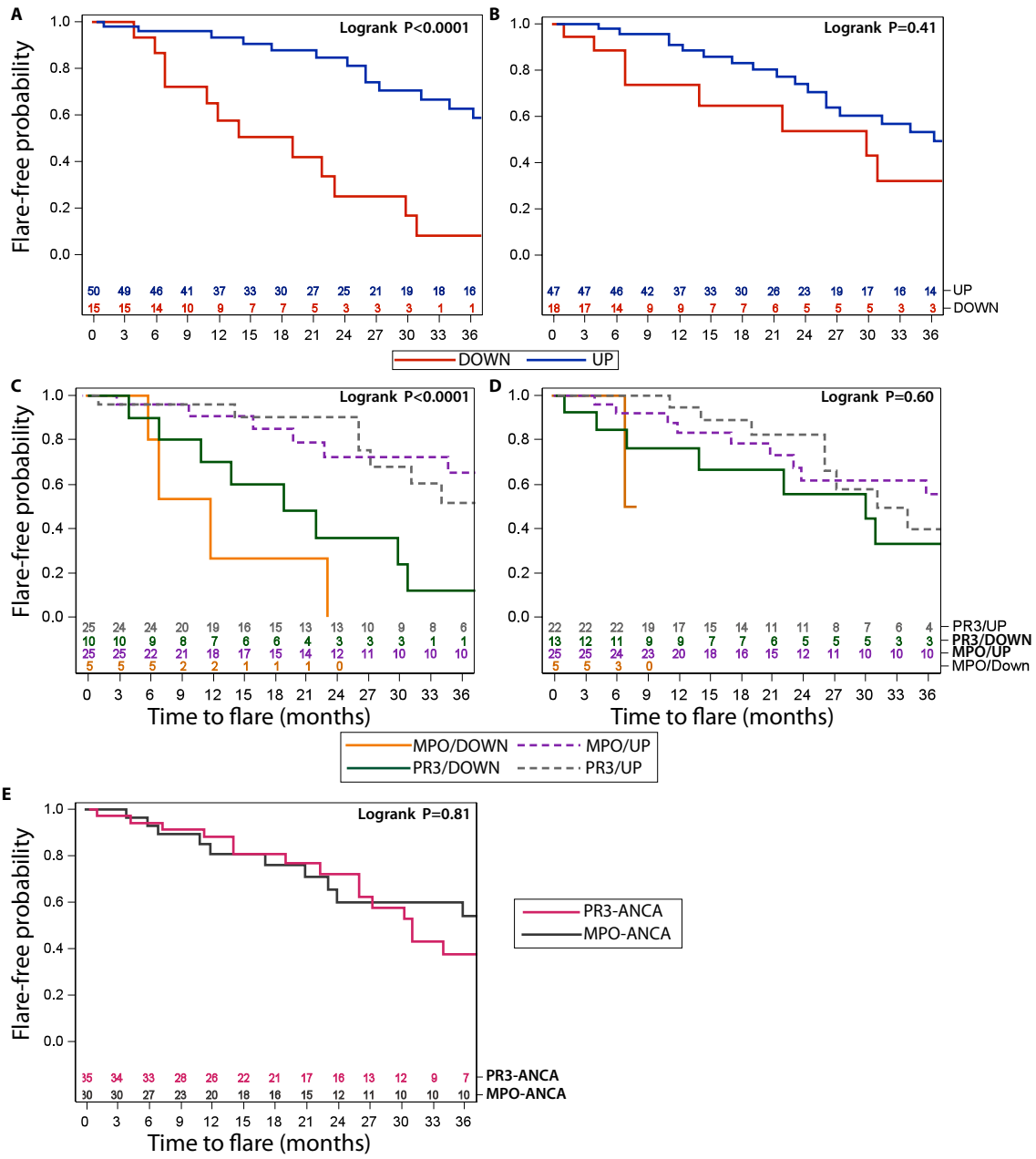

Figure 1.12. DNA methylation and probability of relapse. AAV patients stratified by DNA methylation change and followed until next relapse or last clinic follow-up at the (**A**) *PRTN3* promoter and (**B**) *MPO* CGI/exon 5-6; decreased DNA methylation in red, increased DNA methylation in blue. AAV patients stratified by DNA methylation change and serotype and followed until next relapse or last clinic follow-up at the (**C**) *PRTN3* promoter and (**D**) *MPO*

CGI/exon 5-6; MPO-ANCA patients with decreased DNA methylation in orange, MPO-ANCA patients with increased DNA methylation in dashed purple, PR3-ANCA patients with decreased DNA methylation in green, PR3-ANCA patients with increased DNA methylation in dashed gray. (**E**) AAV patients stratified by serotype and followed until next relapse or last clinic follow-up at the *PRTN3* promoter; PR3-ANCA patients in pink, MPO-ANCA patients in black. Numbers at bottom of graphs correspond to the number of patients in each group who have not relapsed and have been followed up in clinic. P-values <0.05 are considered significant.

Risk of relapse is highest in AAV patients with decreased DNA methylation at the *PRTN3* promoter

Proportional hazard models of time to relapse showed that patients with decreased DNA methylation at the *PRTN3* promoter were 4.55 times more likely to relapse, regardless of serotype (95% CI 2.09, 9.91; p=0.0001). The change in DNA methylation at *MPO* CGI/exon 5-6 was not predictive of relapse with a hazard ratio of 1.41 (95% CI 0.62, 3.20; p=0.41) (Table 1.6). When multivariate analyses were performed to control for additional variables including age, autoantigen gene expression, diagnosis (microscopic polyangiitis-MPA, granulomatosis with polyangiitis-GPA), organ involvement and serotype, the hazard ratio for relapse among patients that decreased DNA methylation versus patients that increased methylation at the *PRTN3* promoter remained significant and did not change from the univariate analysis (Table 1.6). Based on this analysis, the change in DNA methylation at the *PRTN3* promoter indicates the likelihood of relapse among patients with AAV.

The role of the most commonly prescribed therapy in this cohort, corticosteroids, specifically prednisone, in DNA methylation was examined. Fifty-seven of 184 patient blood

samples in this study were collected while the patient was taking prednisone. DNA methylation of active samples on and off prednisone (n=34 and 46, respectively) show a comparable mean methylation at CpG 7, 8 of the *PRTN3* promoter (p=0.14) (Figure 1.13), indicating DNA hypomethylation is largely independent of prednisone therapy in patients with active disease.

In this cohort, polymorphonuclear cell (PMN) counts were only available for 57% (104/184) of the samples, including 22 patient pairs. In this small subset of patients, I found the same association between DNA methylation and PMN count in both the *PRTN3* promoter and *MPO* CGI/exon 5-6; yet only the *PRTN3* promoter was found to be predictive of stable remission. These preliminarily findings suggest PMN count does not explain the observation that a change in DNA methylation at the *PRTN3* promoter predicts relapse probability.

As described above, the change in DNA methylation was calculated by taking the average methylation across all CpGs covered by an amplicon, but individual CpGs show dynamic changes. I tested whether changes in DNA methylation at dynamic CpGs in *MPO* CGI/exon 5-6 associated with different flare-free rates or likelihood of relapse. Unfortunately, none of the CpGs tested were predictive. Within the *PRTN3* promoter, none of the 13 individual CpGs interrogated were found to be more predictive of relapse than the mean DNA methylation change; however, the hazard ratio for relapse among patients that decreased versus patients that increased methylation at CpG 13 was 3.43 (95% CI 1.56, 7.56; p=0.0022) (Table 1.6). This pinpoints a single cytosine residue where a change in DNA methylation may be prognostic.

		***PRTN3* Promoter**		***PRTN3* Promoter CpG13**		***MPO* CGI/exon 5, 6**	
	Controlled variables	HR(95%CI)	p-value*	HR(95%CI)	p-value*	Hazards ratio	p-value*
Methylation		4.55(2.09, 9.91)	0.0001	3.43(1.56, 7.56)	0.0022	1.41(0.62, 3.20)	0.41
	Serotype[1]	1.43(0.64, 3.21)	0.38	1.29(0.59, 2.86)	0.52	1.07(0.46, 2.49)	0.88
	Age[2]	0.99(0.97, 1.01)	0.36	0.99(0.97, 1.01)	0.28	0.99(0.97, 1.02)	0.59
	Diagnosis[3]	2.16(0.99, 4.75)	0.0541	1.97(0.90, 4.31)	0.0906	2.39(1.09, 5.24)	0.0289
	PRTN3 expression change[4]	1.60(0.62, 4.15)	0.34	1.39(0.54, 3.57)	0.50	1.08(0.43, 2.73)	0.87
	MPO expression change[4]	0.99(0.39, 2.54)	0.99	0.85(0.34, 2.14)	0.73	0.68(0.27, 1.70)	0.41
	Corticosteroid Therapy[5]						
	Active samples	0.93(0.43, 1.99)	0.84	0.91(0.42, 1.95)	0.80	0.74(0.35, 1.55)	0.42
	Remission samples	1.44(0.50, 4.12)	0.50	0.98(0.37, 2.60)	0.97	0.82(0.31, 2.16)	0.68
	Organ involvement[6]						
	Lung	0.97(0.46, 2.07)	0.94	1.10(0.52, 2.32)	0.81	1.07(0.51, 2.28)	0.85
	Upper Respiratory	1.52(0.70, 3.27)	0.29	1.67(0.78, 3.56)	0.18	1.75(0.81, 3.77)	0.15
	Kidney	0.93(0.31, 2.86)	0.91	0.73(0.26, 2.00)	0.54	1.53(0.53, 4.41)	0.43

*P values were calculated using each hazard model adjusted by DNA methylation (*PRTN3* Promoter, *PRTN3* Promoter CpG13 or *MPO* CGI/exon 5-6).

[1]MPO-ANCA (n=30) compared to PR3-ANCA patients (n=35); [2]Age at time of first sample (usually active); [3]GPA (n=27) compared to all grouped MPA (n=29), Renal Limited (n=7) and EGPA (n=2); [4]Expression at time of disease remission, subtracted from expression at time of disease activity; positive changes compared to negative changes; [5]Samples on prednisone (active n=34; remission n=23) compared to those not taking prednisone (active n=43; remission n=84); [6]Lung involvement (n=33); upper respiratory involvement (n=36); kidney involvement (n=50).

Table 1.6. Hazard Ratios for the role of methylation change in flare-free probability at *PRTN3* and *MPO*. Hazard ratio, 95% CI and p-value for decreased DNA methylation change at the *PRTN3* promoter (mean methylation and methylation at CpG 13) and *MPO* CGI/exon 5-6 taking into account additional variables including serotype, age, diagnosis, corticosteroid therapy and organ involvement.

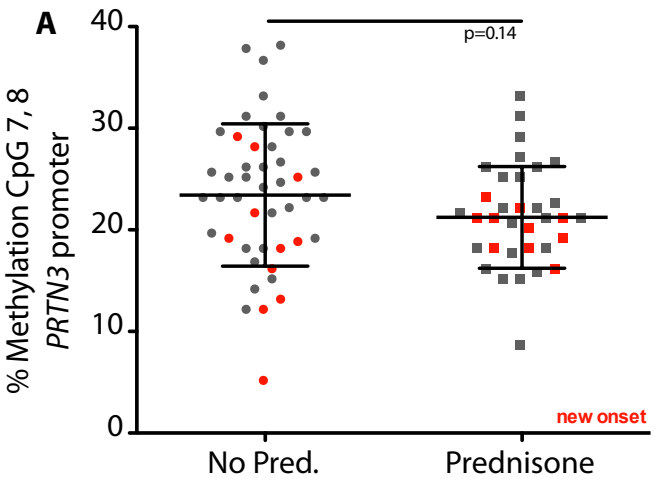

Figure 1.13. Role of prednisone in DNA methylation. (**A**) DNA methylation at CpG 7, 8 of the *PRTN3* promoter for all samples collected from active patients prescribed prednisone (circles) and patients not prescribed prednisone (squares); red indicates new onset active AAV patients. Bars shown are median DNA methylation with interquartile range; $p<0.05$ is considered significant.

Discussion

In this study I measured DNA methylation in patients with AAV during active disease and remission to determine the dynamics of this epigenetic modification during disease. The initial rationale for investigating DNA methylation was based on preliminary data that indicated *DNMT1* mRNA expression was reduced in AAV patients. I confirmed *DNMT1* mRNA was reduced in patients with active disease, and demonstrated *DNMT1* was expressed at levels comparable to healthy individuals when patients were in remission. AAV is not the only autoimmune disease characterized by decreased expression of *DNMT1* (*60, 64*).

One of our primary measurements in this study was the change in DNA methylation from active disease to remission, which revealed two groups of patients: those that increase methylation, and those that decrease methylation. Upon entering remission this dichotomy has striking consequences. First, at all loci studied in *MPO* and *PRTN3*, except for the *PRTN3* promoter, DNA methylation changes segregated patients by serotype. More PR3-ANCA patients decrease DNA methylation at *PRTN3* CGI exon 5 and *MPO* CGI exon 5-6 than MPO-ANCA patients. This suggests that in addition to prior studies indicating genetic differences between MPO-ANCA and PR3-ANCA patients (*118*), differences in ANCA specificities may also have an epigenetic component. Second, DNA methylation changes divided patients by those with autoantigen expression that correlated with DNA methylation and those with expression that did not correlate with methylation. The correlation between autoantigen gene expression and DNA methylation was much greater for patients with increased methylation compared to patients with decreased methylation; it is plausible that DNA methylation regulates transcription of autoantigen genes only among some patients. Finally, most intriguing is the finding that DNA methylation changes indicated the likelihood a patient would relapse. At the *PRTN3* promoter, a

decrease in DNA methylation upon disease remission indicates a higher probability a patient will relapse in the future regardless of ANCA serotype, compared to patients that increased methylation in disease remission. Thus, an increase in DNA methylation at the *PRTN3* promoter marks stable remission.

A comprehensive search for a prognostic biomarker of disease remission in AAV would include epigenetic changes because the epigenome is labile and susceptible to immunological challenges and environmental exposure, which may trigger disease relapse. The role of DNA methylation, histone modifications and other epigenetic modifications are increasingly explored in autoimmune diseases (*64*). Specifically, abnormal DNA methylation of immune cells is known to contribute to the etiology of systemic lupus erythematosus (SLE) (*119-124*), RA (*125-131*) and MS (*132-136*). Hypomethylation of gene promoters in SLE patients has been shown to associate with disease activity (*137, 138*). Hypomethylation of interleukin-6 and interleukin-10 promoters has been suggested to play a role in the pathogenesis of RA (*139, 140*).

I found prognostic value in quantifying DNA methylation changes at the *PRTN3* promoter in patients with AAV. It is likely that an epigenetic modification such as DNA methylation impacts the regulation of autoantigen gene expression in AAV. In patients with decreased methylation an alternative epigenetic modification could explain why expression of the autoantigen genes does not appear to be influenced by DNA methylation; however, in this group of patients I observed a relatively small change in expression.

Markers of disease status in patients with AAV have been found in other cohort studies (*141-143*). When I controlled for additional variables, including markers of disease status, none of the following demographic metrics were found to correlate with stable remission better than change in methylation at the *PRTN3* promoter in AAV patients: serotype, diagnosis, age,

autoantigen gene expression, organ involvement or corticosteroid therapy. Despite the majority of patients taking some form of immunosuppressive therapy, none of the patients in this study were taking known epigenetic modifiers, such as hydralazine. Future studies measuring DNA methylation exclusively in patients off therapy will need to be carried out to definitively address whether therapy contributes to changes in methylation. Our hazard ratio analysis found that corticosteroid use, specifically prednisone, did not alter the predictive value of DNA methylation change at the *PRTN3* promoter.

I measured DNA methylation in two platforms at three additional genes (*ELANE*, *LTF* and *BPI*) chosen as controls because of similarities to *MPO* and *PRTN3* in AAV. Along with *MPO* and *PRTN3*, the expression of these genes is elevated in patients with AAV, and circulating antibodies against elastase and lactotransferrin have been identified as causes of perinuclear ANCA staining pattern. Anti-elastase antibodies have been reported in patients with SLE and drug-induced SLE (*144-146*). ANCA directed against BPI, an endotoxin-binding protein of polymorphonuclear granulocytes, are associated with a cytoplasmic ANCA staining pattern and have been reported in patients with IBD as well as those with cystic fibrosis (CF) (*113, 147*). Generally, DNA methylation changes observed at these genes were not similar to those measured at *MPO* and *PRTN3*, which I conclude demonstrates the specificity of DNA methylation changes at *MPO* and *PRTN3*. Differences in DNA methylation pattern between the methylation platforms at *ELANE* are likely due to differences in probe location. The genome-wide DNA methylation platform measured CpG sites across *ELANE* while local methylation studies only examined CpGs in the CGI/exon 2.

The *PRTN3* promoter is unique in that it does not show differences between serotypes. An increase in DNA methylation (from disease activity to remission) at the *PRTN3* promoter is

predictive of stable remission in both MPO- and PR3-AAV patients. A scarcity of CpGs within the *MPO* promoter prevented the study of DNA methylation at the *MPO* promoter in the three platforms utilized here; however, it remains possible that DNA methylation changes at this promoter are similar to those seen at the *PRTN3* promoter. Interestingly, the only prognostic value for DNA methylation was found in the promoter of *PRTN3*; none of the CGIs examined in *MPO*, *PRTN3*, *ELANE* and *LTF* were predictive of stable remission. Changes in DNA methylation at CpGs in the *PRTN3* promoter may disrupt binding sites for transcription factors. Within the *PRTN3* promoter I was able to identify a single CpG that was predictive comparable to the mean DNA methylation change. This CpG (13) resides within the consensus binding sites for two transcription factors: upstream stimulatory factor (USF*)* and specificity protein 1. This suggests a location where DNA methylation could disrupt transcription factor binding, and previous studies in autoimmunity have demonstrated the importance of methylation at individual CpGs. For instance, hypomethylation at two CpG sites in the promoter of interferon-induced protein 44-like (*IFI44L*) was found to be a highly sensitive and specific diagnostic marker in patients with SLE compared to healthy controls and other autoimmune diseases (*148*).

A previous study reported that in patients with active AAV compared to healthy individuals the *MPO* and *PRTN3* genes had reduced levels of the histone modification H3 lysine 27 trimethylation (H3K27me3), which is associated with gene silencing. H3K27me3 was measured at regions within *MPO* and *PRTN3* in neutrophils from 15 patients, and there was a modest correlation between the level of H3K27me3 and *MPO* or *PRTN3* gene expression (*10*). H3K27me3 mediated silencing of *MPO* and *PRTN3* genes was explored further *in vitro* using myelo-monocytic cell lines. Here, I measured DNA methylation at regions within *MPO* and *PRTN3* from 82 patients, where 65 were paired active-remission samples, and found that changes

in DNA methylation at *PRTN3* promoter were predictive of relapse probability. Both studies describe a role in AAV for epigenetic alterations at autoantigen genes. These observations may be linked mechanistically since DNA methylation/DNMT1 and H3K27me3/Polycomb Repressive Complex 2 act together at the same genes in cancer and embryonic stem cells (*149*).

While this study investigated the link between DNA methylation and expression or the maintenance methyltransferase, *DMNT1*, other enzymes are also responsible for DNA methylation. Ten-Eleven Translocation family enzymes (coded by genes *TET1*, *TET2* and *TET3*) are involved in the repression of pro-inflammatory cytokines (*150*); decreased expression of *TET2* mRNA as well as protein has been reported in the peripheral blood mononuclear cells (PBMCs) of patients with multiple sclerosis (MS) (*133*). DNA methyltransferase and TET family enzymes are involved in the formation of a 5-methylcytosine and 5-hydroxymethylcytosine, respectively. The transcribed gene bodies generally associate with 5-hydroxymethylcytosine (*151-154*). Down-regulation of *DNMT1* and *TET2* in the PBMCs of patients with MS has been linked to aberrant DNA methylation of the gene promoters (*133*). Future studies in patients with AAV would examine the expression of the *TET* genes as well as DNA methylation at the promoters of both *TET* and *DNMT* genes. Unpublished data from our group did not find differential expression of *DNMT3a* or *DNMT3b* when comparing patients to healthy controls.

A limitation of this study is the use of total leukocytes containing varying percentages of different cell types. Among hematopoietic cell types, DNA methylation is known to differ; therefore, it is possible changes in the proportions of a particular cell type could drive the methylation pattern I observed. Neutrophils are likely candidates for altering cell proportions because neutrophil counts fluctuate during inflammation and neutrophils play a key role in the

pathogenesis of AAV (*92*). To this end, the role of neutrophil counts in disease prognosis has been investigated. A cohort of 64 patients with GPA studied in 1993 found leukocyte count to be a significant predictor of survival free of renal failure (*155*). However, a recent study by Hogan et al. reported that while a rise in neutrophil count in combination with C-reactive protein levels and PR3-ANCA titers was predictive of relapse, neutrophil count alone was not an informative biomarker (*156*). In contrast, our study shows that change in DNA methylation at the *PRTN3* promoter is sufficient to indicate the likelihood of relapse. In future studies testing the contribution of different cell types to DNA methylation changes in patients with AAV will elucidate critical cell populations responsible for the methylation changes I observed. Significant progress in generating comprehensive functional methylome maps of individual leukocyte subsets has already been made along with the identification of cell-subset specific hypomethylated regions that correlate with gene transcription levels (*157*).

I recognize the absolute changes in DNA methylation measured here are relatively small. It is possible that small cell populations could be driving DNA methylation changes in a heterogeneous population of leukocytes. Future measurement of longitudinal DNA methylation in healthy controls would establish a normal standard deviation over time that can be used as a threshold for determining aberrant methylation changes in patients. Development of a receiver operating characteristic curve would establish sensitivity and specificity of changes in DNA methylation prior to clinical implementation. A replication cohort of paired patients would allow for the validation of these findings. A recent publication on paired AAV patients was smaller in size and duration of follow-up (*158*), yet it is difficult to obtain access to a cohort with accompanying extensive clinical follow-up comparable to the one reported here. In the absence

of a replication cohort, a blinded validation study would need to be carried out to demonstrate the predictive value for *PRNT3* promoter methylation in patients with AAV.

Improved understanding of epigenetic alterations in AAV patients would increase the understanding of molecular mechanisms involved in disease pathogenesis and inform disease prognosis. Recent analysis of data collected from the rituximab versus cyclophosphamide/azathioprine for AAV (RAVE) trial found serial PR3-ANCA titers to be useful in anticipation of severe relapses; specifically in patients with renal involvement, alveolar hemorrhage or in patients treated with rituximab (*159*). There remains a need for a prognostic indicator that is applicable to AAV patients, as a whole. Previous work to identify potential biomarkers of disease relapse has been limited to cohorts comprised of patients with the same diagnosis or the same serotype (*4, 159*). Studies with paired patient samples and thorough clinical data are optimal for determining the value of a potential prognostic indicator.

To test whether changes in DNA methylation are associated with disease state and prognosis I studied a longitudinal cohort of heterogeneous AAV patients, including those with an MPA or GPA diagnosis as well as equal numbers of both ANCA serotypes, alongside their corresponding clinical information. Currently clinical criteria distinguish the disease status of patients; I characterized DNA methylation patterns at the autoantigen genes, *MPO* and *PRTN3,* and show DNA methylation changes further stratify disease remission status. In patients with ANCA-associated vasculitis I show DNA methylation changes at the *PRTN3* promoter are a potential biomarker of stable remission.

Chapter 2

THE SEARCH FOR CELL TYPES RESPONSIBLE FOR AUTOANTIGEN GENE EXPRESSION IN AAV

Despite the relative ease in isolating leukocyte DNA and RNA for methylation and expression studies, the limitations caused by alterations in cell heterogeneity are not to be overlooked. To this end, I focused significant research efforts on measuring DNA methylation in cell types responsible for expression of autoantigen genes in patients with AAV. Surprisingly, these studies led us to a cell type capable of high autoantigen gene expression and present in our CD4-enriched population, yet lacking CD3 and CD4 on the surface. Characterization of this cell type is still underway; here I present evidence that it is not a progenitor cell but possibly a subtype of monocyte.

Introduction

Our interest in neutrophils and monocytes in the setting of ANCA-associated vasculitis stems from the known aberrant expression of autoantigen genes, *MPO* and *PRTN3*, by these cell types (*8, 160*). Multiple experiments have shown that ANCA activate both neutrophils and monocytes and cause them to attack vessels, leading to vasculitis (*71*). ANCA bind antigens in the primary granules of neutrophils and the peroxidase-positive lysosomes of monocytes (*82*). A timeline of vascular and extravascular lesions in AAV would begin with neutrophil-rich

necrotizing inflammation; these neutrophils quickly undergo apoptosis and necrosis before being replaced by monocytes, macrophages and T cells (*82*). Monocytes, once activated by ANCAs, release pro-inflammatory cytokines monocyte chemoattractant protein-1 and interleukin 8 (IL-8, a neutrophil chemoattractant) (*91*). Cytoplasmic-ANCA (c-ANCA) plays an important role inducing monocyte IL-8 release by binding to monocyte PR3 and cross-linking Fcγ receptors; this process recruits and targets neutrophils. Previous research has suggested that epigenetic modifications associated with gene silencing may be dysfunctional and play a role in the aberrant transcription of *MPO* and *PRTN3* in mature neutrophils circulating in the peripheral blood (*10*).

Monocytes make up a mere 3-8% of leukocytes; any aberrations in DNA methylation or mRNA expression would likely be muted in studies of total leukocytes. Monocytes are involved with adaptive immunity and antigen presentation to activate T cells (*161*).

In addition to the highly studied roles of neutrophils and monocytes in the disease pathogenesis of AAV, this autoimmune response is facilitated by insufficient T cell regulation (*82*). Regulatory T cells (Tregs) are immunosuppressive and function to down-regulate the induction and proliferation of effector T cells. Tregs maintain tolerance to self-antigens and are identified by the surface markers: CD4, CD25 and Foxp3 (transcription factor forkhead box P3). Regulatory T cells prevent autoimmunity resulting from excessive activation of naïve and effector T cells, both of which respond to pathogens (*162, 163*). The transcription factor Foxp3 governs Treg development and function; mutations in or deletions of the gene encoding Foxp3, *FOXP3*, result in severe autoimmune disease along with failure to make CD25+CD4+ Tregs (*164, 165*). The promoter of human *FOXP3* contains several transcription factor binding sites including nuclear factor of activated T cells (NF-AT) and AP-1 (which is involved in a wide range of cellular processes). These transcription factor binding sites positively regulate *FOXP3*

expression after triggering the T cell receptor (TCR) (*166*). Within the non-coding part of *FOXP3*, there are CpGs that are demethylated in Tregs but methylated in naïve and effector T cells; there is an inverse occurrence of acetylated histones in these cell types (*167*). These studies provide evidence that the expression of *FOXP3* must be stabilized by epigenetic modifications to support the development of a permanent suppressor cell lineage of regulatory T cells.

Exploring the role of purified populations of cells isolated from the peripheral blood in disease pathogenesis is becoming more feasible as new platforms make isolating cells more accessible and EWAS becomes more widely utilized. There is a need for genome-wide DNA methylation data combined with gene expression analysis from purified cell subsets; to this end, a collaboration of research groups recently created a functional genome-wide methylome map of five different cell types isolated from healthy individuals (*168*). This research lays the groundwork for understanding where aberrant epigenetic profiles exist in a disease setting. Here, I examine the gene-specific DNA methylation profiles in neutrophils, monocytes and CD4+ T cells along with mRNA expression of these genes and are able to further corroborate these data in an autoimmune setting. The objective of this study was to characterize the DNA methylation patterns in purified cell types collected from the peripheral blood of paired active and remission AAV patients.

Materials and Methods

Study Design

AAV patients were enrolled at UNC–Chapel Hill clinics and followed in the Glomerular Disease Collaborative Network (*101, 102*). Patients and healthy volunteers were recruited, according to the guidelines of the Institutional Review Board (IRB study #97-0523) by the University of North Carolina Office of Human Research Ethics. Study subjects gave informed, written consent and participated according to UNC IRB guidelines. De-identified patient and healthy control samples were assigned randomly to plates for DNA methylation analysis and run in duplicate on separate plates. Investigators who quantified the results were blinded with regard to the type of patient or control being analyzed.

Patient Cohort

Patients were diagnosed according to the Chapel Hill Consensus Conference (*103, 104*). ANCA serotypes were determined by indirect immunofluorescence and antigen-specific PR3 and MPO ELISA (*105*). Disease activity was determined by the 2003 BVAS in conjunction with clinical signs of activity. In this study, patients with a BVAS of 0 and no clinical or laboratory evidence of active disease were considered to be in remission. Active disease was defined as a BVAS >0 with clinical and/or laboratory evidence of disease (*106*). A total of 32 patients with AAV and 13 healthy controls were included in this study based on the availability of paired active/remitting disease samples, clinical data and laboratory data. Patients with suspected or confirmed drug-induced forms of AAV, that were ANCA negative by ELISA, or had overlapping disease were excluded. Patients taking known epigenetic modifiers were also excluded from this study. Patient demographics were similar between healthy controls and AAV

patients with regard to age, gender and race and are a subset of the cohort described in Tables 1.1 and 1.4.

In these longitudinal studies I selected patients for whom samples were available at a time of disease activity and remission and were, on average, three months apart. These criteria were established prospectively. No outliers have been excluded from this study. In addition to the presence of DNA and RNA samples for each patient, I also ensured there was adequate clinical information including BVAS and a list of immunosuppressant therapies the patient was taking at the time of each sample collection.

Isolation of immune cell types

Peripheral blood was collected in two 10ml sodium heparin tubes (14-18mls of blood). For every 5mls whole blood, 1ml of HetaSep™ (Stem Cell Technologies) was added before centrifugation (92g, 6min, no brake). The nucleated cells above the red blood cell pellet were placed over Histopaque 1077 (Sigma) and centrifuged (400g, 30min, no brake). The buffy coat containing PBMCs was washed before isolating cell types using magnetic microbeads for cell sorting using. The Human CD14 Positive Selection Kit was used to sort out the CD14+ monocytes and then the CD4 T cell Enrichment Kit was used to sort the CD4+ T cells from the CD14-depleted PBMCs as previously described (EasySep™ , Stem Cell Technologies) (*168, 169*).

In parallel, neutrophils were isolated from the two red blood cell/granulocyte pellets remaining after the Histopaque spin. After washing, neutrophils were isolated from each of these pellets in a separate manner either for RNA extraction or DNA extraction. For DNA extraction, lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$, 0.1mM EDTA) was added to the pellet and incubated on ice for 30 minutes (*168*). After washing, 600µl RLT buffer from the AllPrep

DNA/RNA Mini Kit (Qiagen, Chatsworth, CA) was added to the neutrophil pellet (350µl for the monocyte and CD4 T cell-enriched pellets) and frozen at -80°C, according to the manufacturer's instructions. The Qiagen AllPrep DNA/RNA Mini Kit was used to isolate neutrophil DNA as well as both DNA and RNA from the CD14+ monocytes and the CD4-enriched T cells. For RNA extraction from the neutrophils, 5mls of DI water was added to the pellet and inverted several times to lyse the red blood cells, next the same volume of 2X PBS was added and the sample was mixed and centrifuged (300g, 10min). STAT-60 (Tel-Test "B", Friendswood, TX, USA) was added to the neutrophil pellet for RNA extraction.

Peripheral blood was also sampled from healthy individuals taking granulocyte colony-stimulating factor (G-CSF); these blood samples were processed as described above to isolate the CD4 T cell-enriched population for flow cytometry and mRNA expression studies.

DNA methylation studies

DNA from monocyte, neutrophil and T cell-enriched cell populations was bisulfite-converted in duplicate using the EZ DNA methylation kit (Zymo Research, Orange, CA). DNA methylation was measured in the bisulfite-treated DNA samples using the EpiTyper MassARRAY (Agena, La Jolla, CA). Here, targeted MALDI-TOF mass spectrometry (EpiTYPER®, Agena Bioscience) was carried out at three amplicons within *MPO* and *PRTN3*. Primer pairs were designed using EpiDesigner software (www.epidesigner.com) (Table 1.2). A cohort of 32 unique AAV patients and 13 healthy individuals were run on this platform in duplicate. In accordance with the standard protocol and following amplification of 650ng of bisulfite-converted DNA, the PCR products underwent the SAP treatment and T-cleavage reaction in preparation for quantitative analysis of DNA methylation. Mean DNA methylation was measured by averaging the CpGs in each individual amplicon.

Taqman mRNA expression studies

For quantitative RT-PCR, cell type RNA was analyzed from 15 PR3-ANCA patients, 17 MPO-ANCA patients and 13 healthy controls. Quantitative detection of *MPO* and *PRTN3* mRNA levels from patient samples was determined using a standard curve. The standard curve for *MPO* mRNA levels was generated using HL60 cells, a cell positive for *MPO* mRNA, diluted with Jurkat cells, a cell line negative for *MPO* mRNA. The standard curve for *PRTN3* mRNA levels was generated using THP-1 cells, a cell positive for *PTRN3* mRNA, diluted with Jurkat cells, a cell line negative for *PRTN3* mRNA. *MPO* and *PRTN3* mRNA levels for patients and healthy donor samples were determined by $2^{-\Delta\Delta Ct}$ calculations and expressed relative to standard curves. Primers and probes for *MPO* and *PRTN3* were previously published and Cytochrome c oxidase (*COX5B*) was used as mRNA internal control (*8*). Quantitative detection of *MPO* and *PRTN3* mRNA levels was determined by $2^{-\Delta\Delta Ct}$ calculations, with *COX5B* as the mRNA internal control, and expressed as fold change of reference control samples. Primers and probes were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). Quantitative RT-PCR assays were performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems), using the TaqMan EZ RT-PCR kit (Applied Biosystems).

Flow Cytometry

The CD4 T cell-enriched population was washed and resuspended in HBSS, 2% FBS, 0.1% sodium azide and stained with surface markers (Table 2.1). Cells were acquired on a BD LSRII and data was analyzed by FlowJo software (Tree Star, Inc.). After staining for CD3 and CD4, cells negative for both markers in the CD4 T cell-enriched population were sorted on a Becton Dickinson FACSAriaII equipped with FACSDiva 7 software (BD Biosciences). Cytospun samples were stained with hematoxylin and eosin.

Surface Marker	Source
CD1c	Biolegend
CD3	Biolegend
CD4	Biolegend
CD8	Biolegend
CD11b	Biolegend
CD11c	Biolegend
CD13	eBioscience
CD14	eBioscience
CD16	eBioscience
CD33	Biolegend
CD34	Biolegend
CD45	eBioscience
CD56	Biolegend
CD64	Biolegend
CD68	Biolegend
CD123	Biolegend
CD141	Biolegend
CD161	eBioscience
HLA-DR	BD Pharmingen

Table 2.1. Surface markers measured by flow cytometry in CD4-enriched population.

RNA Sequencing

The CD4-enriched populations collected from two longitudinal AAV patient pairs were RNA sequenced and differentially expressed genes were analyzed. I investigated differentially expressed genes in these pairs using Ingenuity Pathway Analysis (IPA) software package (Ingenuity Systems).

Statistical Analysis

Comparisons between two independent groups were done using Wilcoxon rank sum test. Bonferroni corrections were used in situations with multiple comparisons between groups. Mean DNA methylation was measured by averaging the CpGs in each individual amplicon. Methylation at individual CpGs was found to be either static or dynamic in a pattern mirroring that shown in the mean DNA methylation. Log transformed correlation for DNA methylation and the expression of autoantigen genes was analyzed by Spearman correlation coefficients.

Results

Increased *PRTN3* expression in monocytes and neutrophils of AAV patients

Using purified cell populations, I measured the expression of autoantigen genes, *MPO* and *PRTN3*, along with DNA methylation at loci within these genes. Previous research indicates aberrant expression of these genes stems from monocytes and neutrophils. Total leukocyte studies (Chapter 1) confirmed increased mRNA expression of both genes in some patients with active disease, compared to healthy individuals. In a small cohort of patients I see a trend of increased *MPO* expression in neutrophils isolated from AAV patients during disease activity (Figure 2.1A). There is a 7.5-fold increase of *PRTN3* mRNA expression in neutrophils isolated from AAV patients with active disease, compared to healthy individuals (Figure 2.1C). In monocytes there is no difference in *MPO* mRNA expression between healthy controls and active AAV patients, though remission patients do show a trend of increased expression (Figure 2.1B). Expression of *PRTN3* is increased five-fold in monocytes isolated from active AAV patients (Figure 2.1D). Thus, there is a significant increase of *PRTN3* expression in both neutrophils and monocytes isolated from patients with active disease.

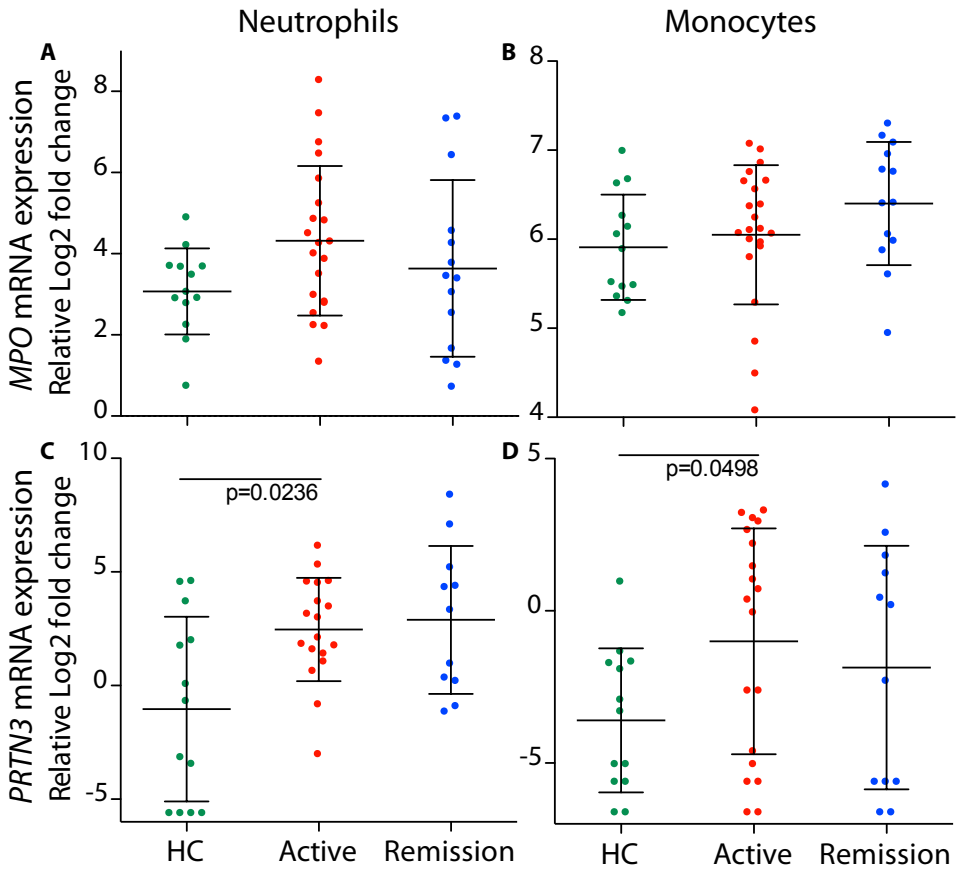

Figure 2.1. Relative *MPO* and *PRTN3* expression in monocytes and neutrophils. Relative *MPO* expression of healthy controls (green), active patients (red) and remitting patients (blue) in (**A**) neutrophils and (**B**) monocytes. Relative *PRTN3* expression in (**C**) neutrophils and (**D**) monocytes. Bars shown are mean and standard deviation; only $p<0.05$ are considered significant and shown.

Static DNA methylation of neutrophils at the *PRTN3* promoter and CGI/exon 5

I measured DNA methylation in purified neutrophils and monocytes at three of the same loci interrogated in total leukocytes: the *PRTN3* promoter, *PRTN3* CGI/exon 5 and *MPO* CGI/exon 7. At the promoter of *PRTN3* I found less than ten percent methylation in neutrophils,

with few variations between healthy controls and patients with AAV (Figure 2.2A). Despite an increase in relative expression of *PRTN3* mRNA in neutrophils (Figure 2.1C) there was no correlation between methylation and expression in neutrophils within this small patient cohort (Figure 2.2C). DNA methylation of CD14+ monocytes at the PRTN3 promoter was only slightly higher than neutrophils, but here AAV patients appear to be hypomethylated compared to healthy controls (Figure 2.2B); this pattern mirrors that seen in total leukocytes (Figure 1.1D). At the *PRTN3* promoter, DNA methylation and *PRTN3* mRNA expression correlate in monocytes, despite the relatively small cohort (Figure 2.2D).

Comparable to the *PRTN3* promoter, DNA methylation of neutrophils at *PRTN3* CGI/exon 5 is low and unchanging between AAV patients and healthy controls (Figure 2.3A). DNA methylation at this locus in *PRTN3* does not correlate with *PRTN3* mRNA expression (Figure 2.3C). Monocyte DNA methylation in patients with AAV is decreased when compared to healthy controls and *PRTN3* mRNA expression correlates with the DNA methylation (Figure 2.3B,D). Together these preliminarily data indicate that while both neutrophils and monocytes isolated from AAV patients are capable of expressing *MPO* and *PRTN3*, DNA methylation is only dynamic in monocytes at loci within *PRTN3* and this methylation correlates well with mRNA expression of *PRTN3*.

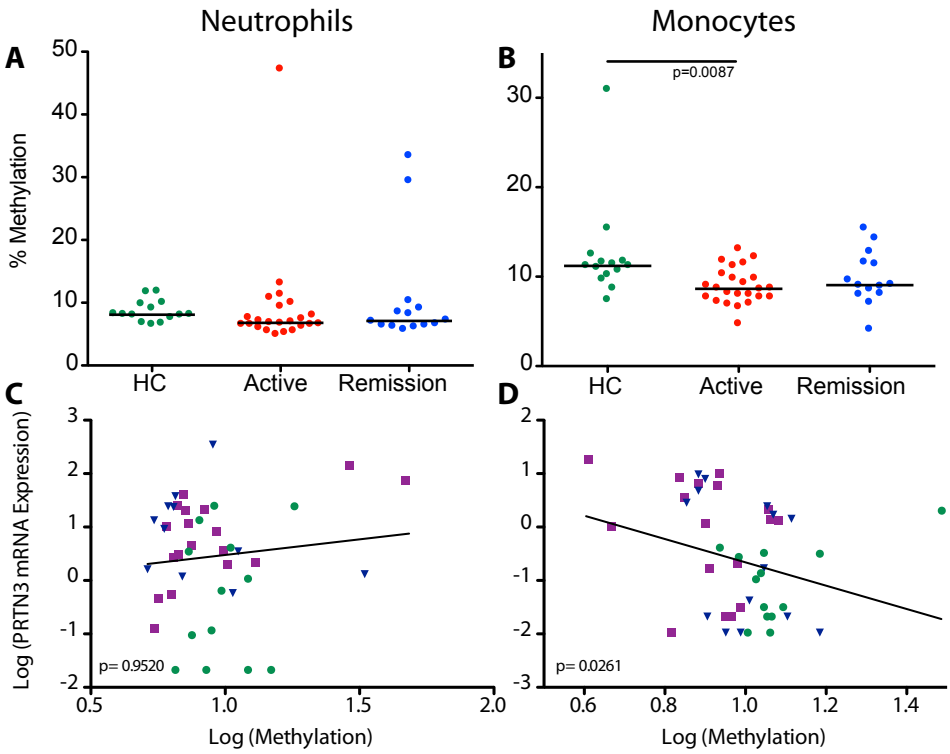

Figure 2.2. Neutrophil and monocyte methylation and expression at *PRTN3* promoter. Mean cross-sectional methylation at *PRTN3* promoter in (**A**) neutrophils and (**B**) monocytes. Healthy controls (green circles), MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares). Log transformed correlation between methylation at the *PRTN3* promoter and *PRTN3* mRNA expression in (**C**) neutrophils (n=42; r= -0.0096) and (**D**) monocytes (n=45; r= -0.3315).

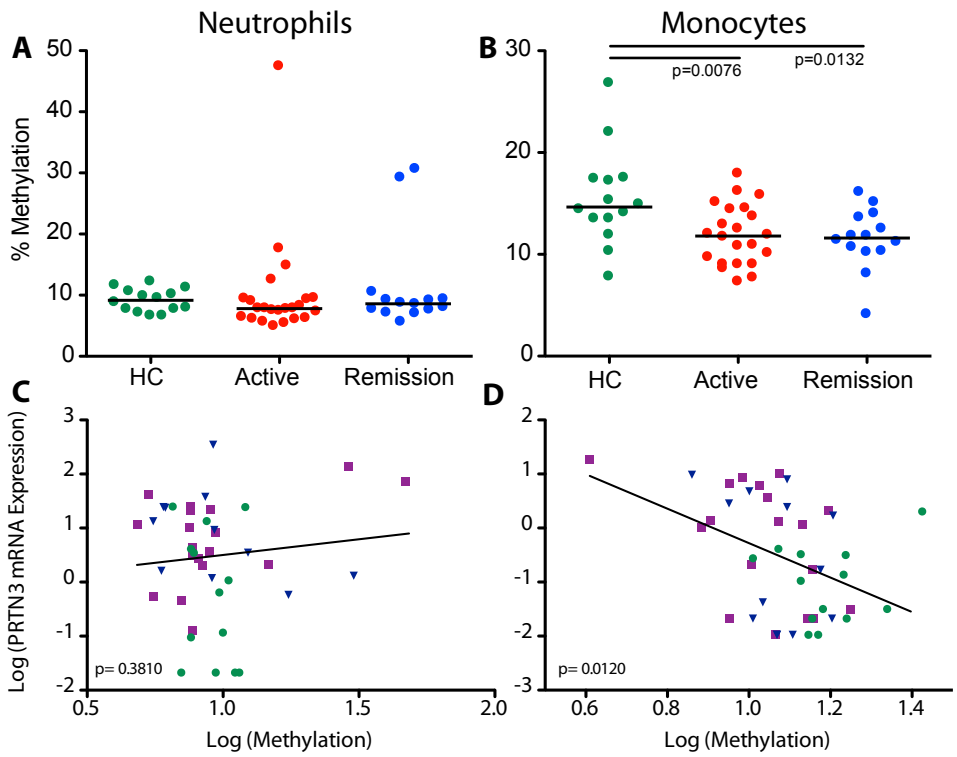

Figure 2.3. Neutrophil and monocyte methylation and expression at *PRTN3* CGI/exon 5. Mean cross-sectional methylation at *PRTN3* CGI/exon 5 in (**A**) neutrophils and (**B**) monocytes. Healthy controls (green circles); MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares). Log transformed correlation between methylation at *PRTN3* CGI/exon 5 and *PRTN3* mRNA expression in (**C**) neutrophils (n=42; r= -0.1387) and (**D**) monocytes (n=44; r= -0.3756).

Neutrophil and monocyte DNA methylation at *MPO* CGI/exon 7 does not correlate with expression

Previously I found dynamic DNA methylation at *MPO* CGI/exon 7 in leukocytes isolated from AAV patients compared to healthy controls. The methylation at this locus correlated with

MPO mRNA expression in total leukocytes. DNA methylation in purified neutrophils at *MPO* CGI/exon 7 was nearly identical to methylation at the *PRTN3* loci, low and unchanging between patients and healthy controls (Figure 2.4A); here the DNA methylation did not correlate with *MPO* mRNA expression (Figure 2.4C). In monocytes there is a significant decrease in DNA methylation in active AAV patients compared to healthy controls at *MPO* CGI/exon 7, despite the overall methylation remaining low compared to total leukocytes (Figure 2.4B). Given that MPO mRNA expression did not change between healthy controls and patients (Figure 2.1B) it is not surprising that DNA methylation at *MPO* CGI/exon 7 does not correlate with expression of this gene (Figure 2.4D).

CD4-enriched population shows high autoantigen gene expression

The third cell type I isolated from AAV patients was CD4+ T cells; these cells were negatively enriched from a CD14-depleted population. While purity of this population was high (>90%) in healthy individuals and most patients in disease remission, the quality of the isolation was diminished in many patients with active disease and for this reason I refer to this fraction as CD4-enriched cells. I measured mRNA expression of *PRTN3* in these cells and saw a significant 15-fold increase in expression in AAV patients with active disease compared to healthy controls (Figure 2.5A). The *PRTN3* promoter was >40% methylated in these cells and active patients tended to have less methylation than healthy controls (Figure 2.5B). The *PRTN3* mRNA expression correlated well with DNA methylation at the promoter indicating that the cells in this fraction may contribute to the correlation seen in total leukocytes (Figure 2.5C).

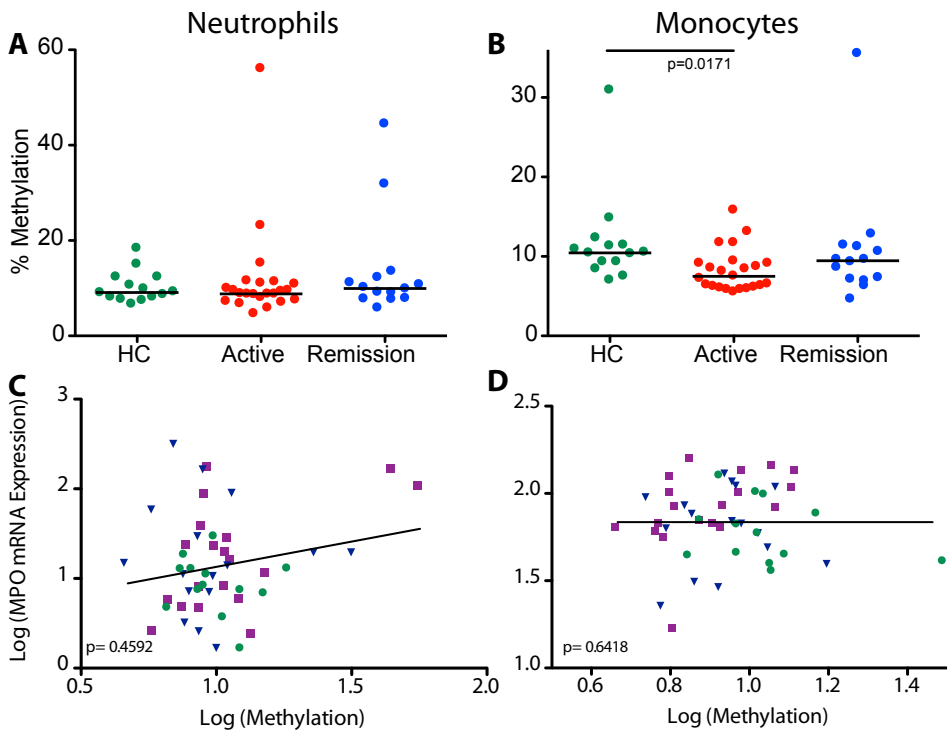

Figure 2.4. Neutrophil and monocyte methylation and expression at *MPO* CGI/exon 7. Mean cross-sectional methylation at *MPO* CGI/exon 7 in (**A**) neutrophils and (**B**) monocytes. Healthy controls (green circles), MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares). Log transformed correlation between methylation at *MPO* CGI/exon 7 and *MPO* mRNA expression in (**C**) neutrophils (n=48; r= 0.1094) and (**D**) monocytes (n=48; r= 0.06887).

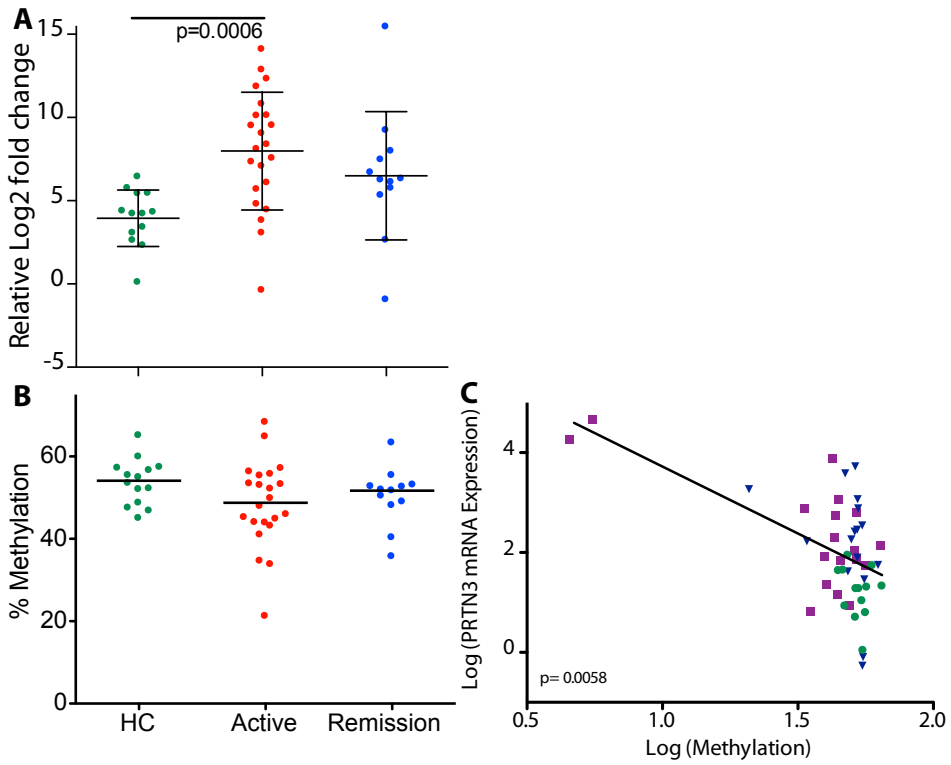

Figure 2.5. DNA methylation and expression in the CD4-enriched population. (**A**) Relative *PRTN3* mRNA expression in the CD4-enriched population for healthy controls compared to AAV patients. Bars are mean ± standard deviation. (**B**) Mean cross-sectional methylation at the *PRTN3* promoter in the CD4-enriched population. (**C**) Log transformed correlation between methylation at the *PRTN3* promoter and *PRTN3* mRNA expression of the CD4-enriched population (n=47; r=-0.3964). Healthy controls (green circles), MPO-ANCA patients (blue triangles) and PR3-ANCA patients (purple squares).

CD4-enriched population does not contain progenitor-like cells

Cell sorting the CD4-enriched population isolated from active AAV patients prior to measuring mRNA expression in each of the sub-populations confirmed the high expression of autoantigen genes, *MPO* and *PRTN3,* was originating from a cell lacking both CD3 and CD4 on the surface and was thus not a T cell. This population of CD3-/CD4- cells present in our CD4 enrichment was both small (anywhere from 4-20% of the cells in the CD4 enrichment from active AAV patients) and confounding. Not all AAV patients with active disease have high expression of the autoantigen genes, even when measuring expression in total leukocytes. Additionally, the population of CD3-/CD4- cells in healthy individuals is a mere 7% of the total population of enriched T cells and does not express *MPO* or *PRTN3*. To further study this population of *MPO* and *PRTN3* expressing cells I identified a population of healthy donors treated with granulocyte colony-stimulating factor (G-CSF) as a possible surrogate sample source. G-CSF is a glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells and then release them to the peripheral blood; it also stimulates the proliferation and differentiation of neutrophils (*170*).

The CD4-enriched cell fraction was isolated from G-CSF donors using the same protocol as for AAV patients and the cells were stained with surface markers (Table 2.1) and quantified using flow cytometry. At first glance, G-CSF donors appeared to be an adequate surrogate for studying these CD3-/CD4- cells as the CD4-enriched population showed consistently high expression of the autoantigen genes and the size of the CD3-/CD4- cell fraction was comparable to active AAV patients (19% in G-CSF donors, 17.8% in AAV patients) (Figure 2.6A,F,K; Table 2.2). However, G-CSF samples were found to express approximately 20 times the level of *MPO* and *PRTN3* seen in AAV patients with active disease; further analysis with surface cell markers

show the presence of progenitor-like cells staining for CD33, CD34 and CD13 (not shown) in the CD3-/CD4- fraction sorted from G-CSF donors; AAV patients and healthy individuals contained minimal staining for all three of those markers (Figure 2.6B,C,G,H,L,M; Table 2.2). Cytospinning the CD3-/CD4- fraction of this cell population in G-CSF donors revealed a heterogeneous population containing cells that resemble monocytes yet none of these sample sources showed significant staining for canonical monocyte surface markers, including CD14 (Figure 2.7). Together, these data indicate that the CD3-/CD4- fraction of cells in G-CSF donors were likely progenitor cells and that AAV patients with active disease do not contain progenitor cells in the CD4-enriched isolation.

In an effort to characterize the CD3-4- fraction in the CD4-enriched population of AAV patients with active disease I sought to determine if these cells contained surface markers characteristic of monocytes, granulocytes or peripheral blood dendritic cells (Table 2.1). Of five active AAV patients analyzed, the CD3-/CD4- population contained an average of 27.4% HLA-DR^+ cells (human leukocyte antigen, D related) (range: 5-69%) and 57.8% $CD45^+$ cells (range: 40-76%) (Figure 2.6D,E,I,J,N,O; Table 2.2). I saw up to 66% of the CD3-/CD4- population stain double positive for HLA-DR and CD45 (not shown). Given the prior positive selection for CD14 monocytes, it seemed unlikely that the CD4-enriched population would contain CD14+ monocytes and I was able to confirm minimal CD14 and CD16 surface staining by flow cytometry. At this point I conclude that the CD3-/CD4- fraction of cells found in the CD4-enriched population contains heterogeneous staining for CD45 and HLA-DR; however, limitations in the sample size and population size of these cells make determining which sub-population expresses *MPO* and *PRNT3* mRNA very challenging.

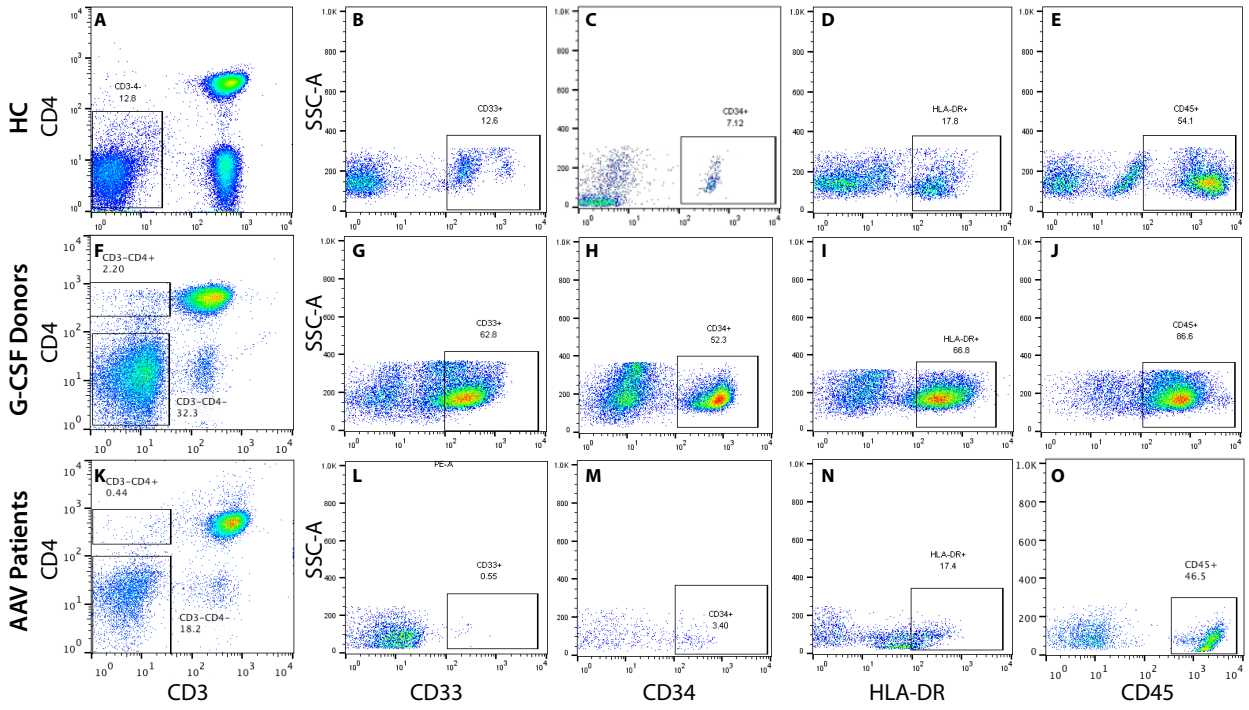

Figure 2.6. Flow cytometry surface staining in CD4-enriched populations. (**A-E**) Healthy controls, (**F-J**) G-CSF donors, (**K-O**) AAV patients with active disease. (**A,F,K**) CD3-/CD4- population in each sample type; (**B,G,L**) CD33 staining; (**C,H,M**) CD34 staining; (**D,I,N**) HLA-DR staining; (**E,J,O**) CD45 staining.

Sample Source	N	$CD3^-/CD4^-$ cells	CD33	CD34	HLA-DR	CD45	Ave *MPO* mRNA Exp	Ave *PRTN3* mRNA Exp
HC	6	7.3	15.3	7.1	25.0	72.0	30.0	15.9
G-CSF	7	19.0	61.5	57.0	40.0	87.0	16,708.4	13,035.6
AAV	5	17.8	1.0	2.0	27.4	57.8	811.4	649.8

Table 2.2. CD4-enriched population in different sample sources. The CD4-enriched population in healthy individuals, G-CSF donors and AAV patients was investigated using flow cytometry to determine the size of and surface markers on the CD3-/CD4- cells; numbers shown are percentages of the CD3-/CD4- population. The expression of *MPO* and *PRTN3* mRNA in the total CD4-enriched population were measured.

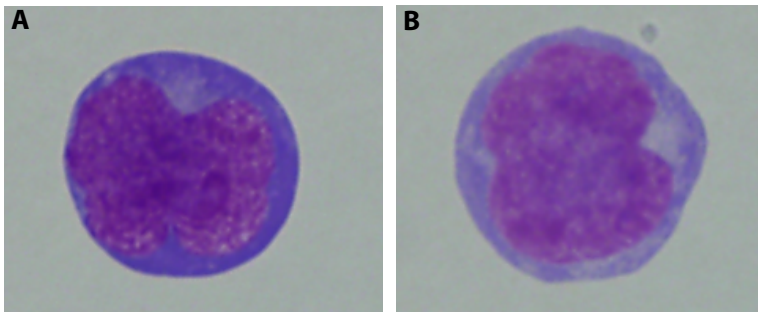

Figure 2.7. Cells from the CD3-/CD4- fraction isolated from G-CSF donors.

RNA sequencing of paired CD4-enriched samples in patients with AAV

After determining that G-CSF donors were not an adequate surrogate for studying the CD3-/CD4- fraction present in the CD4-enrichment I searched for a platform that would allow us to quantify gene expression changes between paired AAV patient samples at times of disease activity and remission. I sequenced two paired CD4-enriched RNA samples in which the expression of *MPO* and *PRTN3* was high in the active samples. This small pilot study allowed for the confirmation of active *MPO* and *PRTN3* transcripts unique to AAV patients with active disease in this CD4 enriched population (Figure 2.8A,B). Expression of *CD4* in this population remained constant regardless of disease activity in these AAV patients (Figure 2.8C). RNA sequencing revealed which genes were most differentially expressed between disease states in these two paired patients. Ingenuity Pathway Analysis was utilized to create a preliminary map of pathways either up-regulated or down-regulated in the differentially expressed genes (Figure 2.9).

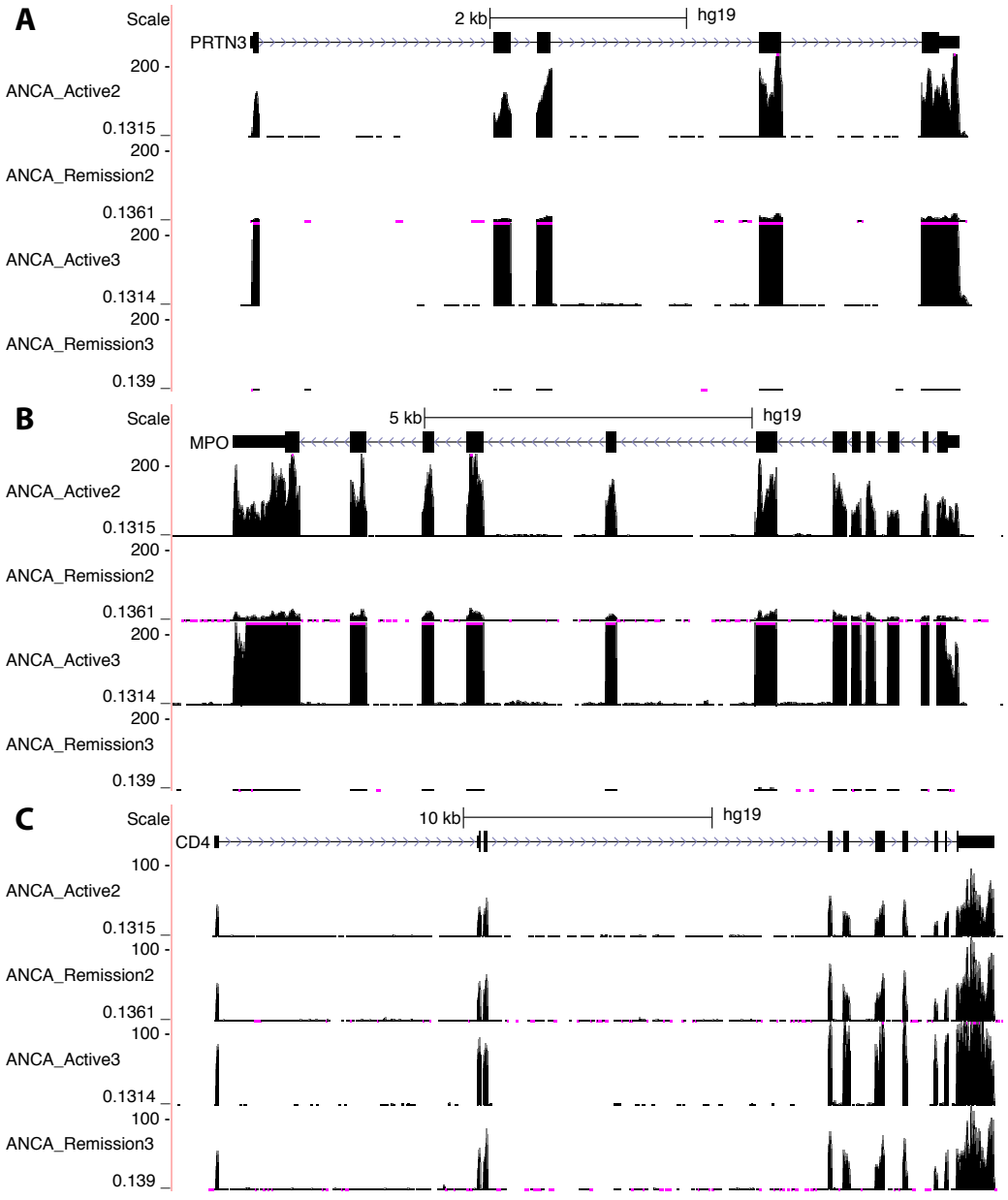

Figure 2.8. RNA sequencing of paired CD4-enriched populations from AAV patients. These browser images show gene expression in the CD4-enriched population isolated from two paired AAV patients in states of disease activity and remission for (**A**) *PRTN3*, (**B**) *MPO* and (**C**) *CD4* in the form of mapped read profiles from RNA-seq data.

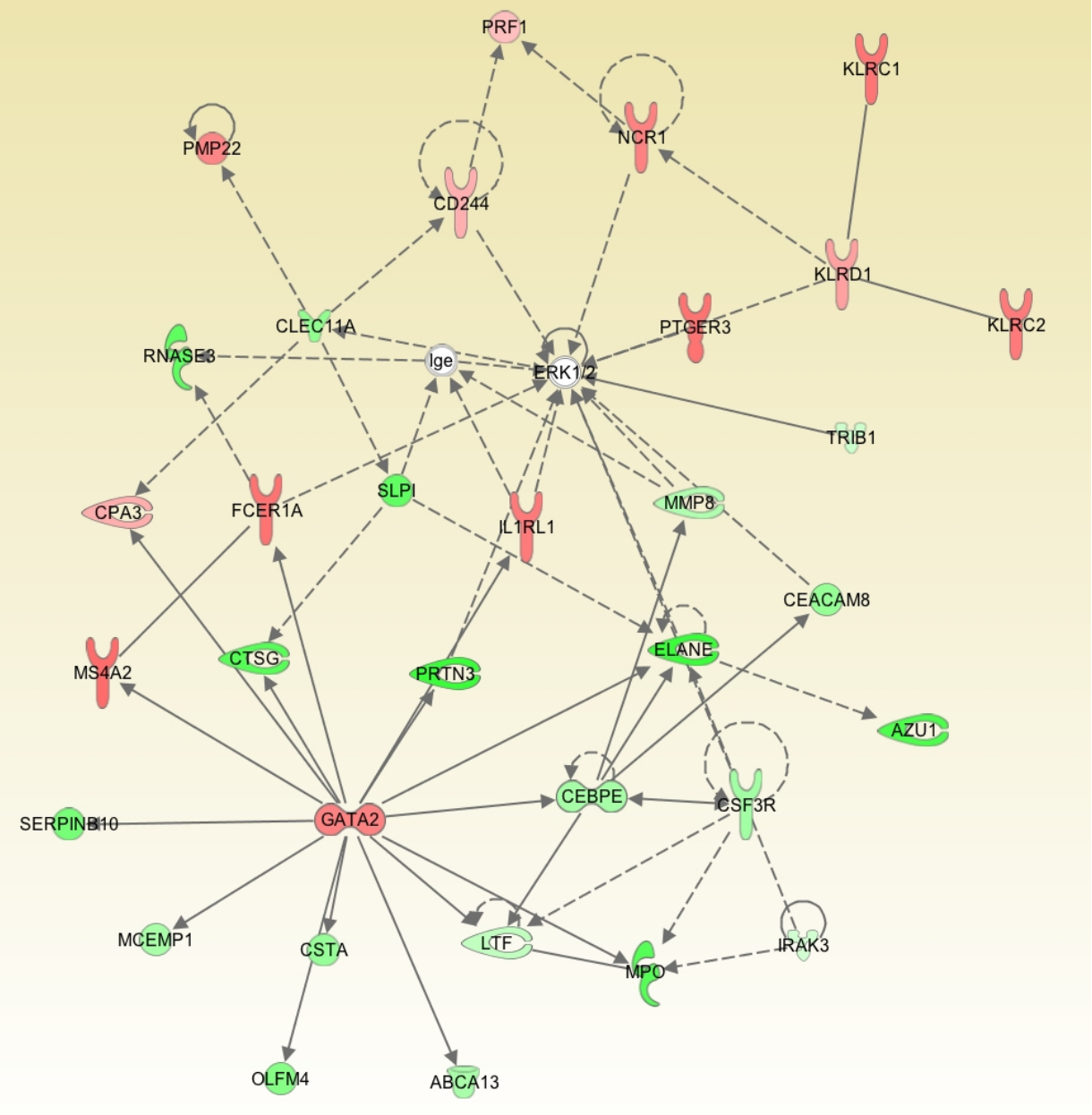

Figure 2.9. Pathway analysis of CD4-enriched populations from AAV patients. Longitudinal AAV pairs from two patients exhibiting high autoantigen gene expression corresponding disease activity. The differentially expressed gene signatures were mapped showing down-regulated genes (green) and up-regulated genes (red). Ingenuity Pathway Analysis software package (Ingenuity Systems).

Discussion

Studies on purified cell populations are needed to elucidate the complete pathogenesis of AAV and the role of epigenetic modifications. There is ease in the collection and isolation of leukocytes, but dynamic alterations in cell heterogeneity, as a product of both disease pathogenesis and therapeutic interventions, add a level of uncertainty to these studies. The combination of known aberrant expression of autoantigen genes, *MPO* and *PRTN3*, by neutrophils and monocytes in patients with AAV (*8, 160*), combined with limitations in blood volume from patients led us to focus on isolating neutrophils, CD14+ monocytes and CD4+ T cells from a small cohort of AAV patients at times of disease activity and remission. I measured expression of *MPO* and *PRTN3* along with DNA methylation at these genes in each of these three populations.

Neutrophils generally make up a majority of leukocytes, but the DNA methylation at *MPO* and *PRTN3* in these cells does not correlate with the expression of these autoantigen genes. Thus, DNA methylation in neutrophils is likely not driving the aberrant autoantigen gene expression characteristic of AAV. It remains possible that histone modifications may impact the expression of *MPO* and *PRTN3* seen in neutrophils from patients with AAV. Static, low DNA methylation in neutrophils suggests that this large cell population is muting the dynamic DNA methylation patterns occurring in other cell populations.

While CD14+ monocytes comprise a significantly smaller fraction of total leukocytes than neutrophils, elevated *PRTN3* expression has been reported in monocytes from patients with AAV (*160*). The DNA methylation at both *PRTN3* loci, the promoter and the CGI overlapping exon 5, is decreased in patients with active disease and correlates with mRNA expression of *PRTN3*. These data suggest that DNA methylation of *PRTN3* in CD14+ monocytes may impact

the expression of *PRTN3* in patients with AAV. Comprehensive methylome maps of various murine hematopoietic cell populations showed that myelopoiesis and lymphopoiesis result in different terminal DNA methylation patterns (*171*). Myeloid commitment is characterized by less global DNA methylation than lymphoid commitment, a finding I corroborate at gene-specific locations.

Perhaps most surprisingly, I saw extremely high mRNA expression of both *MPO* and *PRTN3* in the CD4-enriched population, isolated from patients with AAV during disease activity. In this enriched population I saw a correlation between mRNA expression of *PRTN3* and DNA methylation at the *PRTN3* promoter. While dysregulation of T cells in AAV patients has been studied in the past, these cells are not known to express these autoantigen genes. Further studies revealed the presence of a CD3-/CD4- fraction of cells in this T cell-enriched population expressing *MPO* and *PRTN3*. While this fraction is expanded in active patients, not all patients express these genes during disease activity. Ultimately, I found considerable differences between this CD3-/CD4- fraction of cells within the CD4-enriched population isolated from active AAV patients and G-CSF donors. The G-CSF donors contain a heterogeneous population that included cells with progenitor surface markers; while the cells in this CD3-/CD4- fraction from patients with AAV show minimal surface expression of these markers.

Recent studies exploring the prevalence of low density granulocytes has described these cells as a source of granulocyte gene expression signatures in PBMCs isolated from AAV patients with active disease as well as SLE patients (*172, 173*). Flow cytometry isolation of these cells has defined them as CD14low with either CD15 or CD10 surface staining. It is possible that the cell type I have isolated may be a low density granulocyte as I have seen minimal CD14 surface staining and have not measured CD15 or CD10 by flow cytometry.

DNA methylation by DNMT1 has been found to have a direct role in regulation of the self-renewal of HSCs and the commitment to lymphoid lineages (*174, 175*). Multiple studies have been carried out characterizing the specific epigenetic modification patterns that correlate with active and repressed chromosomal regions in hematopoietic stem cells (HSCs) and differentiated cell populations (*176-178*). One such study isolated CD34+ HSCs, CD14+ monocytes and neutrophils from cord blood and compared them to CD34+ HSCs isolated from adult G-CSF donors. These and other studies identify specific epigenetic regulated pathways associated with HSC differentiation and provide evidence that these cells use DNA methylation to silence the myeloid differentiation program (*174, 175, 178*). These studies can also help to explain why G-CSF donors are not an adequate surrogate sample source in studies of patients with active AAV.

After confirming that the CD3-/CD4- fraction of the CD4-enriched population in AAV patients is distinct from that seen in G-CSF donors, I worked to further characterize the surface markers on this sub-population of interest. Additional surface staining and flow cytometry confirmed the presence of a wide range of CD45+ and HLA-DR+ surface staining on the heterogeneous fraction of CD3-/CD4- cells in active AAV patients. Given the prior CD14 positive selection, I was able to confirm a lack of CD14+ monocytes in the CD4-enriched population, by flow cytometry. Our efforts to determine which sub-population expressed *MPO* and *PRTN3* mRNA was limited by the population size of these cells and blood volume that could be collected from patients with active AAV.

The identification of cell-subset-specific hypomethylated regions (HMRs) that correlate with gene transcription levels suggests HMRs may regulate corresponding cell functions. DNA methylation impacts cellular differentiation during hematopoiesis; myeloid commitment is

associated with less global DNA methylation than lymphoid cells (*171, 179*). In turn, the commitment of a cell to a lymphoid lineage is associated with higher levels of DNA methylation. Given the impact of DNA methylation on gene expression, the extent of hypomethylation may reflect a degree of transcriptional plasticity. In healthy adults, neutrophils are fully differentiated and have stable transcriptional profiles and are characterized by extensive genome-wide hypomethylation compared to lymphoid cells. Lymphoid lineages generally encompass a more heterogeneous population that includes naïve cells with the ability to further differentiate and are characterized by much higher genome-wide methylation than neutrophils.

Future studies would include collection of these purified cell populations from additional paired patients with AAV for epigenome-wide DNA methylation studies in neutrophils and monocytes along with additional RNA sequencing of the CD4-enriched populations followed by a pathway analysis. Preliminarily, I have begun to utilize a single-cell gene expression platform (Fluidigm C1) to identify the individual cells in this CD4 enrichment containing high *MPO* and *PRTN3* expression from this patient population; however, obtaining the ideal active AAV patient for this experiment early enough to allow for time to process the sample and cell sort the CD3-/CD4- population is logistically challenging.

Here I provide evidence that neutrophil expression of *MPO* and *PRTN3* is not regulated by DNA methylation; it remains likely that histone modifications play a more substantial role, as previously documented by our group (*10*). I show preliminary evidence of two separate populations of cells with aberrant expression of *MPO* and *PRTN3* as well as altered DNA methylation profiles at loci within *PRTN3*. These CD14+ monocytes and the CD3-/CD4- fraction of cells found in the CD4-enriched isolation could be driving the altered DNA methylation profiles seen in total leukocyte studies of Chapter 1. In a small cohort of patients I was able to

identify this CD3-/CD4- fraction of cells as a heterogeneous population containing a range of CD45 and HLA-DR on the surface. HLA-DR is a major histocompatibility complex (MHC) class II cell surface receptor used by antigen presenting cells to present protein fragments (processed antigen) to T cells. In healthy individuals, HLA-DR is found on a number of cells including B cells, dendritic cells, macrophages and monocytes as well as precursor T cells and CD4+ T cells. CD45, the leukocyte common antigen, is an essential regulator of T cell antigen receptor-mediated activation; CD45 is found on a wide range of cell types including granulocytes, lymphocytes, macrophages, monocytes and dendritic cells. Together these markers appear to suggest this cell fraction could include monocytes, though I was unable to identify any other monocyte surface markers via flow cytometry in this patient population.

SUMMARY

The presented body of work has focused on the role of DNA methylation in the pathogenesis of ANCA-associated vasculitis. Chapter 1 characterizes DNA methylation alterations at AAV-related autoantigen genes, *MPO* and *PRTN3,* in peripheral blood leukocytes. This longitudinal analysis showed that DNA methylation at *MPO* and *PRTN3* was reduced in patients with active disease and associated with mRNA expression of these genes. Remarkably, I discovered that gene-specific DNA methylation changes are associated with disease remission and relapse. Dynamic DNA methylation patterns subdivided a longitudinal cohort of patients into those who increased methylation while in remission and those who decreased methylation. A Kaplan-Meier estimate of relapse showed that patients who increased DNA methylation while in remission, at the *PRTN3* promoter, had a significantly greater probability of maintaining stable remission, while patients who decreased DNA methylation were more likely to relapse. Thus, the DNA methylation status at the *PRTN3* promoter may be a prognostic marker useful in managing patient therapy. This finding is important beyond the ANCA vasculitis community and may be applicable to other autoimmune diseases where the autoantibody and autoantigen are known.

Chapter 2 describes DNA methylation patterns at *MPO* and *PRTN3* in purified monocytes and neutrophils as well as a CD4-enriched population isolated from patients with AAV. Both monocytes and neutrophils were found to have low DNA methylation at these two genes and increased *PRTN3* mRNA expression in patients with active disease. Monocytes were hypomethylated at both genes in patients with active AAV compared to healthy controls. At both loci interrogated in *PRTN3*, DNA methylation in monocytes correlated with the mRNA expression of *PRTN3*. These data suggest that DNA methylation does not regulate AAV-related

autoantigen gene mRNA expression in neutrophils of patients with AAV; instead histone modifications may play a larger role in regulating *MPO* and *PRTN3* expression in neutrophils.

The presence of a CD3-/CD4- cell fraction in the CD4-enriched population with very high expression of both *MPO* and *PRTN3* indicate the possibility of an additional cell population contributing to disease pathogenesis. High DNA methylation at the *PRTN3* promoter in the CD4-enriched population shows trends of decreased methylation in patients with active disease. Additionally, the expression of *PRTN3* correlated well with DNA methylation at the *PRTN3* promoter in this CD4-enriched population. Thus, there is reason to believe that dynamic DNA methylation in both monocytes and a CD3- CD4- cell type found in our CD4-enriched population are contributing to the methylation changes seen in leukocytes studied in Chapter 1.

EPILOGUE: DYNAMIC GENE-SPECIFIC DNA METHYLATION IN PATIENTS WITH AAV

Altered leukocyte DNA methylation profiles in AAV

There are a number of common threads that run through autoimmune disorders, with regard to mRNA expression and DNA methylation. The alterations in mRNA expression and DNA methylation I describe in a cohort of patients with AAV have been described at different genes in other autoimmune disorders. Differential *DNMT1* mRNA expression has previously been described in lupus, rheumatoid arthritis and multiple sclerosis (*64*). Promoter demethylation has been described in both PBMCs and cells isolated from the synovial fluid of patients with RA (*180*) and there is evidence for altered DNA methylation in MS as well (*68*). Cell-specific studies cite aberrant DNA methylation profiles in CD4+ T cells of SLE patients (*121, 123, 124*) and Tregs of RA patients (*129*). Thus, it is likely that the findings in Chapter 1 will contribute to a greater understanding of the etiology or prognosis of autoimmunity.

There are both genetic and environmental components involved in the pathogenesis of lupus, RA and MS; it is likely that these environmental components include epigenetic mechanisms. The past ten years have seen an increased focus on elucidating the role of epigenetic mechanisms in disease etiology in hopes that these studies can provide answers where genetics has fallen short.

In a subset of patients with AAV, therapy-induced disease remission is punctuated by periods of disease relapse characterized by DNA hypomethylation at the autoantigen genes and increased mRNA expression of these genes. While specific factors that promote remission or permit disease relapse remain unknown, our longitudinal studies find patients that do not increase DNA methylation at the *PRTN3* promoter upon disease remission are significantly more

likely to relapse. There is a strong correlation between DNA methylation and mRNA expression of *MPO* and *PRTN3* in patients that increase DNA methylation upon disease remission indicating that DNA methylation likely impacts the regulation of autoantigen expression.

Purified cell types with the potential to drive methylation changes in ANCA disease

As much as I aim to design logical, hypothesis-driven experiments, sometimes the most exciting part of research are the unexpected findings. In Chapter 2, I set out to elucidate the role of DNA methylation in purified cell types responsible for autoantigen gene expression by isolating neutrophils and CD14+ monocytes. As hypothesized, CD14+ monocytes show dynamic DNA methylation profiles at loci within the autoantigen genes; *PRTN3* mRNA expression correlated particularly well with DNA methylation at the two *PRTN3* loci interrogated. From this small patient cohort it is easy to speculate that these monocytes are contributing to the dynamic DNA methylation profiles described in the total leukocyte studies of Chapter 1. Preliminarily unchanging neutrophil DNA methylation, despite aberrant autoantigen gene expression in patients with active disease, suggests that perhaps another epigenetic pathway is responsible for the autoantigen gene expression characteristic of neutrophils in AAV. Indeed, I repeatedly mention studies by our own research group demonstrating a role for histone modifications upregulating gene expression; thus, it is possible that different epigenetic mechanisms are responsible for aberrant autoantigen gene expression in monocytes and neutrophils isolated from AAV patients.

Our interest in CD4+ T cells stems both from previous work by researchers in our group corroborating dysregulation of these cells in AAV as well as a number of studies supporting altered DNA methylation in T cells isolated from other autoimmune disorders. The high DNA

methylation A describe at the *PRTN3* promoter of a CD4-enriched population is consistent with previous genome-wide DNA methylation studies (*171*). Despite confirming less pure enrichments in samples from patients with high disease activity, it is unlikely the drop in purity would impact these DNA methylation studies. However, the decreased purity of CD4+ T cells in the CD4-enriched population isolated from AAV patients with active disease corresponded with an expansion of CD3- CD4- cells with the ability to express high levels of autoantigen genes. Chapter 2 contains only the challenging beginnings of how to characterize the cell population present in this CD4-enrichement, but lacking both CD3 and CD4 on the surface. I was able to rule out the possibility that this cell type was a progenitor by comparing this population to that isolated from G-CSF donors. Exhaustive surface staining revealed a heterogeneous population present in this CD3-4- fraction containing a mixture of CD45 and HLA-DR. Given these findings, it is tempting to assume this cell is a monocyte, or at least 'monocytoid,' particularly given that the cells isolated from the G-CSF donors appear to be monocytes after cytospinning; yet all of the canonical monocyte markers tested were negative.

The known characteristics of these cells matches well with previous descriptions of low density granulocytes that cluster with PBMCs upon density centrifugation and contain a granulocyte gene expression profile. These low density granulocytes were first documented in patients with active AAV last year, after previously being reported in SLE patients (*172, 173*). This hypothesis could be tested with additional flow surface staining for CD10 and CD15 in the CD4-enriched population isolated from active AAV patients exhibiting high autoantigen gene expression.

Future directions

The unifying theme of epigenetics in disease is the disruption of normal phenotypic plasticity. Our understanding of the role of epigenetics in AAV is still preliminary; the goal of these and studies in other autoimmune disorders extends beyond characterization of aberrant epigenetic mechanisms and into the realm of immunotherapies including epigenetic modulators. One intensively studied approach to epigenetic therapy involves the use of agents that modify the epigenome globally, like inhibitors of DNA methylation or histone acetylation; however, it is likely that targeted epigenetic modifiers will be needed for these types of therapeutic approaches. DNMT inhibitors are already being investigated in the setting of cancer treatment, specifically azacitidine and decitabine for the treatment of acute myeloid leukemia (*181*). DNMT1 inhibitors could exacerbate AAV, making this disease setting a logical place to begin measuring DNA methyltransferase expression.

Future studies relevant to Chapter 1 would need to confirm the DNA methylation at the *PRTN3* promoter seen in leukocytes from paired AAV patients either via a prospective study or a replication cohort at another institution. Ideally, this study would generate enough statistical power to develop a receiver operating characteristic curve to establish sensitivity and specificity of these DNA methylation changes that support clinical implementation. Additional longitudinal pairs would need to be measured for a receiver operating characteristic curve; a curve generated from the present data divided by methylation change at the *PRTN3* promoter shows an area of 0.65 under the curve (90% CI 0.5004, 0.7996; p=0.0799). The 65 patient pairs favor those with increased methylation (n=50), thus additional pairs with decreased DNA methylation at the *PRTN3* promoter would be needed for future analysis utilizing a receiver operating characteristic curve. Longitudinal studies in healthy controls would enable the establishment of a normal

standard deviation of genome-wide DNA methylation in leukocytes, a resource with utility beyond the AAV community. It would also be informative to determine if any control variables predict relapse or remission in the sub-cohorts of patients with increased DNA methylation and those with decreased DNA methylation at the *PRTN3* promoter. While isolating purified cell types is certainly one way negate the impact of cell heterogeneity in total leukocytes, simply having access to accurate differential cell counts on each leukocyte or lymphocyte sample would allow for alterations in cell populations to be statistically controlled in future studies.

There may be other loci with more profound DNA methylation changes and regulatory consequences, in addition to *MPO* and *PRTN3*. Our EWAS studies in total leukocytes identified a number of loci more differentially methylated than those within *MPO* and *PRTN3*; further studies examining mRNA expression as it relates to DNA methylation of these genes would expand our understanding of the impact of the methylome on ANCA-associated vasculitis.

The data presented in Chapter 2 open the door to a number of future studies, as translational science often does. The paired RNAseq data are representative of only two AAV patients; I have since collected 21 additional pairs, though not all with dynamic autoantigen gene expression. RNA sequencing a larger cohort of paired CD4-enriched samples would allow for a more complete analysis of the gene signature associated with active patients exhibiting high *MPO* and *PRTN3* expression as well as the alterations in gene signature during disease remission. I have isolated monocytes and neutrophils from 25 paired AAV patients and extracted both DNA and RNA from these populations. Genome-wide DNA methylation studies would highlight differentially methylated regions and indicate where future gene-specific DNA methylation efforts should be focused. RNA sequencing purified monocytes and neutrophils was something I initially proposed. It would be interesting to profile the transcriptome in the neutrophil

population as these cells play a critical role in the pathogenesis of AAV yet exhibit static DNA methylation at the autoantigen genes. It is likely the dysregulation of other genes, perhaps those contributing to neutrophil activation, is more pronounced.

Conclusions

By measuring gene-specific DNA methylation of the autoantigen genes, I establish a link between DNA methylation and autoantigen gene expression and relate that to disease status in AAV. Patients with active disease demonstrated hypomethylation of *MPO* and *PRTN3* and increased expression of the autoantigens; in remission DNA methylation generally increased. In patients with increased DNA methylation, *MPO* and *PRTN3* expression correlated with DNA methylation. Patients who increased DNA methylation at the *PRTN3* promoter had a significantly greater probability of a relapse-free period, while patients with decreased DNA methylation were nearly five times more likely to relapse. These changes in the DNA methylation status of the *PRTN3* promoter predict likelihood of stable remission and may explain autoantigen gene regulation.

I found evidence that DNA methylation may regulate *MPO* and *PRTN3* mRNA expression in CD14+ monocytes. There may also be an additional cell type other than neutrophil or canonical monocyte where DNA methylation controls *MPO* and *PRTN3* expression that resides in a CD3-/CD4- fraction following CD4 enrichment.

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