Novel Insights in Ankylosing Spondylitis Biology: 
Mouse and Human Studies

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy 
Institute of Medical Sciences 
University of Toronto 

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Doctor of Philosophy
Institute of Medical Science
University of Toronto
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Abstract

Little is known about the mechanisms underlying the clinical overlap of ankylosing spondylitis (AS) and inflammatory bowel disease (IBD). There is also a lack of reliable serological biomarkers to guide AS management. AS is a complex, male-dominant disease with HLA-B27 being the strongest genetic association. The ank/ank mouse is a murine model with serological and pathological features similar to human AS. Lipocalin 2 (murine Lcn2/human LCN2) is a secreted anti-microbial glycoprotein produced in multiple tissues including joint and gut.

Given the functions of lipocalin 2 in infection, inflammation and bone remodeling, our aim is to assess whether lipocalin 2 is a key mediator of the gut-joint axis in AS and whether it would serve a better marker for treatment outcomes. The association of LCN2 with HLA-B27 and sex in patients is also analyzed.

Chapter 2 shows that aberrant levels of Lcn2 are associated with ankylosis and gut pathology in ank/ank mice, indicating a potential mechanism underlying the overlap of AS and IBD. The interplay of gut inflammation and ankylosis in contributing to elevated Lcn2 levels suggests that the ank/ank mouse is a relevant animal model for the gut-joint interactions, as seen
in AS. Peroxisome proliferator-activated receptor gamma (PPARγ) plays a role in aberrant Lcn2 pathway in this AS model.

Chapter 3 confirms the elevation of serum LCN2 in AS patients. Levels of LCN2 are associated with coexisting IBD and with the degree of ankylosis. The relationship of HLA-B27 positivity and sex with LCN2 implicates a host-microbe interaction underlying the gut-joint axis in AS.

Chapter 4 demonstrates that normalization of LCN2 could be viewed as a treatment target in AS. Serum LCN2 served a more sensitive biomarker for treatment outcome in 89% of AS patients. HLA-B27 status and sex does affect LCN2 as a readout for assessing treatment outcome.

The discovery of aberrant Lcn2/LCN2 reveals a relevant mechanism underlying the gut-joint axis in AS. The effect of PPARγ on aberrant Lcn2 pathway sheds lights on novel therapeutic targets for the disease. More knowledge of the dysregulation of Lcn2/LCN2 pathways will contribute to better AS management and more effective treatment strategies.
Acknowledgments

The journey of PhD is definitely a rewarding experience in my life. Although I am the one receiving the degree, this would not be possible without the support and love from those around, to whom I would love to extend my most sincere ‘thank you’.

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To: Mom and Dad. Thank you for always being there, always understanding and accepting my flaws and always looking out for my best interest. I definitely could not do this without your constantly support in spirits with love.
Contribution

All animal studies are done by myself.

Human studies are teamwork done by Dr. Florence Tsui, Dr. Hingwo Tsui, Dr. Zhenbo Zhang and myself.
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<th>Description</th>
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<tbody>
<tr>
<td>2,5-DHBA</td>
<td>2,5-Dihydroxy Benzoic Acid</td>
</tr>
<tr>
<td>AS</td>
<td>Ankylosing Spondylitis</td>
</tr>
<tr>
<td>ASDAS</td>
<td>Ankylosing Spondylitis Disease Activity Score</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>axSpA</td>
<td>Axial Spondyloarthritis</td>
</tr>
<tr>
<td>BADGE</td>
<td>Bisphenol A Diglycidyl Ether</td>
</tr>
<tr>
<td>BASDAI</td>
<td>Bath AS Disease Activity Index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Proteins</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>COXIB</td>
<td>Cyclooxygenase II Inhibitor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive Protein</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease-Modifying Anti-Rheumatic Drug</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sodium Sulfate</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum-Associated Degradation</td>
</tr>
<tr>
<td>ERAP</td>
<td>Endoplasmic Reticulum Aminopeptidases</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FHC</td>
<td>Free Heavy Chain</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-Wide Association Studies</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hβ2m</td>
<td>Human Beta 2 Microglobulin</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IBP</td>
<td>Inflammatory Back Pain</td>
</tr>
<tr>
<td>ICs</td>
<td>Immune Complexes</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal Epithelial Cell</td>
</tr>
<tr>
<td>LCN2/Lcn2</td>
<td>Lipocalin 2</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Mechanical Back Pain</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage Migration Inhibitory Factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mSASSS</td>
<td>Modified Strokes AS Scoring System</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MTECs</td>
<td>Mouse Tracheal Epithelial Cells</td>
</tr>
<tr>
<td>mTNF</td>
<td>Membrane-bound Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear Factor of Activated T Cell c1</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NGAL/Ngal</td>
<td>Neutrophil Gelatinase Associated Lipocalin</td>
</tr>
<tr>
<td>NIK</td>
<td>NFKB-inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like Receptor</td>
</tr>
<tr>
<td>NOG</td>
<td>Noggin</td>
</tr>
<tr>
<td>nr-axSpA</td>
<td>Non-Radiographic Axial Spondyloarthritis</td>
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</tbody>
</table>
NRS  Numerical Rating Scale
NSAID  Non-Steroidal Anti-Inflammatory Drug
OA  Osteoarthritis
OPN  Osteopontin
Pi  Inorganic Phosphate
PPARγ  Peroxisome Proliferator-Activated Receptor gamma
PPI  Inorganic Pyrophosphate
PPREs  Peroxisome Proliferator Elements
PsA  Psoriatic Arthritis
RA  Rheumatoid Arthritis
ReA  Reactive Arthritis
ROS  Reactive Oxygen Species
Rosi  Rosiglitazoneand
SFB  Segmented Filamentous Bacteria
SIJ  Sacroiliac Joint
SNP  Single Nucleotide Polymorphism
SOST  Sclerostin
SpA  Spondyloarthritis
SPF  Specific Pathogen Free
sTNF  Soluble Tumor Necrosis Factor α
TCR  T Cell Receptor
TLR  Toll-like Receptor
TNAP  Tissue Nonspecific Alkaline Phosphatase
TNFi  Tumor Necrosis Factor α Inhibitor
TNFRI  TNF Receptor I
TRAF3  TNF Receptor Associated Factor 3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>USpA</td>
<td>Undifferentiated Spondyloarthritis</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VSA</td>
<td>Visual Analog Scale</td>
</tr>
<tr>
<td>β2m</td>
<td>Beta 2 Microglobulin</td>
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Chapter 1

Introduction
1.1 Complex disease

1.1.1 What are complex diseases

Complex diseases are medical conditions that arise from an intricate interaction of inherited (nature) and environmental (nurture) factors. The vast majority of diseases are, in fact, categorized as complex diseases, such as Alzheimer's disease, asthma, Parkinson's disease, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and psoriasis reviewed by Hunter (Hunter, 2005).

In our body, there are not only constant interplays within genetic variants and among environmental factors, respectively, but also continual interactions between host and environment (Fig. 1.1). With a healthy state, these interactions are maintained in a balanced manner. However, dysregulation of the host-environment interactions may trigger the onset of complex diseases and persist to play an ongoing role during disease progression.

Genetic predisposition represents part of the risk associated with complex disease phenotypes. Compared to the single gene-controlled diseases, complex diseases are polygenic conditions in which the inheritance patterns are often complex. Complex diseases generally do not follow the standard Mendelian patterns of inheritance. In disease development, the interactions of various genetic susceptibility risk factors may play a more essential role than a single contributory gene.

However, the actual onset and progression of complex diseases are significantly impacted by environmental factors. A genetic predisposition represents only part of the risk, clinical expression of complex diseases may be altered significantly by modulation of the contributory environmental factors and their interaction with genetic susceptibilities.
Figure 1.1 Aberrant gene-environment interaction induces onset of complex diseases. During disease onset and development, there are constant interplays within genetic variants and among environmental factors, respectively, and there are continual interactions between host and environment.

1.1.2 Causes of complex diseases

In general, complex diseases are caused by a combination of genetic and environmental factors, most of which have yet to be identified. It is a challenging task to distinguish a single causal factor and to determine its contribution in a complex disease because (1) the effect of a single factor may be obscured or confounded by other contributing factors; (2) genes and environment both encompass a wide range of variables. Indeed, the complexity of the interplay and combinations of various genetic and environmental risks has posed challenges to research on complex diseases.
1.1.2.1 Genetics

Genetically, the intricacy of most complex diseases is likely due to the involvement and interactions of multiple susceptible genes. In fact, diseases that follow the single gene-dominant or single gene-recessive Mendelian patterns of inheritance are rare. Since the 19th century, many diseases have been discovered which do not obey Mendel’s rules of inheritance, thus prioritizing studies of identification of genetic factors that drive the onset of complex genetic diseases (Hern & Bidichandani, 2004).

Studies of genomics have provided useful insights into the contribution of individual genes/single nucleotide polymorphisms (SNPs) and potential gene-gene interactions to the phenotypes associated with various complex diseases. The Human Genome Project has discovered that SNPs are highly prevalent in the human genome. SNPs may vary from individual to individual and most SNPs have no effect on health or development. Some of them, however, have been associated with human disease. If a SNP occurs in the coding region of a gene which is involved in conferring disease susceptibility, the SNP may disrupt the generation of a functional gene product. Then there is an increased likelihood that this SNP will demonstrate a phenotypic effect. More commonly, SNPs are found in DNA segments between genes, for example in a regulatory region near a gene. In this case, the SNPs may influence directly gene-gene interactions, e.g. functioning as enhancers. Thus by mapping these SNPs and associating them with a particular phenotypic effect, SNPs can be used as genetic markers for assessing disease risk. For instance, if a SNP appears to be more prevalent in affected subjects than healthy
controls, this suggests that the SNP is physically close to the disease-causing mutation or plays a vital role in the expression of the functional genes.

Genome-Wide Association Studies (GWAS) represent major tools for unraveling the genetic basis of complex diseases. Seeking to identify genetic associations with complex diseases, GWAS have conducted comprehensive analysis of SNPs related to complex diseases across the entire human genome, which often provide insights into molecular pathways that lie beneath the disease. However, these studies are not without drawbacks. Some researchers argue that GWAS operate without a context for discerning what is functionally relevant, and hence they are of limited value (G. K. Chen & Witte, 2007). Moreover, the genetic variants found in GWAS can only explain a small portion of the disease risk, because rare variants (0.01%-0.05% frequencies) with intermediate effects will be missed by GWAS (McCarthy et al., 2008; Schork, Murray, Frazer, & Topol, 2009). Fortunately, the latest technologies in sequencing with significant increases in speed and volumes are allowing the discovery of variants with lower frequency (Mardis, 2008). Despite the given limitations, GWAS still serve powerful tools to uncover new sets of genes associated in a variety of complex diseases.

By studying the whole picture, researchers are better positioned to identify causal genes, which may in turn be regulated by environmental factors, and vice versa. By translating the critical findings to clinical settings, GWAS may potentially facilitate the development of personalized medicine and optimal clinical care.

1.1.2.2 Environment

Although genetic risks are of importance for the onset and development of complex diseases, environmental triggers are not negligible. Commonly studied environmental factors
include life styles, smoking, diet, alcohol, exercise, etc. Recently, the gut microbiome is becoming a key environmental factor in complex disease research.

The human gut is inhabited by trillions of microorganisms and normal health relies on the stability and balance of the interactions of host and intestinal bacteria. In the gastrointestinal tract, the host genetically determined physiological environment shapes the colonization of gut microbiota. In addition, the host immune system is constantly monitoring the potential invasion by pathogens while at the same time tolerating trillions of commensals. Conversely, these microbes actively regulate the host immune response in a mutually beneficial relationship. Various types of cells in the gut, such as intestinal epithelial cells (IECs), mononuclear phagocytes, innate lymphoid cells, and B and T lymphocytes, can be the targets of such microbial modulation.

On the one hand, the microbiota composition is shaped by host factors, including physical (epithelial and mucus layers), biochemical (enzymes and antimicrobial proteins) and immunological (IgA and epithelia-associated immune cells) (Hooper & Macpherson, 2010). Thus host genetic factors play an essential role in shaping the gut microbiome. The similarity of intestinal microbiome in twins has demonstrated that host genome plays a role in shaping the gut flora (Goodrich et al., 2014). Recent studies have highlighted that host genetic polymorphisms and deletions lead to changes in the patterns of bacterial colonization (Benson et al., 2010; Blekhman et al., 2015; P. Lin et al., 2014; Spor, Koren, & Ley, 2011). Those studies have connected certain genetic variants with the quality and quantity of some intestinal bacteria. Thus in the presence of different inherent genetic factors, the composition and abundance of intestinal commensals may vary from individual to individual. In addition, the maintenance of the gut microbiome largely depends on energy available in the gut. One important source of energy is from fermentation and sulphate reduction of host carbohydrates, such as intestinal mucus (Arike & Hansson, 2016;
Tailford et al., 2015). A major component of the mucus layer is highly glycosylated Mucin 2, which provides energy to the gut microbiome (Arike & Hansson, 2016). Thus, the survival of microbes in the gut relies on their phenotypic traits evolved with the energy availability in the host gut (Ruth E Ley, Peterson, & Gordon, 2006). In addition, life style (e.g., smoking and diet), psychological health (e.g., depression) as well as living conditions (urban or rural) and geographical location of the host can all affect the composition of gut microbiome (Biedermann et al., 2013; Jiang et al., 2015; Rodríguez et al., 2015; Tyakht et al., 2013). Although the composition of gut microbiota is relatively stable in each individual, it is still subject to perturbation by life events (Dethlefsen & Relman, 2011). And the recovery of a balanced gut microbiome varies from individual to individual depending on the host genetic and environment factors. Therefore, there is a huge challenge on the global analysis of human gut microbiota composition in health or disease owing to the different genetic background, lifestyle, or diet of the studied subjects. Moreover, the relevance of using animal models for the study of gene-environment interactions in human disease remains questionable. Although the two main phyla of the gut microbiota, Bacteroidetes and Firmicutes, are the same in both human and mice, 85% of bacterial genera found in mice are not present in humans (R. E. Ley et al., 2005).

On the other hand, most microbes co-evolved with the host to regulate the immune system in ways that convey mutual benefits, either via direct interaction with the host immune system or through the production of metabolites. For example, microbiota has been found to play a role in shaping local population of the regulatory/effector T-cell axis (Lathrop et al., 2011). Segmented filamentous bacteria (SFB) can promote Th17 cell expansion, while Bacteroides fragilis and some Clostridia species increase Treg populations (Atarashi et al., 2013; Ivanov et al., 2009; Telesford et al., 2015). In addition, butyrate and propionate, two bacteria metabolites, have been shown to induce the differentiation of colonic/extra-thymic Treg cells in mice, suggesting an anti-
inflammatory function (Arpaia et al., 2013; Furusawa et al., 2013). Most importantly, the interaction between commensal microbiota and the mucosal immune system is essential for proper immunity. The critical role of the microbiota in the development and maintenance of immune homeostasis is evident from the observation of deficient immunity in germ-free animals as reviewed by Round and Mazmanian (Round & Mazmanian, 2009). Therefore, the gut microbiome plays a vital part in maintaining the host immune homeostasis. Immune homeostasis is a dynamic state that keeps the system alert to invasion while avoiding autoimmunity and maintaining tolerance for beneficial commensals. In a number of complex diseases, such as inflammatory bowel disease (IBD) and type II diabetes, dysbiosis (dysregulation of gut commensals) has been identified (Parekh, Balart, & Johnson, 2015). Yet it remains unclear if the dysbiosis is the cause or result from a shift in the immune balance in many scenarios.

In addition to the regulation of host immune system, the gut microbiome provides a variety of beneficial properties to the host due to its large genomic content and metabolic complement. Some of the most crucial roles include maintaining the integrity of the mucosal barrier against pathogens, contributing to digestion of food, extraction of necessary vitamins and minerals (Kamada, Chen, Inohara, & Núñez, 2013; Kau, Ahern, Griffin, Goodman, & Gordon, 2011). It has been proposed that it may even contribute to mood and behavior (Carabotti, Scirocco, Maselli, & Severi, 2015).

1.1.3 Evaluation of gene-environment interactions in complex diseases

Considering the interplay between gene products and by-products of environmental insults in complex diseases, analyzing genetic variants and environmental separately is inadequate. Cross-disciplinary efforts on understanding gene-environment interactions are required to better predict
disease onset and monitor disease development. There are a number of studies which examine the interplay of these factors.

Twin studies represent one of the powerful tools to examine the weight of individual hereditary factors in contributing to a complex disease compared to environmental factors. In particular, monozygotic (MZ) twins share identical genetic background while dizygotic (DZ) twins inherit 50% of their genes. In twins, high concordance rates in MZ and DZ twins of a complex disease demonstrate a strong genetic contribution, while low concordance rates imply environmental involvement (Bogdanos et al., 2012). This can be demonstrated by comparing Crohn’s disease (CD) and ulcerative colitis (UC), the two main manifestations of IBD. Concordance rates in MZ are estimated to 95.4 and 49.5 for CD and UC, respectively, in a number of twin studies, indicating a more essential role of environmental factors in UC (Bengtson, Aamodt, Vatn, & Harris, 2010). Indeed, at least one genetic variant of NOD2/CARD15 is identified in 44% CD patients and 19% healthy twins compared to 2% UC patients (de Lange & Barrett, 2015). Aside from genetic contribution, the difference of gut microbial composition in CD, UC and healthy individuals reflects environmental variability associated with the conditions (Willing et al., 2010). Similar to the strong environmental contribution to UC, the concordance rates of rheumatoid arthritis (RA) are 15% in MZ and 4% in DZ twins, with smoking as a well representing a strong environmental factor (Generali, Ceribelli, Stazi, & Selmi, 2017). In psoriatic disease, different age of onset in concordant MZ twins has provided evidence for the influence of environmental factors since early-onset psoriasis is not more genetically determined (Lonnberg et al., 2016). Thus, to better understand complex diseases, interpretation on gene-environment interplay rather than a single factor alone is necessary.

Other than twin studies, commonly used strategies for gene-environment interactions include family- and population-based designs, as well as cohort (prospective) and case-control
(retrospective) designs (Dempfle et al., 2008). In particular, retrospective studies are simple and relatively inexpensive and less time consuming compared to cohort studies. Case-control studies can be applied to common diseases in existing cohorts for confirmation studies (Dempfle et al., 2008).

To unravel the mechanisms underlying complex diseases, researchers must sort out all the pieces of the puzzle from a black box and start to assemble them one piece at a time. Most importantly, if we consider every factor (genetic or environmental) as just one piece of a complex jigsaw puzzle, we should be aware that different combinations of various number of pieces could possibly lead to the same picture when a puzzle is completed.

### 1.1.4 Clinical implementation of gene-environment interactions in complex diseases

The most important application for gene-environment interactions is personalized medicine, both in prevention and treatment.

Personalized preventive medicine can be applied if the effects of an environmental risk strongly rely on an identified genetic factor. In view of this, it is useful to assess the effect of an environmental exposure on groups with different genetic backgrounds and vice versa. However, it is still a challenge to identify high-risk individuals even when a strong gene-environment interaction is found, because many other confounding factors will be involved in most cases.

Personalized treatment relies on better understanding of gene-environment-drug interactions and genetic individualization of drug responses. Adverse drug reactions could likely be due to various factors including disease determinants, environmental factors and genetic
susceptibility. Since drug response may be dependent on genotype and environmental triggers, a test of real clinical value can be revealed by characterizing the relationship of host-environment interactions and drug response. Moreover, patient care will also be benefitted by novel biomarkers that predict treatment response based on host-environment interactions. If biomarkers can identify patients who will respond positively from those who will be nonresponsive or will exhibit adverse reactions, personalized treatments will be rewarding and will improve health outcomes, including those related to complex diseases.

Therefore, better understanding of gene-environment interaction of complex diseases would be a major contribution to public health and clinical care.

1.2 Ankylosing Spondylitis (AS)

Ankylosing spondylitis (AS) is a complex disease featured by chronic inflammatory arthritis primarily affecting the axial joints. AS is a heterogeneous subset of the spondyloarthritis (SpA) family, which also includes reactive arthritis (ReA), psoriatic arthritis (PsA), IBD-related SpA, undifferentiated SpA (USpA) and non-radiographic axial SpA (nr-axSpA). AS, USpA and nr-axSpA.

The hallmark of AS is new bone formation at sites of joint inflammation. In AS, inflammation and irreversible neo-ossification normally initiate from sacroiliac joints and progress to the rest of the spine over time, leading to a bamboo spine in the most severe cases. Thus, AS patients have significantly decreased quality of life due to pain and stiffness, which results in loss of work productivity (G. Bakland, Gran, Becker-Merok, Nordvag, & Nosent, 2011; Ibn Yacoub, Amine, Laatiris, Abouqal, & Hajjaj-Hassouni, 2011; O’Shea, Riarh, Anton, & Inman, 2010;
The disease mainly strikes males in their peak productive years, with HLA-B27 being the strongest genetic association.

However, AS is an under-recognized disease owing its low prevalence and non-specific symptoms (Bakland, Nossent, & Gran, 2005; Lawrence et al., 1998). At present, the pathogenesis of AS is unclear. There is a lack of diagnostic criteria and there are no curative treatments. In addition, objective and reliable biomarkers for disease monitor are still lacking.

1.2.1 Clinical articular features of AS

In AS, the classic clinical articular features include inflammatory back pain (IBP) and structural changes. Sacroiliac joints (SIJ) and spine are the main affected sites of these events.

Clinically, IBP distinguishes inflammatory spinal arthritis from mechanical back pain (MBP). IBP may persist continually or intermittently throughout disease development, while radiographic evidence is generally detected at a later phase.

1.2.1.1 Inflammatory back pain (IBP)

Inflammatory back pain (IBP) is a common characteristic feature of AS. The earliest symptom of AS is usually back pain reflecting inflammation of the SIJ. The dull pain is normally insidious in onset and difficult to localize. It can be intermittent and unilateral or bilateral (van Tubergen, 2015). At a later stage, the pain progresses to involve lumbar spine, inducing chronic low back pain. Unlike MBP, IBP normally worsens at night and in the early morning and it can be improved after physical activity such as exercise or hot showers (van Tubergen, 2015). Spinal inflammation can evolve into bony ankylosis (structural changes) after many years (J Sieper, Braun, Rudwaleit, Boonen, & Zink, 2002). As the disease progresses, there is a gradual loss of
mobility consistent with the gradual flattening of lumbar spine and development of exaggerated thoracic spine kyphosis (van Tubergen, 2015).

Criteria for identifying IBP are needed for early diagnosis of AS. The diagnosis of AS is often missed or markedly delayed, especially in a primary care setting (Martin Rudwaleit, Khan, & Sieper, 2005), likely due to the fact that back pain is very prevalent in the general population, while AS is not its most common cause. Early criteria for chronic inflammatory back pain were proposed by Calin et al In 1977 (Calin, Porta, Fries, & Schurman, 1977). More recently, the Assessment of Spondyloarthritis International Society (ASAS) has developed new criteria for IBP (J Sieper et al., 2009). These criteria include five parameters: (1) age at onset < 40 years (2) insidious onset (3) improvement with exercise (4) no improvement with rest and (5) pain at night with improvement on getting up. Despite the new standard for identifying IBP being a step towards better criteria contributing to early diagnosis of AS, the clinician’s opinion is often based on subjective evidence.

1.2.1.2 Radiographic changes

Sacroiliitis and spinal ankylosis are two cardinal features of structural changes in AS. Sacroiliitis is regarded as an early radiographic hallmark of the disease. The earliest sign of sacroiliitis is indistinctiveness of the sacroiliac joints. The joints initially widen before they narrow. Bony erosions on the sides of the joint develop, with eventual bony fusion. During the course of AS, structural changes of the spine will be observed subsequent to sacroiliitis. In the spine, small erosions at the corner of vertebral body can be seen in the early stage of spondylitis, which are followed by syndesmophyte formation. This process is the ossification of the outer fibers of the
annulus fibrosis, thus causing the adjacent vertebral to “bridge” one another. The end result can be complete bridging of the vertebral bodies by syndesmophytes, leading to a fused “bamboo spine”.

Radiographic evidence of both SIJ and spines is needed for the assessment of AS, both of which provides insights of disease progression. Moreover, sacroiliitis defined by X-ray provides evidence to make a clinical diagnosis of AS. But in many patients, the onset of clinical symptoms, such as IBP, may occur many years before the detection of radiographic changes, which results in a delay of diagnosis. Recently, the diagnosis of AS has been benefitted greatly from the application of magnetic resonance imaging (MRI). MRI is an excellent tool to demonstrate sacroiliitis and enthesitis in patients in whom clinical suspicion of early disease is high but standard radiography of sacroiliac joints is normal. MRI may detect evidence of bone edema and even bony erosion that are not detectable by conventional radiographs (Oostveen, Prevo, den Boer, & van de Laar, 1999). Although there are drawbacks, the introduction of MRI has aided early diagnosis of AS.

1.2.1.3 Peripheral and other articular features

Although the main affected site associated with AS is the axial skeleton, peripheral joints are occasionally involved. Peripheral arthritis is seen in up to 30% of patients with AS. Classically, it is an asymmetrical oligoarthritis with less than 6 joints affected at a time. Limb joint involvement is mostly seen in the hip joints and shoulders, and less frequently in temporomandibular and knee joints (Bennett, Ohashi, & El-Khoury, 2004; J Sieper et al., 2002). Hip joint involvement, a marker of severity of AS, is discovered more commonly in younger patients. Peripheral joint ankylosis is detected in few cases.

Interestingly, despite the bone-forming tendency in AS, osteoporosis and osteopenia are not uncommon (Carter & Lories, 2011; Magrey & Khan, 2010). AS patients are at four times higher risk of vertebral fractures, compared to the general population (Finkelstein, Chapman, &
Mirza, 1999; Westerveld, Verlaan, & Oner, 2009). These fractures may be underdiagnosed as patients may fail to distinguish acute fracture-type pain from their usual inflammatory pain (Montala et al., 2011). Males and patients with decreased functional capacity have lower bone density (van der Weijden et al., 2011).

1.2.2 Extra-articular features of AS

The most common extra-articular manifestations associated with AS are IBD, uveitis and psoriasis.

A significant clinical overlap exists between IBD and AS. Up to one third of IBD patients develop articular disease featuring AS (Palm, Moum, Ongre, & Gran, 2002). Conversely, approximately 10% of AS cohorts are identified with clinical IBD (Martin Rudwaleit & Baeten, 2006). Importantly, there is frequently asymptomatic subclinical gut inflammation in 40-60% of patients with AS, both macroscopically and microscopically (Altomonte et al., 1994; De Vos et al., 1989; Leirisalo-Repo, Turunen, Stenman, Helenius, & Seppälä, 1994; Mielants, Veys, Cuvelier, & de Vos, 1988; Mielants, Veys, Cuvelier, & De Vos, 1988). Gut inflammation in AS can involve any part of the intestine, but predominantly in the terminal ileum. An early onset of disease and more severe spinal restriction are commonly observed in primary AS compared to those with IBD-associated AS (Perez Alamino et al., 2011). Furthermore, remission of joint inflammation is commonly associated with remission of gastrointestinal inflammation. Nevertheless, persistence of peripheral joint inflammation is usually associated with persistence of bowel involvement (C. Cuvelier et al., 1987; C. A. Cuvelier et al., 1995).

Although the pathogenesis of concurrent IBD and AS remains unclear, these clinical entities share common risk factors and potential pathogenic pathways. The current working models explaining the coexistence of AS and IBD include genetic susceptibility overlaps, such as those
loci found in IL-23/IL-17 pathways (Cortes et al., 2013), and shared immune response and immune cells linking the gut-joint axis (Gracey, Qaiyum, et al., 2016). Early evidence of shared mechanisms came from patients with gastrointestinal infections by *Campylobacter* spp, *Yersinia* spp, *Salmonella* spp, and *Shigella* spp, who later on developed ReA with features similar to AS (Leirisalo-Repo, 1998). Up to 20% of these ReA patients eventually progressed into AS. More recently, dysregulation of gut microbiome (*dysbiosis*), which is a feature of IBD, has been discovered in AS patients and in the rat model of AS (Asquith et al., 2016a; Costello et al., 2015). This indicates that dysbiosis in AS may serve as a potential environmental trigger to disease onset.

Aside from IBD, uveitis and psoriasis are two other major extra-articular manifestations of AS. AS-associated acute anterior uveitis, occurring in 25-40% of AS patients, is typically unilateral and often recurrent (Max, Lorenz, & Mackensen, 2010). Ocular symptoms include eye pain, redness, irritation and blurring of vision. It is more common among HLA-B27 positive patients in AS. Psoriasis is present in approximately 10% of AS cohorts (S Brophy et al., 2001). In addition, there are some other less common extra-articular diseases such as pulmonary fibrosis, renal, cardiovascular and neurological comorbidities (C.-W. Lin, Huang, Chiu, Ho, & Pan, 2014; Nurmohamed, van der Horst-Bruinsma, & Maksymowych, 2012).

### 1.2.3 Classification criteria of AS

Currently, there is no diagnostic criteria for AS. Rheumatologists make the diagnosis of AS with reference to two classification criteria based on both clinical and radiographic features of AS: modified New York criteria and ASAS classification criteria of Axial Spondyloarthritis. These two criteria cover patients at different stages of disease progression as demonstrated in Fig. 1.2. The modified New York criteria identifies AS patients only when radiographic modifications are
detected, while ASAS criteria classified a broader range of patients not limited to AS but also other forms of axial SpA (axSpA).

1.2.3.1 Modified New York Criteria

The modified New York Criteria (Table 1.1) combining clinical and radiographic criteria represent the most commonly used standard for the classification of AS (van der Linden, Valkenburg, & Cats, 1984). Briefly, a diagnosis of AS is made if radiographic sacroiliitis is associated with low back pain or restriction of spinal mobility or chest expansion. Sacroiliitis is scored from 0 to 4 as: 0-normal; 1-suspicious but not definite abnormality; 2-minimal subchondral sclerosis; 3-definite sclerosis and joint erosions; and 4-ankylosis (Barozzi, Olivieri, De Matteis, Padula, & Pavlica, 1998). According to the modified New York criteria, there should be X-ray evidence of bilateral grade II or at least unilateral grade III sacroiliitis to make a diagnosis of AS (van der Linden et al., 1984).

Concern has been raised about the specificity of bilateral grade II sacroiliitis due to the difficulty to distinguish grades I and II sacroiliitis, which is currently the borderline between AS and no AS. Indeed, a recent study by Omar et al. has shown a low agreement rate for scoring grade II sacroiliitis between two experienced rheumatologists, including views from both the AP pelvis and Ferguson (Omar et al., 2017). Another study demonstrated the error rate in designating grades I and II was estimated up to 18% of cases (Gofton, Lawrence, Bennett, & Burch, 1966). However, the specificity would increase significantly if only patients with grade III sacroiliitis had been included. The presence of grade II sacroiliitis bilaterally by radiography has continued to be used to retain some degree of sensitivity. To meet the demand for more sensitive criteria for identification of AS, the ASAS group has developed new criteria for classifying axSpA.
Figure 1.2 Spectrum of Axial Spondyloarthritis. axSpA spectrum of disease, from inflammation to structural damage. This illustration shows the different radiographic stages between modified New York criteria and ASAS Assessment of axSpA international Society.

### Spectrum of Axial Spondyloarthritis

<table>
<thead>
<tr>
<th>Axial SpA (ASAS criteria)</th>
<th>Ankylosing Spondylitis (modified New York criteria)</th>
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<tbody>
<tr>
<td>Non-radiographic stage (X-ray negative) (MRI positive sacroiliitis Or MRI negative HLA-B27 positive)</td>
<td>Radiographic stage (X-ray-positive sacroiliitis)</td>
</tr>
<tr>
<td>Radiographic stage (X-ray-positive sacroiliitis and/or spinal changes)</td>
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#### 1.2.3.2 ASAS Criteria for Axial Spondyloarthritis

The proposed new criteria for axSpA (Table 1.2) by ASAS allowed earlier diagnosis of disease with the application of MRI (M Rudwaleit, Landewé, et al., 2009; M Rudwaleit, van der Heijde, et al., 2009). According to the modified New York criteria, X-ray evidence of bilateral grade II or unilateral grade III sacroiliitis is required to make a diagnosis of AS, resulting in a delay of up to 8 to 10 years since bone-building is a slow process (Aggarwal & Malaviya, 2009; Calin, Elswood, Rigg, & Skevington, 1988; Dincer, Cakar, Kiralp, & Dursun, 2008). In the new ASAS
criteria for axSpA, MRI evidence of inflammation in the sacroiliac joints is sufficient to make a diagnosis in the right clinical context. Another novelty of this classification is a non-imaging arm with HLA-B27 taken into account. A patients with HLA-B27 but no radiographic evidence of sacroiliitis can now potentially be classified as SpA (M Rudwaleit, Landewé, et al., 2009; M Rudwaleit, van der Heijde, et al., 2009).

However, the ASAS criteria include not only patients at an early stage of AS, but also those with USpA and nr-axSpA (Fig. 1.2). These ASAS criteria may pose challenges to AS research as they may introduce a more heterogeneous patient population not limited to AS (van Tubergen, 2015).

Although neither of the above criteria considers the structural changes of the spine, it is in practice an important reference for disease progression analysis. A scoring system named mSASSS (modified Strokes AS scoring system) is established to evaluate the degree of spinal ankylosis. mSASSS assesses chronic spinal changes by scoring the anterior vertebral edge of each vertebra between the lower edge of C2 and the upper edge of T1, and between the lower edge of T12 and the upper edge of S1. mSASSS is scored on a scale from 0 to 72 with higher scores associated with more advanced ankylosis.
Table 1.1 Modified New York criteria for ankylosing spondylitis

I. Clinical criteria

a) Low back pain and stiffness for more than 3 months, which improves with exercise, but is not relieved by rest.

b) Limitation of motion of the lumbar spine in both the sagittal and frontal planes.

c) Limitation of chest expansion relative to normal values corrected for age and sex

II. Radiographic criterion

Sacroiliitis grade 2 bilaterally or sacroiliitis grade 3-4 unilaterally

A diagnosis of AS is made if the radiographic criteria is associated with one clinical criteria.

Table 1.2 ASAS classification criteria for axial Spondyloarthritis

Entry Criteria: Patients should be 45 years of age or younger and have back pain for more than 3 months for this criteria to be applicable.

These patients can be classified as axial SpA if they have either

A. Imaging evidence of sacroiliitis and at least 1 other SpA feature OR

B. HLA-B27 and at least 2 other SpA features

Imaging evidence: Definite sacroiliitis according to modified New York criteria or MRI showing definite bone marrow edema or osteitis in sacroiliac joints.

SpA features: Inflammatory back pain; Arthritis; Enthesitis; Uveitis; Dactylitis; Psoriasis; Inflammatory bowel disease; Good NSAID response; Family history of SpA; 10. HLA B27; Elevated CRP
1.3 **AS is a complex disease**

Like the diseases mentioned in section 1.1, AS is a complex disease influenced by both genetic and environmental factors. The heterogeneity of AS lies in the fact that different combinations of genetic risks and environmental factors may lead to the same disease outcome – AS.

However, the pathogenesis of AS remains unknown. On the one hand, the specific genes and environmental factors, and the number and forms of their combinations contributing to AS have yet to be fully elucidated. On the other hand, gene-gene interactions as well as gene-environment interactions underlying AS remain to be fully investigated. In AS, genetic susceptibility only provides predisposition for disease development. Environmental factors (e.g. bacterial triggers) are required to initiate the onset of disease.

1.3.1 **Genetics of AS**

1.3.1.1 **Early family and twin studies**

It has been long recognized that there is a strong genetic association with AS from the results of family and twin studies. The recurrence risk of sibling with AS has been reported to be as high as 9.2% compared to 0.1% in the general population (Brown, Laval, Brophy, & Calin, 2000). Similarly, in twin studies, the concordance of AS in monozygotic twins is roughly double that of dizygotic twins (Järvinen, 1995). The heritability of AS is estimated to be in excess of 95%, indicating a strong genetic association in AS (Brown et al., 1997; O. B. Pedersen et al., 2008).
1.3.1.2 Role of HLA-B27 in AS

In early 1970s, the discovery of a robust association with human leukocyte antigen (HLA)-B27 was the cornerstone in the genetic studies of AS (Brewerton et al., 1973; Schlosstein, Terasaki, Bluestone, & Pearson, 1973). The first two studies, discovering the connection of HLA-B27 with AS, reported an 88% and 96% of association, respectively (Brewerton et al., 1973; Schlosstein et al., 1973). HLA-B27 has been found to be present in 96% of patients with AS, compared with 4% of unaffected individuals (Brewerton et al., 1973). However, the mechanisms on the association of HLA-B27 with AS remain unknown. Several theories have been proposed regarding the immunological roles of HLA-B27 in AS:

(1) **Arthritogenic peptide hypothesis.**

Since the classical role of HLA-B27 is to present peptides to CD8+ T cells as an major histocompatibility complex (MHC)-I molecule, it was assumed that certain peptides presented by HLA-B27 in some way resulted in an arthritogenic immune response. The mechanism underlying this theory postulates that presentation of distinctive peptides specifically by HLA-B27 induces a CD8+ T cell-mediated cascade, which results in joint inflammation (Fiorillo et al., 1997; Tanigaki et al., 1994).

However, there has been no single B27-specific arthritogenic peptide discovered so far. HLA-B27 does seem to present a restricted group of peptides with an arginine at position 2 from the N-terminus (Jardetzky, Lane, Robinson, Madden, & Wiley, 1991). HLA-B27-restricted T cell responses have been detected both in the blood of patients with AS and in the joints of patients with ReA (Appel et al., 2004; Fiorillo, Maragno, Butler, Dupuis, & Sorrentino, 2000; Hermann,
Yu, Meyer zum Büschenfelde, & Fleischer, 1993). But the presence of such a response does not establish its pathogenicity. In support of the arthritogenic peptide hypothesis, there is recent evidence of shared sequences in the T cell receptors in CD8+ cells in AS (Faham et al., 2017). In this study, deep repertoire sequence analysis discovered that 15 independent motifs were identified to be enriched in the B27-positive AS patients as compared to B27-positive healthy controls. But a convincing case for a single B27-specific arthritogenic peptide has yet to be discovered. In addition, rats expressing human HLA-B27 develop AS-like features regardless of the presence or absence of CD8+ T cells (Schittenhelm, Sian, Wilmann, Dudek, & Purcell, 2015; Joel D Taurog et al., 2009). This appears to be against the hypothesis of arthritogenic HLA-B27-antigen presentation leading to disease.

Of course, the lack of qualitative changes does not exclude the possibility of the contributory role of quantitative differences in the peptide repertoire to the disease risk. Although peptide searching alone might not help solve the mystery of HLA-B27 in AS, it does help us better understand the HLA-B27 binding biology (López de Castro, 2010).

In view of the fact that the search for arthritogenic peptides over several decades has yielded no conclusive outcome, some researchers shifted their focus on other possibilities of HLA-B27 in AS pathogenesis. The Bowness and Colbert groups first reported abnormal properties of HLA-B27, including HLA-B27 heavy chain dimerization on the cell surface and misfolding in the endoplasmic reticulum (ER) (Allen, O’Callaghan, McMichael, & Bowness, 1999; Mear et al., 1999). Such configuration changes might contribute to disease risk and pathogenesis.
(2) Immune responses mediated by HLA-B27 dimerization.

A study by Bowness et al. discovered that the expression of HLA-B27 dimers, on the surface of antigen presenting cells, promoted both natural killer (NK) and T cell survival and proliferation (Bowness et al., 2011). These T cells were increased in numbers and promoted an IL-17 response. It is observed that HLA-B27 has a greater tendency to form homodimers of beta 2-microglobulin (β2m)-free heavy chains (FHCs) than other class I alleles in cells lines and certain cell types from the blood and joints of AS patients (S D Khare, Hansen, Luthra, & David, 1996; Kollnberger et al., 2004; Raine et al., 2006; Tsai et al., 2002). Studies on cells from HLA-B27tg rats further confirmed this finding (Kollnberger et al., 2004; McHugh et al., 2014; Tran et al., 2004). While T cell receptors (TCRs) do not bind to HLA-B27 FHC, KIR3DL2, an innate immune receptor, has an unusually high affinity to HLA-B27 FHC (Kollnberger et al., 2002). KIRD3DL2-expressing NK cells and T cells are abundant in HLA-B27-positive AS patients (Chan, Kollnberger, Wedderburn, & Bowness, 2005). While the interaction between KIR3DL2 and HLA-B27 has been shown to play a role in AS, the relative contribution of cell-surface-expressed FHC of HLA-B27 to AS pathogenesis has yet to be established.

(3) Unfolded protein response.

The misfolding theory of HLA-B27 in the ER has also been proposed. As revealed by crystallographic analysis, HLA-B27 is typically composed of a heavy chain non-covalently linked to β2m (Madden, Gorga, Strominger, & Wiley, 1992). During the peptide binding process in the ER and peptide presentation on the cell surface, MHC I molecules must fold and associate with β2m. However, the substantially slower folding rate of HLA-B27 in the ER results in an enhanced oxidization of the usually free cysteine residue at position 67 (Cys67) (Dangoria et al., 2002; Mear et al., 1999). The oxidized Cys67 commonly forms disulphide bonds with another Cys67 on
another HLA-B27 heavy chain, leading to B27 heavy chain dimers that can stabilized without β2m (Allen et al., 1999). Normally, misfolded HLA-B27 heavy chains are degraded by endoplasmic reticulum-associated degradation (ERAD) (Mear et al., 1999), but in certain circumstances the accumulation rate exceeds the elimination rate.

The misfolded HLA-B27 heavy chains accumulated in the ER will activate an inflammatory pathway named the unfolded protein response (UPR). Under normal circumstances, UPR is considered to be protective, helping promote correct folding of ER-synthesized proteins. However, ongoing ER stress can trigger pro-inflammatory cytokine release (e.g. IL-23) and apoptosis of the affected cells (Colbert, DeLay, Klenk, & Layh-Schmitt, 2010; DeLay et al., 2009; Hetz, 2012). There is a potential link between the inflammatory response dependent on HLA-B27-induced UPR and bone remodeling (Layh-Schmitt, Yang, Kwon, & Colbert, 2013; Smith et al., 2008). HLA-B27tg rats studies confirmed the association of disease susceptibility with HLA-B27 misfolding and subsequent UPR (Tran et al., 2004; Turner et al., 2005). Human studies, however, fail to show such correlation. No evidence indicates there is a UPR-dependent response in the synovial, gut or blood tissues of AS patients (Neerinckx, Carter, & Lories, 2014; Zeng, Lindstrom, & Smith, 2011). Alternatively, these human studies revealed the association of HLA-B27 misfolding with autophagy, which is the other pathway responsible for misfolded protein elimination in ER (Horiuchi, Mitoma, Harashima, Tsukamoto, & Shimoda, 2010).

To date, the exact role of HLA-B27 in the pathogenesis of AS remains unclear. A more systematic examination of dimerization and misfolding theories is needed and definitive links between the aberrant forms of HLA-B27 and AS pathogenesis have yet to be established.
Although the HLA-B27 is strongly associated with AS development, HLA-B27 by itself is insufficient to cause disease. Only 2% of HLA-B27-positive individuals develop AS, indicating other genetic as well as environmental factors may play a role (Jurgen Braun et al., 1998). Despite the high odds ratio (OR=171) of HLA-B27 to the development of AS, HLA-B27 only counts for one fourth of the genetic risk for AS (Brown et al., 1996). Indeed, the additional susceptibility genes related to AS identified by GWAS have implied potential gene-gene interactions in disease progression. Moreover, increasing attention has been focused on environmental factors associated with HLA-B27 contributing to AS.

### 1.3.1.3 Genome-Wide Association Studies (GWAS)

In the recent decade, the use of GWAS is of great help to unravel the complex genetics of AS. Aside from the robust HLA-B27 association with AS, GWAS have identified 48 non-MHC loci which are potentially contributing to AS (Ellinghaus et al., 2016). Based on their known functions of the respective proteins, these susceptibility genes have been clustered into groups including antigen peptide handling, IL-23 cytokine pathways, Nuclear Factor kappa B (NFkB) activation, DNA methylation, bacterial sensing in the gut, gut mucosal immunity and TCR signaling (Ellinghaus et al., 2016). Below are some example genes discovered by GWAS.

With the discovery of the association of IL23R with AS, trials of IL-17 blockage have been implicated in AS. It is notable that *IL23R*, the gene encoding the receptor for the cytokine IL-23, is involved in the activation of a wide range of pro-inflammatory responses. *IL23R* is a common susceptible element shared by both AS and IBD. In a phase II study, biologic treatment blocking IL-17 has been shown to have beneficial effect similar to TNFi (Baeten et al., 2013). Other genetic risks associated with AS that play a role in IL-23 pathway include *CARD9, EOMES, ICOSLG,*
IL1R1, IL1R2, IL6R, IL7R, IL12B, IL27, PTGER4, RUNX3, TBX21, TYK2 and ZUMIZI (Cortes et al., 2013).

The identification of endoplasmic reticulum aminopeptidases (ERAP) 1 and its association with HLA-B27 by GWAS have shed light on gene-gene interaction in AS (Cortes et al., 2013; Wellcome Trust Case Control Consortium et al., 2007). Among the GWAS-identified non-MHC I susceptibility genes, ERAP1 is the gene most strongly associated with HLA-B27 (Evans et al., 2011; Robinson, Costello, et al., 2015). Expressed in the ER, ERAP 1 acts as a “molecular ruler” that cleaves peptides down to optimal length for MHC-class I protein presentation to immune effector cells (Chang, Momburg, Bhutani, & Goldberg, 2005). Therefore, HLA-B27 may play a role in AS by a mechanism involving ERAP1, likely by aberrant peptide presentation. ERAP1 has been associated with the development of AS in HLA-B27-positive cases through interacting with HLA alleles (Bowness et al., 2011). Although there have been controversial results on ERAP1 effect on HLA-B27 cell surface expression, it has been shown that different ERAP1 variants did alter HLA-B27 FHCs (L. Chen et al., 2016; Haroon, Tsui, Uchanska-Ziegler, Ziegler, & Inman, 2012). Thus, the protective variants of ERAP 1, which prevent conformational changes of the molecule, are required for proper peptide cleavage and reduction of abnormal peptide presented in the ER to HLA class I molecule (Alvarez-Navarro, Martín-Esteban, Barnea, Admon, & López de Castro, 2015).

At this point, there is not enough evidence to support a conclusive theory explaining the mechanistic interaction of ERAP1 and HLA-B27. But it has been noted that in the case of influenza infection, generation of preferred B27-epitopes is distinctly dependent on ERAP1 (Akram, Lin, Gracey, Streutker, & Inman, 2014). In the absence of ERAP, there is a significant reduction in the cytotoxic T cell response to B27/NP383-391 epitope in influenza A-infected HLA-B27tg mice.
In addition to ERAP1, genetic associations of ERAP2 and HLA-B27 have been identified in GWAS studies with AS (Cortes et al., 2013; Wellcome Trust Case Control Consortium et al., 2007). Both ERAP1 and ERAP2 belong M1-aminopeptidases which are a family of metalloproteinases. Both ERAPs share a 50% identical sequence (Birtley, Saridakis, Stratikos, & Mavridis, 2012; Saveanu et al., 2005). Similar to ERAP1, ERAP2 has been associated with abnormal HLA-B27 expression and an increase MHC-I free heavy chains and activation of UPR response (Z. Zhang et al., 2017). In addition, ERAP2 is associated with HLA-B27-negative AS (Robinson, Costello, et al., 2015). Similar to ERAP1, loss of function variants of ERAP 2 are protective for AS development (Andrés et al., 2010). However, ERAP2 SNPs did not influence HLA-B27 associated ER stress (Robinson, Lau, et al., 2015).

Another level of gene-gene interactions in AS is presented by the pathogenic effect of interaction between ERAP1 and ERAP2 in HLA-B27-related AS via the altered interactions with MHC-I. Acting as an n-terminal aminopeptidase, ERAP2 may form heterodimers with ERAP1 (Birtley et al., 2012; Saveanu et al., 2005). The heterodimers show different peptide cleavage functions from either ERAP1 or ERAP2. Other than forming heterodimers, ERAP2 also works in concert with ERAP1 in trimming peptides, given location and functional overlap of these two molecules (Saveanu et al., 2005). In particular, inhibition of ERAP1 or ERAP2 alone decreased about 10% MHC-I surface expression, while the suppression of both aminopeptidases resulted in 20% decrease. Yet larger studies are required to enhance our knowledge on ERAP-dependent HLA-B27-related pathogenesis of AS. The absence of ERAP2 expression in mice has limited mechanistic studies of this interaction in AS pathogenesis.

Despite the abundant information generated by GWAS, the studies do have pitfalls, including selection bias in SNPs, limitation of cohort size, and lack of detailed clinical parameters and well-characterized disease outcomes (Cortes & Brown, 2011; Ellinghaus et al., 2016;
Visscher, Brown, McCarthy, & Yang, 2012). The GWAS-identified susceptibility genes only account for 29% of the heritability of AS, with HLA-B27 accounting for 25% of total heritability (Smith & Colbert, 2014). There is still a large proportion of heritability undiscovered in AS. Moreover, although studies have documented sexual dimorphism in AS, sex effect has not been taken into account in GWAS analysis of AS. Up to 15% of quantitative trait loci can be sex-specific (Dimas et al., 2012; Gilks, Abbott, & Morrow, 2014).

1.3.2 Sexual dimorphisms in AS

Being one of the genetic factors, sex difference has long been neglected in clinical and biomedical studies with both human and animals, which has led to potential shortcomings in health care and patient management (Danska, 2014; Fish, 2008). For instance, from 1997 to 2000, 80% of drugs had to be recalled from the US market owing to serious risks for females (Danska, 2014). Despite earnest efforts by many funding agencies to mandate sex as a variable in biomedical and pre-clinical research, females are still under-studied in clinical trials (Geller, Koch, Pellettieri, & Carnes, 2011). More progress has to be made to distinguish such differences in mechanistic studies.

AS has been recognized as a male-dominant disease since the 1600’s when Bemard Connor first documented skeletal changes that resembled AS (Blumberg, 1958). In the early 1900’s, the most frequently quoted sex ratio in AS was ten to one in favor of males (West, 1949). This ratio, however, underestimated AS in females. Although the reports on sex difference in AS are still limited, with increasing interest in this aspect, it is now generally recognized that males are normally affected two to three times more frequently than females (Will, Edmunds, Elswood, & Calin, 1990). In contrast, there is no sexual dimorphism in other categories of SpA, such as nr-
Several reasons have been proposed for underestimation of AS in females. First of all, the persistent belief that AS is an exclusively male disease may contribute to the under-diagnosis or late recognition of this disease in females (W. Lee, Reveille, & Weisman, 2008)]. Secondly, reluctance to expose female reproductive organs to X-ray could be another reason. The current improved classification criteria with the use of MRI offer better opportunities for the screening and diagnosis of female patients with AS. Last but not least, it is of importance to note that sex bias in AS is not merely present in prevalence numbers. The differences in clinical and radiographic features in male vs female AS may play a vital role in a longer diagnostic delay (Calin et al., 1988).

Clinically, AS males have an earlier age of symptom onset and a longer mean disease duration at diagnosis than females (W. Lee et al., 2007; van der Horst-Bruinsma, Zack, Szumski, & Koenig, 2013). Interestingly, women have lower baseline C-reactive protein (CRP) levels (13.1 vs 20.9 mg/l) and a lower proportion of HLA-B27 positivity (76.3% vs 85.2%) compared with men (van der Horst-Bruinsma et al., 2013). It is more common for males to develop extra-articular manifestations, such as gut inflammation, than females in AS (Van Praet et al., 2013). On the contrary, females appear to bear more systemic burden present by greater pain scores and higher disability than males. This is revealed through baseline data demonstrating females with more back pain (high BASDAI) and lower quality of life (high ASQoL) (Arends et al., 2015; Gran & Husby, 1990; Lee et al., 2007; van der Horst-Bruinsma et al., 2013).

Radiographically, females have been reported to show more structural changes in the cervical spine than men (Baraliakos, Listing, von der Recke, & Braun, 2011; Roussou & Sultana, 2011), which may contribute to the under-diagnosis of female with AS. On the contrary, a more
rapid rate of radiographic progression and worse radiographic severity has been observed in males (W. Lee et al., 2007; van der Horst-Bruinsma et al., 2013).

In addition to clinical and radiographic differences of the disease, females also have poorer response rates to treatment (Gran & Husby, 1990; van der Horst-Bruinsma et al., 2013). Hence it indicates that different sexes of patients might involve different mechanistic pathways of disease onset and development, which should be taken into account when making treatment decision.

Despite the sex differences noted in the AS clinic, the small number of female patients in most studies of AS provides few mechanistic clues about it. Originally, common hypotheses on altered sex hormone levels and X-link genes were proposed. Unfortunately, no direct links have been found (Giltay, van Schaardenburg, Gooren, Popp-Snijders, & Dijkmans, 1999; Gordon, Beastall, Thomson, & Sturrock, 1986; Hoyle, Laval, Calin, Wordsworth, & Brown, 2000; Mahendira et al., 2014; Spector et al., 1989). For instance, in male patients, the levels of testosterone were similar in AS vs healthy controls. Similarly, in females, pregnancy or the use of oral contraceptive did not affect the disease. Moreover, no X-linked genes have been identified to be associated with AS. Therefore, there is no clear mechanism for the sex-bias of AS. However, recent studies focusing the sex-biased immune response shed some lights on some possibilities. Male AS patients are discovered to have higher pro-inflammatory cytokines than females (W.-N. Huang, Tso, Kuo, & Tsay, 2012). A recent study by Gracey et al. revealed that there was sexual dimorphism in the Th17 but not Th1 axis of AS (Gracey, Yao, et al., 2016). In male patients, the frequency of IL-17A and Th17 cells was increased compared to female AS patients. Interestingly, this skew was independent of HLA-B27 status. On the other hand, the fact that male patients are more susceptible to infection (Libert, Dejager, & Pinheiro, 2010; Washburn, Medearis, & Childs, 1965) may link this bias to the shifted of gut microbiome in AS. Although sex-biased effects on gut microbiome in AS are not clear, such differences have already been found in patients with
obesity (Haro et al., 2016a). As there is close relationship between host immunity and gut bacteria, the microbial effect on sex-biased immune response may be another explanation. Yet, more studies are needed to unravel the sex difference in AS.

1.3.3 Bacterial triggers in AS

The involvement of environment in the development of AS has long been suggested, although a definitive link is yet to be established (Altomonte et al., 1994; S Brophy et al., 2001; De Vos et al., 1989; Leirisalo-Repo et al., 1994; Mielants, Veys, Cuvelier, & de Vos, 1988; Mielants, Veys, Cuvelier, & De Vos, 1988; Perez Alamino et al., 2011). Despite the unusually high heritability in AS, the monozygotic twin concordance rate is 75% (Brown et al., 1997), indicating that environmental factors might serve as a disease trigger.

Recently, there is increasing evidence supporting the role of intestinal microbiome in AS. One study demonstrated a dysregulated gut microbial composition in the terminal ileum in AS patients compared to healthy controls (Costello et al., 2015). AS patients had a higher abundance of Lachnospiraceae, Ruminococcaceae, Rikenellaceae, Pophyromonadaceae, and Bacteroidaceae, and a decrease in Prevotellaceae and Veillonellaceae. Further analysis showed that interactions between these indicator species within the microbial colonizers shaped the AS gut microbial community signature. Moreover, most of the AS patients with active disease exhibited an elevated serum IgA levels, implying the possibility of microbial translocation and intestinal barrier failure (Ebringer & Wilson, 1996). The “leaky gut” theory is supported by a recent observation of adherent and invasive bacteria in the gut of AS patients with intestinal inflammation, as well as dysregulation of zonulin, a component of the gut vascular barrier (Ciccia et al., 2017). Interestingly, it has been noted that breastfeeding, which is believed to modulate early colonization of gut microbiota, may protect against the development of AS (Montoya et al., 2016).
This findings implicate a protective role of gut commensals to AS development. Breast milk likely regulates the infant’s gut flora directly by transmitting maternal bacteria or indirectly through interaction with the infant’s immune system.

The increasing interest in the link between intestinal bacteria and AS has altered conventional thinking about HLA-B27 pathogenesis of AS. Since more than 95% of HLA-B27 individuals remain healthy, environmental triggers seem necessary in HLA-B27 pathogenesis. Although the specific interaction of HLA-B27 and intestinal microbiome in AS is poorly understood, there is support of bacteria-driven AS in genetically predisposed individuals and animals. Studies showed that there is a higher tendency of HLA-B27-positive individuals to develop into AS from ReA triggered by intestinal infection with *Shigella, Salmonella, Yersinia,* and *Campylobacter* or urogenital infection with *Chlamydia* (Schwimmbeck, Yu, & Oldstone, 1987; Scofield, Warren, Koelsch, & Harley, 1993; van Bohemen et al., 1986). Furthermore, the HLA-B27tg rats were free from arthritis when housed in a germ-free environment but acquired disease after relocated to specific pathogen free environment. This suggests that the potential interaction of gut bacteria with the host HLA-B27 in AS development. Although the mechanisms underlying this remain unclear, a distinctive signature of HLA-B27 on the gut commensals has been revealed by a recent animal study comparing three groups of bacteria including *Firmicutes,* *Proteobacteria,* and *Akkermansia muciphila* (Asquith et al., 2016b). More data is needed to address the roles of bacterial stress in stimulating HLA-B27-associated AS.

Little is known about specific functions of gut microbiome in AS at the molecular levels. However, microbial studies have provided clues on the impact of bacteria on immune responses as well as on bone health. The bacterial influence on immune response has been elucidated in section 1.1.2.2. A direct evidence of the relationship between gut microbes and bone is the studies on bone mineral density (BMD) in germ-free vs. conventionally raised mice (Sjögren et al., 2012).
In germ-free animals, higher trabecular bone density, higher rates of bone formation and lower rates of osteoclasts per bone perimeter were observed. These mice also had lower expression of pro-inflammatory cytokines IL-6 and TNFα in bone tissues. In line with these findings, normalized bone mass and frequency of T-lymphocytes and osteoclast precursor cells were discovered in germ-free mice conventionalized with gut microbiota from conventional animals (Bäckhed et al., 2004). A number of probiotics intervention studies in rat and mouse models further confirmed the effect of gut bacteria on bone (Villa, Ward, & Comelli, 2017). Several mechanisms have been proposed for the link between gut microbiome and bone including gut-derived serotonin, maturation of the immune system during development, and lipopolysaccharide (LPS)-induced systemic inflammation (Cani et al., 2007; Zhou et al., 2017).

To date, no single bacteria has been determined to be causative of AS, however, this does not exclude the possibility of a combination of different commensals/pathogens that would lead to disease. Furthermore, in a complex disease like AS, merely studying aberrant host immune response or dysbiosis alone is not enough. There is a constant and dynamic interaction between the host and gut microbiome throughout disease onset and development. Mediators which can reflect such interactions might be of interest.

With greater understanding of the relationship between gut microbiota and AS, there is an emerging interest in novel AS therapies targeting the gut microbiome. These potential therapies include antibiotics, probiotics, prebiotics, dietary manipulation, and fecal microbial transplantation (Sinead Brophy et al., 2008; Ebringer & Wilson, 1996; Fuentes & de Vos, 2016; Jenks et al., 2010; Ogrendik, 2007; Pamer, 2014; Šturdík, Hlavatý, & Payer, 2016). Given limited numbers of clinical trials, evidence supporting the benefit of these new therapies is still inconclusive in the field of AS and related diseases, such as IBD (Sinead Brophy et al., 2008; Jenks et al., 2010). Among these treatments targeting gut microbiome, fecal transplants seem to be the most effective. However,
there are concerns about the safety of its application. Numerous number and different combinations of bacteria, viruses and parasites in the intestinal content might not be harmful to the donor, while potential pathogens as well as healthy microbes may create unexpected outcomes in recipients (Alang & Kelly, 2015). Further studies are required to elucidate the exact composition of the ecosystem being administered which may thus enhance the overall safety of this approach. Moreover, with the presence of different inherent genetic factors, the composition and abundance of intestinal commensals may vary from individual to individual, as does the efficacy of treatment interventions targeting the gut microbes. To explore the possibility of using microbiome as a novel treatment target, large cohorts of patients and controls with matched genetic, clinical and microbiome data will be needed.

1.4 Animal models of AS

Studies of AS have been hindered by limited availability of biopsies of spinal lesion from patients. Hence, animal models are needed to gain insight into cellular and molecular mechanisms of disease. Although none of these models exactly mimics the human disease, information on certain aspects will aid in unravelling the mysteries of AS. An ideal animal model of AS should present the key articular features of AS including inflammation and ankylosis in the spinal and sacroiliac joints, as well as extra-articular manifestations (e.g. uveitis, colitis and psoriasis). A number of murine models are available for AS studies, mainly focusing on the role of HLA-B27 and those mechanisms underlying inflammation as well as ankylosis. Table 1.3 summarizes both articular and extra-articular features of some common animal models of AS.
<table>
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<tr>
<th>Focused mechanisms</th>
<th>Animals</th>
<th>Articular symptoms</th>
<th>Extra-articular symptoms</th>
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<td>SIJ</td>
<td>Spine</td>
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<td>HLA-B27tg mouse</td>
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<td>Inflammation</td>
<td>hTNFtg &amp; TNF&lt;sup&gt;ΔARE&lt;/sup&gt; mouse</td>
<td>A &amp; C</td>
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<td></td>
<td>SKG mouse (after curdlan)</td>
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<tr>
<td>Ankylosis</td>
<td>Aged DBA/1 male mouse</td>
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<td>ank/ank mouse</td>
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<td>A &amp; B</td>
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(A: Inflammation; B: Ankylosis; C: Bone erosion; nr: not reported)

(Braem, Carter, & Lories, 2012; Fritsche, Nairz, Libby, Fang, & Weiss, 2012; R. E. Hammer, Maika, Richardson, Tang, & Taurog, 1990; Holmdahl, Jansson, Andersson, & Jonsson, 1992; S D Khare, Luthra, & David, 1995; Sanjay D. Khare et al., 2001; Nairz et al., 2015; Nairz, Haschka, Demetz, & Weiss, 2014; Shashidharamurthy et al., 2013; Sweet & Green, 1981; Y. Zhang et al., 2014)
1.4.1 HLA-B27tg rat

The HLA-B27tg rats is a well-established rat model for AS. A high copy number of HLA-B27 (55-150 copies) with a proper ratio of human β2m (hβ2m) is necessary to induce disease manifestations similar to human SpA (R. E. Hammer et al., 1990). Irrespective of sex, about 90% of these animals with high copy number of B27 develop arthritis.

Since its creation in 1990, HLA-B27tg rats have provided vital insights into the AS disease, including HLA-B27 misfolding and subsequent induction of UPR (DeLay et al., 2009), irrelevance of thymic-derived T cells, dependence on CD4+ other than CD8+ T cells (Glatigny et al., 2012; Hoentjen et al., 2007; May et al., 2003; J D Taurog et al., 1994) and the link with Th17 axis in inflammation (DeLay et al., 2009).

In addition, the correlation between HLA-B27 and bacteria has long been recognized. HLA-B27tg rats bred in a germ-free environment are free from colitis and arthritis, indicating that gut and joint inflammation are interconnected through the role played by the microbiome on immune homeostasis (J D Taurog et al., 1994). Interestingly, exposure of these animals to a specific pathogen free (SPF) environment enables the re-establishment of both inflammatory manifestations (Rath et al., 1996). Recently, through 16S rRNA gene sequencing, Lin et al. has identified differences in the cecum bacteria of HLA-B27tg rats, compared with wild-type controls (Lin et al., 2014). Further analysis revealed that there was an increase in *Prevotellaceae* and a decrease in *Rikenellaceae* in the HLA-B27tg rats (Lin et al., 2014). Moreover, an inflammatory cytokine signature precedes the development of clinical bowel inflammation and dysbiosis in HLA-B27rg rats (Asquith et al., 2016b). Despite the current link between HLA-B27 and gut microbiome on immune homeostasis and disease onset, more research is needed to gain better understanding of AS from this model.
1.4.2 HLA-B27tg mouse

Unlike the HLA-B27tg rats, the full spectrum of SpA has not been observed in the HLA-B27tg mouse. The HLA-B27tg mouse has only one copy of the transgene and an hβ2m on an endogenous MHC deletion background. Disease onset in HLA-B27tg mice appears to be environment dependent as animals only develop male-dominant symptoms in conventional environment but not in SPF environment (Khare et al., 1996; Khare et al., 2001). Moreover, immunization of aggrecan were demonstrated to induce limited peripheral arthritis in these animals (Kuon et al., 2004).

1.4.3 hTNFtg and TNFΔARE mouse

The hTNFtg and TNFΔARE mouse involves overexpression of TNF. In these models, signaling through TNF receptor I (TNFRI) is a prerequisite for the development of both arthritis and Crohn’s-like IBD (Kontoyiannis, Pasparakis, Pizarro, Cominelli, & Kollias, 1999). Furthermore, early studies suggested that tissue stroma-residing cells are the necessary TNF targets for the onset of arthritis (Kontoyiannis et al., 1999; Kontoyiannis et al., 2002). However, it is not clear why stromal cells are preferentially activated at certain locations such as entheses rather than at other sites.

1.4.4 Aged DBA/1 male mouse

The DBA/1 mouse has been a model for RA. However, when cohousing 10-12-week-old male DBA/1 mice, 50% to 100% of these mice developed short-lasting unilateral arthritis of the extremes (ankles and toes) and digit ankylosis at about 26 week of age (Lories, Matthys, de Vlam, Derese, & Luyten, 2004). Despite the clinical swelling, there is no detectable inflammatory
infiltrate in the synovium of these mice. Moreover, in the aged DBA/1 male mice, bone morphogenic proteins (BMP) signaling is activated and systemic over-expression of noggin (NOG) prevents and rescues enthesopathy (Lories, Derese, & Luyten, 2005).

1.4.5 SKG mouse

Recently, SKG mouse has been recognized as a model for AS. SKG mice remain well in SPF conditions until exposed to curdlan, a β-1,3-glucan derived from yeast, fungal and bacterial cell walls, after which they develop systemic arthritis (Yoshitomi et al., 2005). This provides evidence for the participation of microbes in AS pathogenesis. With spontaneous mutation in ZAP-70, SKG mouse has impaired TCR signaling owing to an altered thymic selection of autoreactive T cells. Such T cells are arthritogenic CD4+ T cells leading to the development of autoimmune disease (Ito et al., 2014). Moreover, IL-23 was found to be increase in both ileum and serum of the animals and blocking of IL-23 signalling significantly altered disease progression (Benham et al., 2014).

1.4.6 ank/ank mouse

ank/ank mutant is another mouse model of skeletal disorders which has been an informative model for AS. Homozygous mutant mice have progressive ankylosis as presented by a flat-footed gait at a younger age and the loss of mobility spreads from limbs to axial spines at a later stage (Sweet & Green, 1981). The ank/ank mouse has a nonsense mutation of the ank gene locus to proximal mouse chromosome 15 that truncates the COOH-terminal regions of the Ank protein and greatly reduces its activity (Ho, Johnson, & Kingsley, 2000). Ank is a transmembrane protein
expressed not only in joints but other tissues. Ank regulates the transport of inorganic pyrophosphate (PPi). Since PPi inhibits the crystallization of inorganic phosphate ions (Pi) with calcium and thus suppresses the formation of hydroxyapatite crystals, appropriate extracellular PPi levels are required to prevent pathologic calcification (Johnson & Terkeltaub, 2005). The other source of PPi in the extracellular matrix is through the degradation of adenosine triphosphate (ATP) by tissue nonspecific alkaline phosphatase (TNAP) or ectonucleotidases, mainly NNP-1 (Terkeltaub, 2001). Therefore, the mutation of ank gene in the mouse leads to a diminished production of extracellular PPi (Fleisch, 1981; Sweet & Green, 1981). As a result, although homozygous ank/ank mice are normal at birth, they can be recognized as soon as 4 weeks given their inability to cling to cage cover. Full-blown ankylosis can be observed by 16 weeks of age. The lifespan of ank/ank mice is about 24 weeks owing to a rapid development of progressive bone formation in all the joints from peripheral to axial skeletons. However, it is still controversial whether the human homolog of ank, ANKH, plays a role in AS (Liu, Cui, Zhou, Zhang, & Han, 2013; Pimentel-Santos et al., 2012; Söderlin, Börjesson, Kautiainen, Skogh, & Leirisalo-Repo, 2002).

A number of mechanistic studies have been done in ank/ank mice to unravel possible pathways involved in human disease. Phenotypically, although bone proliferation is observed much later in the spine than in digits, molecular imaging showed that ankylosis of these animals developed simultaneously in distal and axial joints (Las Heras et al., 2011). Interestingly, ank/ank phenotype is independent of HLA-B27 status, nonresponsive to non-steroidal anti-inflammatory drug (NSAID) indomethacin treatment, and independent of a T-cell-mediated immune response (Krug & Mahowald, 1991; Krug & Taurog, 2000; Krug, Wietgrefe, Yofterberg, Taurog, & Mahowald, 1997). On the contrary, ank/ank knockout animals demonstrated a rescue of the ankylosis phenotype by the deletion of NLRP3, implicating the essential role of inflammasome
NLRP3 in this model of ankylosis (C. Jin et al., 2011). Moreover, the elevated Wnt/β-catenin signaling observed in chondrocytes of ank/ank mutant mice is also a feature of human AS (Las Heras et al., 2012).

Importantly, the ank/ank mouse is the first AS model that led to the discovery of a novel serological feature in patients. Tsui et al. has identified elevated levels of NOG and sclerostin (SOST)-IgG immune complexes (ICs) in the serum of ank/ank mice (Tsui, Tsui, Las Heras, Pritzker, & Inman, 2014). The increase of NOG and SOST-IgG IC in the serum was subsequently confirmed in AS patients. AS had long be considered seronegative disease compared to RA. The discovery of this serological feature is a cornerstone in understanding disease pathogenesis and may shed lights on better disease management, as well as new therapeutic approaches.

Collectively, ank/ank mutant mice would serve a suitable model to study the disease mechanism, given the articular and serological similarities to human AS. Though little is known about extra-articular features of this model, with better knowledge of intestinal inflammation, ank/ank mice could be a relevant model for investigating the basis of gut-joint coexistence in AS.

Given the number of models discussed above, there will be limitations using animals to solve the puzzle of AS pathogenesis. Since AS is a complex disease, the unraveling of disease pathogenesis largely depends on better understanding of the gene-environment interactions, rather than either perspective alone. However, it is challenging to establish an animal model fully mimicking the interplay between human genes and environment in the disease. In animals, there will be different compositions of intestinal microbiota as well as genetic background, thus potentially leading to a different gene-environment interaction compared to the real scenario in
human diseases. Although intestinal bacteria can be humanized in gnotobiotic mice (germ-free mice inoculated by human gut microbiota samples) or a disease genetic risks can be expressed or silenced in animals, the gene-environment interaction may still be skewed by other potential confounding murine factors. Thus, animal models could only answer questions from a certain perspective.

Taken together, there are no perfect animal models mirroring human AS. With similarity in some aspects, the experimental models provide opportunities to unravel the basis of certain characteristics of the disease. Lessons learnt from animal models may hold the promise of early diagnosis, better management or even an ultimate cure for AS.

### 1.5 Potential of lipocalin 2 (LCN2/Lcn2) involved in AS pathogenesis

Considering the characteristic features (inflammation and ankylosis) and the environmental triggers (e.g. gut dysbiosis) in AS, pleiotropic factors involved in processes which include infection, inflammation and bone remodeling could be novel pathways underlying AS pathogenesis. Lipocalin 2 is one such molecule, which is an anti-microbial protein participating in both inflammation and bone homeostasis. Lipocalin 2 is a secreted glycoprotein produced in multiple tissues by various cells types. Human lipocalin 2 is denoted by upper case LCN2 or NGAL, while its mouse homolog is denoted by lower case (Lcn2 or Ngal) (Kjeldsen, Cowland, & Borregaard, 2000). These abbreviations will be applied in the following sections referring to human or animal studies.
1.5.1 LCN2/Lcn2 biology

Human LCN2, also named as neutrophil gelatinase associated lipocalin (NGAL) or oncogene 24p3, is a 25 kDa secreted glycoprotein encoded by a gene located at the chromosome locus 9q34.11. The LCN2 gene has seven exons that produce at least five functional transcripts. The most common one encodes a 198 amino acid protein. Mouse Lcn2 is encoded by a gene on chromosomes locus 2 27.0 cM with six exons coding for two functional transcripts (Heise, Rentsch, Braeuer, Friedrich, & Quintel, 2011; Holmes, Paulsene, Jide, Ratledge, & Strong, 2005; Kjeldsen et al., 2000).

Human LCN2 proteins are composed of two main domains: a 20 amino acid N-terminal signal peptide and a lipocalin domain which makes up most of the length of the molecule. The ligand-binding region sits in the lipocalin domain, comprised of short stretches of lipophilic amino acids, enabling the protein to bind and transport small hydrophobic molecules (Chu, Lin, Huang, & Chen, 1998). The stretches of amino acid residues at the binding region are highly conservative among different species. This is of importance to studies using mouse models investigating the function of human LCN2 as there is little similarity in the amino acid sequence of the entire human LCN2 protein compared to mouse (62%) or rat (63%) Lcn2 proteins.

The structure of LCN2 proteins enable its anti-microbial properties. A recent study on the three-dimensional structures of LCN2 revealed a significant polar nature of the binding cavity of this molecule, suggesting a possible mechanism whereby LCN2 interacts with bacterial and mammalian proteins named siderophores (Goetz et al., 2002). Siderophores are small molecules produced by microorganisms as well as host cells to bind specifically to ferric iron, which is important to bacterial survival and host iron homeostasis (Bao et al., 2010).
1.5.2 LCN2/Lcn2 production and resolution

Human LCN2 was first detected in neutrophilic granules (Le Cabec, Calafat, & Borregaard, 1997). Subsequently, using in situ hybridization, northern blot analyses and immunohistochemistry, LCN2 was then identified in multiple tissues, such as bone marrow, colon, liver and kidney (Chakraborty, Kaur, Guha, & Batra, 2012). In addition, with the development of polyclonal antibodies for the detection of LCN2, it was demonstrated that LCN2 was present in the plasma of healthy humans with a mean level of 72 ng/ml (Kjeldsen, Johnsen, Sengeløv, & Borregaard, 1993). Type of cells identified to produce LCN2 include circulating macrophages, chondrocytes, osteoblasts, epithelial cells and adipocytes (J B Cowland & Borregaard, 1997; Moreno-Navarrete et al., 2010; Sethi et al., 2008).

Similar to human LCN2, Lcn2 in mice was also found in various tissues and cell types (Aigner et al., 2007; J B Cowland & Borregaard, 1997; H. L. Huang, Chu, & Chen, 1999; Owen, Roberts, Ahmed, & Farquharson, 2008). Mouse Lcn2 was first discovered from a culture of infected murine kidney cells associated with matrix metalloproteinase (MMP)-9 (Hraba-Renevey, Türlar, Kress, Salomon, & Weil, 1989). Although it is not yet known the major producer of the human circulating LCN2 in humans, Xu and coworkers demonstrated that liver is the main source of increased serum mouse Lcn2 upon bacterial infection in the hepatocyte-specific Lcn2 knockout mice (Lcn2\(^{Hep^{-/-}}\)) (Xu et al., 2015). Moreover, these authors discovered that hepatocytes contributed to 25% of the low basal serum level of Lcn2 protein by comparing Lcn2\(^{Hep^{-/-}}\) and global Lcn2 knockout mice. In mice, intestinal epithelial cells and neutrophils to a lesser extent are two other main sources of Lcn2.

Contrary to the active research examining the production of LCN2/Lcn2, little is known about the LCN2/Lcn2 resolution and degradation after upregulation during various physiological
processes within the host. LCN2/Lcn2 is a secreted protein which is internalized through endocytosis. There are two major cell surface receptors identified for LCN2/Lcn2 so far: megalin (Barasch et al., 2016) and 24p3R (Devireddy, Gazin, Zhu, & Green, 2005). Megalin is the first characterized LCN2/Lcn2 receptor, and is a multi-ligand endocytosis receptor primarily expressed by kidney epithelia to facilitate renal Lcn2 reabsorption (Hvidberg et al., 2005). The majority of human circulating LCN2 (50-70%) is captured and filtered by the kidney (Axelsson, Bergenfeldt, & Ohlsson, 1995). Similarly, the majority of mouse circulating Lcn2 is filtrated by kidney, followed by reabsorption from the lumen of the proximal tubule depending on megalin, whereupon Lcn2 is degraded (Barasch et al., 2016). The second LCN2/Lcn2 receptor is 24p3R, which belongs to the organic cation transporter family and is expressed in many tissues (Devireddy et al., 2005; Shao et al., 2016). The binding of Lcn2 by 24p3R in cancer cells is associated with promotion of iron uptake and induction of apoptosis.

As multiple roles are played by LCN2/Lcn2 in various physiological processes, LCN2/Lcn2 can be regulated by a number of factors depending on the specific settings, as discussed below.

1.5.3 Biological roles of LCN2/Lcn2

Since LCN2/Lcn2 has emerged as a pleiotropic molecule involved in a series of biological processes including immunity, cell apoptosis, cancer, and metabolism, its roles in infection, inflammation and bone homeostasis also provide clues for its potential involvement in the comorbidities of gut inflammation and ankylosis seen in AS. These three perspectives will be the focus of this section.
1.5.3.1 LCN2/Lcn2 in infection and host iron homeostasis

Upon bacteria invasion, the transcription, translation and secretion of mouse Lcn2 is mediated by toll-like receptors (TLRs) on mammalian cells (Flo et al., 2004). Srinivasan et al. demonstrated that Lcn2<sup>−/−</sup> mice were hyper-sensitive to LPS compared to their wild-type counterparts (Srinivasan et al., 2012). Consistently, LPS-induced TLR4 activation appeared to be essential for human LCN2 gene expression (Cowland, Muta, & Borregaard, 2006; Cowland, Sørensen, Sehested, & Borregaard, 2003).

LCN2/Lcn2 has been regarded as a pivotal component of the innate immune barrier in the acute response to infection, mainly due to its function of binding to bacterial siderophores. Siderophores are ferric iron-binding proteins produced by bacteria which are dependent on iron for survival. Examples of bacterial siderophores include enterobactin from <i>E.coli</i> and bacillibactin from <i>Bacillus</i> species (Martin, Savory, Brown, Bertholf, & Wills, 1987; Miethke & Skerra, 2010). Within the host, there is scarce free iron due to the binding of any available free iron by the host iron chelators, such as transferrin, ferritin and lactoferrin (Carrano & Raymond, 1978). However, compared to the endogenous iron chelators, siderophores have much higher affinity for iron, ferric iron in particular, which allows siderophores not only the bind to free iron but also extract iron bound by host chelators.

During infection, Lcn2 in mice binds to both iron-laden and iron-free siderophores and transports them into mammalian cells to limit the availability of iron, thus preventing the growth and invasion of pathogens (Bao et al., 2010). Studies in Lcn2<sup>−/−</sup> mice demonstrated increased susceptibility of these mice to infections by Gram-negative bacteria such as <i>Klebsiella</i> or <i>Salmonella</i> <i>spp</i> (Flo et al., 2004). Similar findings were seen in a study using infection by Chlamydia pneumoniae (Candido et al., 2016). Consistently, the bacterial growth was increased
by the addition of anti-Lcn2 antibody whereas suppressed by the addition of recombinant Lcn2 in the infected Lcn2−/− mice vs wild-type controls. With the addition of an iron chelator, desferoxamine, these Lcn2−/− mice benefit and demonstrate reduced LPS-induced toxicity and mortality (Srinivasan et al., 2012). As an acute phase protein, murine Lcn2 can be elevated in the serum within 4 hours following an intraperitoneal injection of E.coli (Flo et al., 2004). Similarly, an X-ray crystallography study demonstrated that siderophores are ligands of human LCN2 (Goetz et al., 2002). The binding affinity of LCN2 to siderophores is largely attributed to ionic strength between the positively charged amino acids (Arg81, Lys125 and Lys134) at the cavity of LCN2 molecule and the negatively charged side chains of siderophores (Borregaard & Cowland, 2006; Holmes et al., 2005).

Interestingly, apart from direct sequestering siderophores to prevent pathogen invasion, the role of mouse Lcn2 in iron homeostasis indirectly limits bacterial growth. On the one hand, the involvement of Lcn2 in macrophage iron homeostasis ensures host defense against Salmonella typhimurium (Nairz et al., 2015). This process is pivotal for host innate immune response to bacterial infection. As a central player in acute inflammation, macrophages are regulated by Lcn2. One of the mechanisms by which macrophages participate in host immune response is through modulating iron supply. By phagocytosis of senescent red blood cells, macrophages restrict the survival of extracellular pathogens while fostering inflammation. Macrophages are a major source of free iron in the body. Iron intake and release are closely related to the polarization of macrophages, a process which is regulated by Lcn2 via the modulation of a feed-forward activation of NF-κB/STAT3 loop (Guo, Jin, & Chen, 2014). In Lcn2−/− mice on a high-fat diet, there was upregulation of cd11c (an M1 marker) and downregulation of arginase 1 (an M2 marker) in adipose tissue and liver. Lcn2 promotes M1 macrophage polarization in a mouse cardiac ischaemia-reperfusion injury model (Cheng et al., 2015; Yoo et al., 2013). On the other hand, the alteration
of iron homeostasis in the lumen may affect the composition of intestinal commensal microbiome. A microbiome pyrosequencing analysis revealed an iron-induced shift in bacterial compositions in mice fed with low iron diets (Kortman et al., 2015). The lack of Lcn2 resulted in an increased abundance of *Alistipes* spp. in IL-10−/− mice, contributing to a severe colonic inflammation (Moschen et al., 2016).

Although known to be protease resistant, human LCN2 can be degraded by some bacterial proteases (Kjeldsen, Johnsen, Sengelov, & Borregaard, 1993; Le Cabec et al., 1997). So the long-term antimicrobial response relies on the ability of epithelial cells not only to produce LCN2 upon bacteria invasion, but also to take up LCN2 into intracellular space to prevent the degradation of LCN2 by microbial proteases (Borregaard & Cowland, 2006).

In addition to the protective role during pathogen invasion, Lcn2 in mice takes part in host iron homeostasis thus monitoring a series of physiological processes. Lcn2 has been identified to bind and deliver 2,5-DHBA (2,5-dihydroxy benzoic acid) to shuttle iron across cellular membranes (Bao et al., 2010; Devireddy, Hart, Goetz, & Green, 2010; Nairz et al., 2014). 2,5-DHBA is a recently identified mammalian siderophore, which has been characterized to mediate cytosolic iron homeostasis, modulate cellular levels of reactive oxygen species (ROS), facilitate iron transport into mitochondria, and regulate cellular apoptosis (Davuluri et al., 2016). Moreover, Lcn2 stabilizes the iron/chelator complex, as demonstrated by the increase of intracellular labile iron in Lcn2-deficient mice. Thus, aberrant Lcn2 levels might hamper host iron homeostasis leading to dysregulation of various physiological processes, and also contribute to dysbiosis causing subsequent inflammatory immune response.
1.5.3.2 LCN2/Lcn2 in inflammation

There is an interplay of LCN2/Lcn2 and inflammatory molecules as well as immune cells. LCN2/Lcn2 is highly regulated under different inflammatory conditions and functions as a pro-inflammatory or anti-inflammatory molecule depending on different contexts.

On the one hand, apart from being an acute phase protein upon bacterial infection, mouse Lcn2 plays a role in inflammatory response. For example, iron withheld in the gut epithelia may aggravate inflammation. By limiting iron-mediated oxidative stress, Lcn2 modulates the host pro-inflammatory cytokine production and may improve gastrointestinal health especially during experimental IBD (Horniblow et al., 2016). Yet it is still controversial whether Lcn2 is pro-inflammatory or anti-inflammatory because of its dependence on the local context of the cells. Both Lcn2Hep−/− and global Lcn2−/− mice demonstrated an increased susceptibility to infection with K. pneumoniae or E. coli, indicating an anti-inflammatory role for Lcn2 (Xu et al., 2015). Similarly, it is discovered that dextran sodium sulfate (DSS)-induced colitis stimulates Lcn2 expression in a microbial- and TCR-dependent manner and that mice deficient of Lcn2 were more susceptible to DSS colitis (Singh et al., 2016a). However, the pro-inflammatory property of Lcn2 is presented by 50% reduction of acute skin inflammation with less immune cell infiltration in Lcn2−/− mice (Shashidharamurthy et al., 2013).

On the other hand, human LCN2 can be secondarily modulated by ongoing inflammation. Axelsson et. al. were among the first to investigate the regulation of LCN2. They found a strong upregulation of LCN2 in neutrophils after exposure to the cytokine granulocyte monocyte colony stimulating factor (GMCSF) (Axelsson et al., 1995). Initial cloning and sequencing studies revealed, on the promoter of both human LCN2 and mouse Lcn2, there are binding sites of two inflammation-related transcription factors, NFkB and CCAAT/enhancer binding protein (C/EBP) (J B Cowland & Borregaard, 1997; Shen, Hu, Goswami, & Gaffen, 2006). This implies the
association of LCN2/Lcn2 with inflammation. Proteomic analysis of FRO thyroid cancer cells expressing IκBαM which is a super-inhibitor of NFκB confirms that NFκB is a positive regulator of human LCN2 (Iannetti et al., 2008). Mouse Lcn2 can also be induced by several pro-inflammatory cytokines and growth factors (Aujla et al., 2008; Bando et al., 2007; Bu et al., 2006; Jack B Cowland et al., 2003; Raffatellu et al., 2009; Xu et al., 2015; Zhao, Elks, & Stephens, 2014). TNFα is a strong inducer of both murine Lcn2 and human LCN2 (Zhao et al., 2014).

In particular, during infection by an enteropathogen, there is a complex defense mechanism involving antigen presenting cells, T-cells and cytokines to mount Lcn2 expression in intestinal epithelial cells. For example, in a cecal infection model by *Salmonella typhimurium*, mouse Lcn2 was induced by the inflammatory cytokines IL-17 and IL-22 (Raffatellu et al., 2009). The induction involved phagocytosis of the bacteria, followed by activation of antigen presenting cells and release of IL-18 and IL-23, and finally activation of T-lymphocytes and secretion of IL-17 and IL-22. Pre-treatment of these two cytokines in mouse tracheal epithelial cells (MTECs) significantly upregulated Lcn2 production while significantly decreased the proliferation of *Klebsiella pneumoniae* in vitro. This was mediated by Lcn2 as MTECs from Lcn2−/− mice failed to suppress *K. pneumoniae* growth despite pre-treatment with either or both cytokines (Aujla et al., 2008). Moreover, it has also been shown that IL-17, synergistically with IL-22/TNFα, triggered a further increase in Lcn2 synthesis by intestinal epithelium (Stallhofer et al., 2015) and in bone cells (Shen, Ruddy, Plamondon, & Gaffen, 2005), compared to IL-17 alone.

1.5.3.3 LCN2/Lcn2 in bone homeostasis

The identification of LCN2/Lcn2 in articular cells and tissues has led researchers to study its role in bone remodeling. Recently, human LCN2 and murine Lcn2 has been discovered in chondrocytes stimulated by IL-1b, leptin, adiponectin, LPS, and dexamethasone (Conde et al.,
2011; Owen et al., 2008). In addition, MMP-9/LCN2 complexes were detected in the synovial fluid from patients with knee osteoarthritis (OA), which is believed to be involved in matrix degradation (Gupta, Shukla, Cowland, Malemud, & Haqqi, 2007). Yet these investigations are still at the early stage.

It is still controversial whether mouse Lcn2 promotes osteoclastogenesis or osteoblastogenesis. On one hand, Lcn2 favours osteoclastogenesis since over-expression of Lcn2 in mice altered bone development and turnover via a negative effect on bone formation, affecting growth plate development and interfering with osteoblast differentiation (Costa et al., 2013). These Lcn2 transgenic mice were smaller with a thinner layer of cortical bone and a decreased trabecular number. In these mice, there is a positive effect on bone resorption by the enhanced osteoclast compartment. Recently Lcn2 has been characterized as a new osteoblast mechano-responding gene regulating bone homeostasis. Rucci et al. discovered that in mice Lcn2 was the most upregulated gene under simulated microgravity conditions where bone loss is common (Rucci et al., 2015a). This is confirmed by healthy human subjects confined to bed rest as well as mice undergoing experimentally-induced mechanical unloading. Moreover, Lcn2-expressing osteoblasts exhibited an increase of the Rankl/Opg ratio and IL-6 mRNA, suggesting that Lcn2 could link poor differentiation of osteoblasts to enhanced osteoclast stimulation. On the other hand, some studies revealed Lcn2 as an anti-osteoclastogenic molecule that exerts its effects by retarding the proliferation and differentiation of osteoclast lineage cells through RANKL signaling pathway (Kim et al., 2015). Consistently, Lcn2 deficiency stimulated the induction of c-Fos and nuclear factor of activated T cell c1 (NFATc1), key transcription factors for osteoclastogenesis, and promoted RANKL-induced inhibitor of kappa B (IκBα) phosphorylation (Kim et al., 2016). Thus, the functions of Lcn2 on bone seems to be context-dependent.
Taken together, LCN2/Lcn2 is actively involved in acute infection, inflammation and bone remodeling. Better understanding of LCN2/Lcn2 functions will shed lights on its potential application in bacteria-related chronic inflammatory bone diseases such as AS.

1.5.4 Association of mouse Lcn2 with peroxisome proliferator-activated receptor gamma (PPARγ)

Recently, the relationship of Lcn2 and peroxisome proliferator-activated receptor gamma (PPARγ) in inflammation and metabolism has been identified in both gut and adipose tissues (Chou et al., 2017; Guo et al., 2013a; D. Jin et al., 2011b; Kundu et al., 2014b; Pereira et al., 2016a; Wang et al., 2007; J. Zhang, Wu, Zhang, LeRoith, et al., 2008). PPARγ is a ligand-inducible transcription factor of the nuclear receptor superfamily regulating genes in relation to cell differentiation, inflammation and lipid metabolism (Debril, Renaud, Fajas, & Auwerx, 2001; Fajas, Debril, & Auwerx, 2001). Given the role of PPARγ on inflammatory and bone homeostasis, as well as its association with Lcn2, PPARγ could possibly be involved in an aberrant LCN2 pathway in AS.

1.5.4.1 PPARγ biology

The structural features of PPARγ cDNA, gene and protein have been identified. There are nine exons of PPARγ gene which extends over more than 100 kilobases of genomic DNA (Fajas et al., 1997). Two isoforms of PPARγ have been discovered: PPARγ1 and PPARγ2. PPARγ1 is encoded by eight exons while PPARγ2 is encoded by seven exons. PPARγ has modular structures containing a N-terminal A/B region with a transactivation domain (AF1), a DNA binding domain
(DBD) with two zinc-finger motifs and a C-terminal ligand binding domain (LBD) with the ligand-dependent transactivation function (AF2) (Poulsen, Siersbæk, & Mandrup, 2012; Tontonoz & Spiegelman, 2008).

Activation of PPARγ signaling requires the recruitment of retinoid X receptor α (RXRα) and the formation of a heterodimer complex with PPARγ receptor at the nucleus. The complex then binds to specific DNA sequence elements called peroxisome proliferator elements (PPREs) to stimulate gene expression (Kliwer, Umesono, Noonan, Heyman, & Evans, 1992). The expression of PPARγ can be modulated by natural ligands such as polyunsaturated fatty acid (Schoonjans, Martin, Staels, & Auwerx, 1997) and eicosanoids (Forman et al., 1995), as well as synthetic chemicals including rosiglitazone (Rosi) and bisphenol A diglycidyl ether (BADGE) (Celinski et al., 2011).

PPARγ is highly expressed in white and brown adipose tissues and the large intestine, with adipocyte being the predominant PPARγ producer (Fajas et al., 1997). Kidney, liver and small intestine have intermediate levels; whereas PPARγ is barely detectable in muscle. While PPARγ1 is expressed in many tissues, PPARγ2 is adipose tissue-specific under normal physiological conditions with less abundant than PPARγ1 (Fajas et al., 1997). The expression of PPARγ is not restricted to adipocytes, but is also found in immune cells, such as B and T lymphocytes, monocytes, macrophages, dendritic cells, and granulocytes. Moreover, PPARγ expression is also found in tumor cells.

1.5.4.2 Role of PPARγ in gut inflammation and bone remodeling

Being a transcription factor of nuclear receptor, PPARγ regulates the expression of genes involving in various processes. Originally, PPARγ was identified as being essential for glucose metabolism. Thus, synthetic PPARγ agonists, the thiazolidinediones (TZDs), are used in type 2
diabetes therapy as insulin sensitizers. More recent evidence implies an important role of PPARγ in controlling inflammation and determining MSC differentiation.

Expression and/or activation of PPARγ has been implicated in the prevention of inflammation in autoimmune and infectious disease. Polymorphisms in PPARγ have been related to susceptibility for IBD (Bank et al., 2014). The association of PPARγ mutation and IBD partly relies on its ability to mediate the M1 to M2 switch of macrophages (Wendelsdorf, Bassaganya-Riera, Hontecillas, & Eubank, 2010). In UC patients, PPARγ was observed to demonstrate a 60% decrease at both the mRNA and protein levels, compared with CD and healthy controls (Dubuquoy et al., 2003). Moreover, the loss of PPARγ in T cells increased disease activity and colonic inflammatory lesions following Clostridium difficile infection in mice (Viladomiu et al., 2012). In rats with experimentally induced colitis receiving Rosi (a PPARγ agonist), the inflammatory reaction was found to be markedly limited; ulceration, edema and infiltration activity were reduced (Celinski et al., 2011).

PPARγ is shown to play a role in mesenchymal stem cell (MSC) differentiation into osteoblasts. MSC are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes and adipocytes (Ankrum, Ong, & Karp, 2014). Between adipogenic and osteoblastogenic lineage, an inverse relationship exists such that differentiation towards an osteoblast phenotype occurs at the expense of an adipocytic phenotype. Traditionally, impaired PPARγ signaling is considered to be in favor of osteoblastogenesis, leading to an increase in bone mass (Akune et al., 2004). Pharmacological inhibition by BADGE or bone-specific PPARγ conditional knockout in mice all revealed significant increase in the number of osteoblasts (Cao et al., 2015; Duque et al., 2013b). However, controversial findings exist. Genetic inhibition of PPARγ S112 phosphorylation in mice reduced bone formation and stimulated marrow adipogenesis (Ge et al., 2018).
Last but not least, PPARγ has been associated with different forms of articular diseases including OA and RA. A recent study showed that adult cartilage-specific PPARγ knockout mice exhibit the spontaneous OA phenotype (Vasheghani et al., 2013). A genetic polymorphism of PPARγ has been related to susceptibility of RA and PsA in a meta-analysis (Y. H. Lee, Bae, & Song, 2014).

1.5.4.3 Association of PPARγ and Lcn2

Given the pleiotropic roles of PPARγ and Lcn2 in infection, inflammation and bone homeostasis, there is the possibility that PPARγ is involved in the aberrant LCN2 pathway in AS.

In the gut, the absence of intestinal PPARγ aggravates acute infectious colitis in mice through an Lcn-2-dependent pathway (Kundu et al., 2014b). In this model, *S. typhimurium* hijacks control of host immune response genes such as those encoding PPARγ and Lcn2 to acquire residence in a host.

In adipose tissues, the relationship of PPARγ and Lcn2 is more complicated involving not only inflammation but also lipid homeostasis and adipose tissue remodeling (Guo et al., 2013a; D. Jin et al., 2011b; J. Zhang, Wu, Zhang, LeRoith, et al., 2008). In primary adipose cells from obese mice and rats, the administration of TZD (a PPARγ agonist) reduced Lcn2 expression, while the addition of Lcn2 induced mRNA levels of PPARγ (J. Zhang, Wu, Zhang, LeRoith, et al., 2008). However, in 3T3-L1 adipocytes, Lcn2 administration attenuated the expression of PPARγ (J. Zhang, Wu, Zhang, LeRoith, et al., 2008). In Lcn2−/− mice with high-fat diet-induced obesity, Rosi-induced body weight and subcutaneous fat gain and liver lipid accumulation were markedly lessened compared to wildtype animals (D. Jin et al., 2011b).
1.6 Treatments of AS

There is no curative treatment for AS currently, highlighting the need for novel treatments based on better understanding of the biological basis of disease. The latest therapeutic targets for AS have been defined as remission/inactive disease and, alternatively, low/minimal disease activity (Smolen et al., 2018). However, there are so far no objective markers to measure disease activity. The current treatments of AS aim to control symptoms, such as pain, stiffness and fatigue, and improve the quality of life of patients, but fail to prevent the process of radiographic damage.

Unlike other inflammatory rheumatic diseases such as RA, the therapeutic options of AS are limited. Depending on whether medications are involved, treatment strategies can be categorized as non-pharmacological treatments, such as physiotherapy and spinal surgery, and pharmacological treatments, including non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and biological treatments. The most commonly used frontline pharmacological treatments for AS patients are NSAIDs (Song, Poddubnyy, Rudwaleit, & Sieper, 2008). DMARDs have a limited role on peripheral arthritis but are not effective in axial symptoms. Patients with an inadequate response to NSAIDs are eligible for biologics treatments like TNFα inhibitors (TNFi). The advent of TNFi represents a significant advance in managing AS clinically, however, they are effective in about 60% of AS patients (Glintborg et al., 2013).

1.6.1 Non-pharmacological management

For the past decade, there are emerging studies showing clinically significant improvement with regular physiotherapy as well as multimodal exercise therapy (Dagfinrud, Hagen, & Kvien, 2008; Ince, Sarpel, Durgun, & Erdogan, 2006). The primary objective of physiotherapy in AS is to assist patients to recover and maintain global health or to improve their physical ability.
Physiotherapy for AS comes in a variety forms including exercise, heat, cold, electrotherapy, and manual techniques. In general, several forms of treatment are combined to provide comprehensive therapy and optimize treatment outcomes.

Spinal surgery is another non-pharmacological treatment option for the incomplete prevention or reversal of radiographic progression by TNFi in AS. Spinal surgery is electively recommended to a small group of AS patients. Given the requirement of preoperative discontinue of TNFi (Krüger, Albrecht, Rehart, Scholz, & Kommission Pharmakotherapie der DGRh, 2014) and the risks associated with operations in these patients, surgery is only considered when conservative options have failed to achieve treatment goals.

### 1.6.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) have been considered to be the cornerstone and frontline treatments of AS since they are highly effective in reducing back pain and stiffness (J. Braun et al., 2011; Dougados, Revel, & Khan, 1998). Until the advent of biologic treatments, NSAIDs were the only effective pharmacological treatments for AS. NSAIDs appear to be more effective in patients with early disease (35% remission rate) compared to those with longstanding disease (12-15% remission rate) (J. Sieper et al., 2008; Désirée van der Heijde et al., 2005). NSAIDs are used based on the patients’ symptoms and can be reduced or discontinued if remission is achieved, as there have been concerns about the safety of their long-term use. Such risks include cardiovascular, gastrointestinal, and renal side effects (I. H. Song et al., 2008). However, with the introduction of selective cyclooxygenase II inhibitors (COXIBs), the adverse effects on the gastrointestinal tract have been decreased (Langman et al., 1999; Mallen, Essex, & Zhang, 2011). Although there were data suggesting that continuous use of NSAIDs may contribute
to reduction of radiographic progression in AS (Wanders et al., 2005), more recent data has challenged this concept (Joachim Sieper et al., 2016).

### 1.6.3 Disease modifying anti-rheumatic drugs (DMARDs)

The disease modifying anti-rheumatic drugs (DMARDs) have been shown to be effective by reducing the inflammatory pain in peripheral joints in AS patients with peripheral arthritis (Braun et al., 2006; Ferraz, Tugwell, Goldsmith, & Atra, 1990; Kirwan, Edwards, Huitfeldt, Thompson, & Currey, 1993). However, they are generally without efficacy in pure axial disease as demonstrated by various randomized controlled trials (Biasi et al., 2000; Gonzalez-Lopez, Garcia-Gonzalez, Vazquez-Del-Mercado, Munoz-Valle, & Gamez-Nava, 2004; Haibel, Rudwaleit, Braun, & Sieper, 2005; Haibel & Sieper, 2010; Sampaio-Barros, Costallat, Bertolo, Neto, & Samara, 2000; Van Denderen et al., 2005; Ward & Kuzis, 1999). Conventional DMARDs include methotrexate, sulfasalazine and leflunomide. There has been ongoing consideration on whether a combination of conventional DMARDs with biologic agents would increase the biological drug survival rate by preventing the anti-drug antibodies against the biological drugs. Nevertheless, most investigations failed to show such an advantage (Breban et al., 2008; Heiberg et al., 2008; Sepriano et al., 2016).

### 1.6.4 Biological Therapy

Currently, biological therapy in AS primarily refers to TNFα inhibitors (TNFi). The initial rationale of blocking TNFα in AS is developed after the discovery of elevated expression of TNFα mRNA and protein in the inflamed sacroiliac joints of patients (MacQueen et al., 2016a). Treatments with TNFi revealed significant improvement in controlling inflammation in the
sacroiliac joints or spine. These agents are effective in 60-70% of patients achieving at least a 50% reduction of pain scores.

To date, there are 5 TNFi agents approved in Europe, USA and most other parts of the world: infliximab, etanercept, adalimumab and golimumab and certolizumab. Most TNFi bind to both soluble (sTNF) and membrane bound forms (mTNF) of TNF, neutralizing its biological activity. Infliximab is a chimeric monoclonal IgG1 antibody formed from human constant and murine variable regions. It reacts with both sTNF and mTNF. Unlike infliximab, etanercept is a recombinant human soluble TNF receptor binding to sTNF predominantly. Adalimumab and golimumab are both fully humanized monoclonal antibodies against TNF, thus reducing the chances of immunogenicity and antibody formation (Poddubnyy & Rudwaleit, 2011).

Although TNFi have greatly benefitted AS disease management, these biological treatments are not a definitive cure for AS. Discontinuation of TNFi generally results in relapse in 75-90% of cases (Baraliakos et al., 2005; Haibel et al., 2013; I.-H. Song et al., 2012). In addition, there are still about 30% of patients demonstrating non-responsiveness, loss of response, or intolerance of these agents (Glintborg et al., 2013). Antibodies may develop against these TNFi, infliximab and adalimumab in particular, therefore dampening the efficacy of treatments. These antibodies can occur as early as less than 6 months of treatment or can develop later (Bartelds, 2011; M K de Vries et al., 2009; Mirjam K de Vries et al., 2007; Kneepkens et al., 2015; Vogelzang et al., 2014).

Thus, attempts have been made in search of new agents targeting other inflammatory pathways, such as IL-6, IL-17 and IL-23 axis (Baeten et al., 2015). However, all these biological drugs target molecules at the downstream level of disease. Better understanding of the underlying mechanism and novel treatments targeting early events would be of great help in improving disease management.
1.7 Current and potential biomarkers for treatment outcome in AS

There is currently a lack of accurate and objective markers which reflect treatment outcome. Current treatment decisions in AS are largely dependent on symptoms, such as pain, stiffness and fatigue, as well as nonspecific serological markers, such as CRP and erythrocyte sedimentation rate (ESR). Novel and reliable biomarkers are in urgent need to aid better disease management.

1.7.1 Current standards for treatment outcome

The current gold standard for assessing treatment response in AS is based on the Bath AS Disease Activity Index (BASDAI, a patient-reported outcome measure), and CRP and ESR (two nonspecific serological markers of inflammation). AS disease remission is defined by a low BASDAI score plus normal CRP/ESR values. However, the reliance on a subjective score and the lack of reliable serological markers pose great challenges in disease management.

BASDAI is calculated from questionnaire composed of questions on how the patients feel about pain in the back, entheses or joints, as well as the duration of the pain, and the severity of early morning stiffness (one of the features of IBP) (Garrett et al., 1994). The score ranges from 0-10 with a score above 4 defined by convention as reflecting active disease. When clinicians determining treatment response, a minimum decrease of 20% or 2 units of BASDAI score is used to define a successful treatment response. Though being the cornerstone in evaluating the treatment efficacy, BASDAI is a subjective score and it is recognized that a high BASDAI score is not limited to AS patients but can also be seen in MBP patients, leading to a low specificity and
sensitivity of this standard. Finally, it contains inherent redundancy in the questions and lacks specificity for inflammatory process.

On the other hand, there is low specificity for CRP and ESR although these markers are widely accepted as serological biomarkers in AS that reflect inflammation as acute phase reactants. CRP is a relatively nonspecific measurement of acute inflammation induced by IL-6. It has been observed that CRP can be associated with secondary/peripheral symptoms in AS (Laurent & Panayi, 1983). A study in 2007 demonstrated the correlation of CRP vs other clinical parameters such as pain, morning stiffness and BASDAI (Visvanathan et al., 2007). However, another study did not find a correlation of CRP with BASDAI score (Juul Pedersen et al., 2011). CRP levels have also correlated with MRI features of inflammation in some studies. However, other studies showed CRP was elevated only in about 30% of AS patients with active disease (Benhamou, Gossec, & Dougados, 2010), and poorly correlated with clinical response of biologic treatments (Mirjam K de Vries et al., 2009). Moreover, CRP may not be useful for the diagnosis because of its low sensitivity in AS (Dougados et al., 1999; Rudwaleit et al., 2009). Recently, high sensitivity (hs)-CRP was found to be better than regular CRP in predicting clinical response to TNFi (Poddubnyy, Rudwaleit, Listing, Braun, & Sieper, 2010). Yet more studies are needed. Similarly, ESR is also a crude measurement as it relies on red blood cell/erythrocyte sedimentation rate, which is affected by the adhesion via fibrinogen or immunoglobulin during inflammation. Thus, any non-specific inflammation would induce ESR changes. Moreover, ESR failed to correlate well with BASDAI score (Juul Pedersen et al., 2011). So, a normal CRP or ESR does not rule out active AS or comprehensively capture active disease. Overall, the relevance of using CRP/ESR in treatment management of AS remains questionable. Better serological markers are required.

To overcome the subjectivity of BASDAI and non-specificity of CRP/ESR, a new scoring system composed of the BASDAI score and CRP/ESR was developed and termed the Ankylosing
Spondylitis Disease Activity Score (ASDAS) (Juhl Pedersen et al., 2011; van der Heijde et al., 2009). It combines five disease activity parameters with only partial overlap, leading to a single score with better truth and enhanced sensitivity to change as compared to single-item variables (Lukas et al., 2009; van der Heijde et al., 2009). ASDAS in combination with CRP as the acute-phase biomarker is the preferred version, and the alternative version utilizes ESR. Aside from the value of CRP or ESR, 4 additional self-report variables are included: (1) back pain (visual analog scale (VSA) 0-10 cm, or numerical rating scale (NRS) 0-10); (2) duration of morning stiffness (VAS/NRS); (3) peripheral pain/swelling (VAS/NRS); and (4) patient global assessment of disease activity (VAS/NRS). Indeed, combined indices may reflect disease more faithfully than single measurement (Tugwell & Bombardier, n.d.; van der Heijde et al., 1992). However, one cannot deny that there may be times when a single index is more appropriate for assessing a specific outcome, because the intervention is directed primarily at one construct and may not necessarily produce a global change. Furthermore, although ASDAS is now commonly used as the standard in clinical trials, combining a patient-reported score with non-specific serological markers may complicate clinical judgement on disease development and proper treatments.

So far it remains a challenge for patient care in AS that no specific and objective biomarkers are reproducibly shown to predict outcome.
1.7.2 Potential biomarkers reflecting treatment outcome in AS

In recent years, to overcome the unmet need in AS management, there are increasing numbers of studies in search of specific biomarkers which may reflect treatment response. Based on the predominant features in AS, potential markers include those targeting inflammation (e.g. cytokines, macrophage migration inhibitory factor (MIF) and osteopontin (OPN)), bone remodeling (e.g. MIF, OPN, MMPs, vascular endothelial growth factor (VEGF), and SOST and NOG), and gut inflammation (e.g. calprotectin and antiflagellin antibodies (anti-CBir1)).

There have been several cytokines demonstrated to be associated with AS with highly variable levels. Such cytokines include interleukin 6 (IL-6), 17 (IL-17), 23 (IL-23), 33 (IL-33) and MIF. IL-6 is a multifunctional cytokine regulating the production of several acute phase proteins, such as serum amyloid A and CRP (Bal et al., 2007; Gratacos et al., 1994; Juhl Pedersen et al., 2011; Romero-Sanchez et al., 2011). Yet there have been controversial results for the role of IL-6 in reflecting disease activity (Juhl Pedersen et al., 2011; Romero-Sanchez et al., 2011; Sveaas et al., 2015). On the other hand, the role of IL-23/IL-17 axis in the pathogenesis of AS has been supported by evidence from GWAS, animal, translational and clinical studies. Elevated serum/plasma levels of IL-17 and IL-23 have been shown in the sera of AS patients (Andersen et al., 2012; W.-S. Chen et al., 2012; Han et al., 2011; G. Li, Wang, Duan, Zeng, & Pan, 2013; X. Li et al., 2013; Mei et al., 2011; Romero-Sanchez et al., 2011; Sveaas et al., 2015) and to be associated with disease activity (W.-S. Chen et al., 2012; X. Li et al., 2013). But conflicting results exist as high IL-17 levels failed to correlate with ESR, CRP, or BASDAI in a recent study (C.-H. Chen et al., 2006). In addition, serum IL-33 is also reported to be increased in AS patients in three independent Chinese studies (Han et al., 2011; G. Li et al., 2013; X. Li et al., 2013). Lastly, macrophage migration inhibitory factor (MIF) is another potent pro-inflammatory cytokine which has been reported to be elevated in serum as well as synovial fluid of AS patients (Calandra &
A recent study by Ranganathan et al. demonstrated a potential mechanism underlying AS: MIF induced TNF production in monocytes, activated β-catenin in osteoblasts and promoted the mineralization of osteoblasts (Ranganathan et al., 2017). However, there are disadvantages of using cytokines as biomarkers: (1) Being downstream molecules in the inflammatory process, cytokines might be not be sensitive enough to change immediately, thus introducing a delay in reflecting disease activity; (2) Their transient nature makes it difficult to capture changes in the blood or any other tissues; (3) Due to the non-specificity of cytokines, their levels can be induced/enhanced by any secondary inflammation.

Osteopontin (OPN) has been a potential serological marker for AS owing to its roles in inflammation and bone remodeling. OPN is a matrix extracellular glycol-phosphoprotein acting as a pro-inflammatory cytokine and is in turn regulated by TNFα (O’Regan, Nau, Chupp, & Berman, 2000). In addition, bone cells secrete OPN during the physiological process of bone remodeling. A study by Choi et al. showed a significantly higher plasma level of OPN in patients with AS (Choi et al., 2008). However, OPN levels failed to correlated with ESR, CRP, nor BASDAI, indicating OPN might be involved in the process of bone remodeling rather than inflammation in AS.

In AS, serum levels of several matrix metalloproteinases (MMPs) have been determined in AS, especially MMP3 (S. Arends et al., 2011; C-H Chen et al., 2006; Keyszer et al., 1999; Mattey et al., 2012b; S. J. Pedersen et al., 2011a; Sun et al., 2014; Wendling, Cedoz, & Racadot, 2008; Woo, Lee, Sung, & Kim, 2007; Yang et al., 2004), MMP8 (C-H Chen et al., 2006), MMP9 (Mattey et al., 2012a), and TIMP-1/2 (C.-H. Chen et al., 2006; Keyszer et al., 1999). There is an increased turnover of extracellular matrix (ECM) proteins, whose degradation relies on MMPs. Originally, MMP3 appeared to reflect treatment response and predict structural changes in AS patients (S.
Arends et al., 2011; Maksymowych et al., 2007; Mattey et al., 2012a). However, a recently study with 92 AS patients treated with etanercept demonstrated MMP3 was not informative for monitoring and predicting response in terms of disease activity and functional assessments (S. Arends et al., 2011). Similarly, another study on 30 potential biomarkers only identified the association of AS disease activity with MMP8 and MMP9, but not MMP3 (Mattey et al., 2012a). With more contradictory results on MMP3 in the assessment of response to treatment, MMP3 as an AS biomarker is falling out of favour.

Vascular endothelial growth factor (VEGF) has become a potential biomarker in AS patients. Produced by fibroblasts, chondrocytes and osteoblasts, VEGF is responsible for regulation of vascular permeability (Ferrara, Gerber, & LeCouter, 2003; Jain, 2003). VEGF is a potent angiogenic vasoactive molecule which is a specific mitogen for endothelial cells. The angiogenesis role played by VEGF is essential for the process of new bone formation, in particular enchondral ossification (Patil, Sable, & Kothari, 2012). Serum levels of VEGF were elevated and correlated with clinical and laboratory markers in both AS and SpA patients (Drouart et al., 2003; S. J. Pedersen et al., 2011b; Susanne Juhl Pedersen et al., 2010; Visvanathan et al., 2007). Moreover, VEGF polymorphisms were found to be associated with disease severity in AS (Seo et al., 2005). Together with IL-6 and CRP, VEGF levels were showed to be significantly decreased in response to infliximab treatment compared with placebo (Visvanathan et al., 2007). Elevated VEGF may also be an predictor of axial radiographic progression in patients with syndesmophytes at baseline (Poddubnyy et al., 2014).

Sclerostin (SOST) and noggin (NOG) became attractive biomarkers in AS for the reason that they modulate mitogenic activity through sequestering BMPs. However, there have been inconsistent results of SOST levels in AS patients compared to controls. On the one hand, some reports showed that SOST is increased in osteocytes (Appel et al., 2009) and decreased in serum
of AS patients from different cohorts, which were significantly associated with new syndesmophytes formation (Appel et al., 2009; Klingberg, Nurkkala, Carlsten, & Forsblad-d’Elia, 2014; Saad et al., 2012). Furthermore, Tsui et al. confirmed the low serum levels of free SOST and NOG in AS patients and, for the first time, showed an increase in SOST- and NOG-IgG ICs in these patients (Tsui et al., 2014). On the contrary, studies identified no differences or higher levels of SOST in AS patients vs healthy subjects (Korkosz et al., 2013; Taylan et al., 2012; Tuyulu et al., 2014).

The comorbidity with IBD has steered research towards searching shared biomarkers between AS and IBD. Established as a biomarker for IBD (Matzkies et al., 2012), the idea of calprotectin as a biomarker in AS is not new. Calprotectin is a heterodimer composed of S100A8 and S100A9 subunits, secreted in inflamed tissues primarily by infiltrating neutrophils. Over two decades ago, calprotectin was found to be a marker of inflammation in ReA (H. B. Hammer, Kvien, Glennås, & Melby, 1995). More recently, some studies found an increase of serum calprotectin in AS patients compared to healthy controls, while others failed to identify such an increase (Klingberg, Carlsten, Hilme, Hedberg, & Forsblad-d’Elia, 2012; Turina, Yeremenko, Paramarta, De Rycke, & Baeten, 2014). Fecal levels of calprotectin are also increased in 41% of patients with AS (de Andrade et al., 2014). Serum calprotectin not only associates with disease activity, but also reflect TNFi treatment response (Turina, Yeremenko, et al., 2014). Moreover, baseline calprotectin serum levels correlated with radiographic progression, with higher level of calprotectin seen in patients with progression in mSASSS (Turina, Sieper, et al., 2014). However, there is limitation of using calprotectin as a marker due to its limited number of source/producers.

Another potential biomarker that links IBD with AS is the anti-flagellin antibodies (anti-CBir1). The prevalence of anti-CBir1 in CD is approximately 50%. A recent study has identified elevated anti-CBir1 levels in 42% of patients with AS-IBD, 20% with AS only and 2% of MBP,
indicating that the presence of anti-flagellin antibody in AS patients without clinical IBD may be an indicator of subclinical bowel inflammation or a predictor of potential future IBD (Wallis et al., 2013). Moreover, the anti-CBir1 levels in AS were associated with elevated CRP and ESR. However, limited studies have been done, thus more investigation is needed before this antibody comes into clinical use.

The investigation of biomarkers in AS is still at the very early stage. Controversial results are not uncommon. A recent study by our lab on extensive serum biomarker multiparametric analysis in golimumab-treated AS patients demonstrated few biomarkers showed correlation with disease activity or MRI changes, which failed to confirm biomarkers found in prior studies which were significantly associated with AS imaging (Inman et al., 2016). Several concerns are proposed. (1) Inflammation is a dynamic and bidirectional process. During different disease processes in AS, inflammation can worsen or improve or even disappear at time of assessment. An ideal biomarker would be specific for AS, which is sensitive to change and truly reflects the inflammatory process of the disease. Neither the previous or future inflammation should influence the readout at the point of analysis. Unfortunately, there are only quantitative rather than qualitative differences in the inflammation identified in AS compared to other forms of chronic arthritis. Hence, biomarkers targeting merely inflammation may not be sufficient. (2) Bone-related candidate biomarkers are not specific to AS but rather to structural damage in bone. This indicates any other causes of skeletal damage would result in change of the readout. Moreover, structural damage is accumulative. Single point analysis may not truly reflect the real process of disease. Data collected over time and sequential analysis of markers from the same patients may offer better opportunity for biomarker validation. (3) AS is a complex disease with interaction between the host and microbiome. Biomarkers which reflect changes of bacteria can be an indirect measure of gut
microbiome. (4) One should not exclude the possibility that combinations of various markers, compared to a single marker, may serve better for the prediction of disease activity and treatment response.

Therefore, an informative biomarker in AS should link the process of inflammation and bone homeostasis, as well as genetic risk factors and bacterial triggers together. Although there are a number of potential biomarkers for AS, biomarkers which fulfill such requirements have yet to be identified. Overall, better insight into the pathogenesis of the disease will form the basis for the discovery of novel biomarkers.

### 1.7.3 LCN2 as a potential serological marker for AS

Recent years, LCN2 has been extensively studied as a potential biomarker in a wide array of human diseases, including inflammatory, infectious, metabolic diseases, cardiovascular, renal, hematologic disorders and solid tumors (Chakraborty et al., 2012). This is largely dependent on its prompt upregulation in biological fluids during disease, its secreted properties, and the robust commercially available immunoassays. To date, LCN2 as a biomarker for AS is largely unknown.

The possibility of LCN2 being a biomarker in AS relies on its potential role as a biomarker in articular and AS-related extra-articular manifestations, including IBD and psoriasis.

LCN2 has been shown to be involved in inflammatory and degenerative articular diseases, including RA and OA (Gómez et al., 2011). In RA, a proteomic analysis revealed that the upregulation of LCN2 in neutrophils was triggered by granulocyte macrophage colony stimulating factor (GM-CSF), followed by the induction of a series of enzymes, including cathepsin D, transitional endoplasmic reticulum ATPase, and transglutaminase 2 in synoviocytes (Katano et al., 2009). These enzymes would lead to synovial cell proliferation and inflammatory cell infiltration.
Moreover, in chondrocytes, a TLR-4 dependent LCN2 expression was boosted by nitric oxide (Gómez et al., 2013). Although LCN2 has been identified to be higher in the synovial fluid of patients with RA than those with OA (Katano et al., 2009), its potential as a biomarker of OA has been proposed in a cartilage degradation model (Wilson, Belluoccio, Little, Fosang, & Bateman, 2008).

In addition, LCN2 has been shown to be relevant to the extra-articular manifestations of AS, including psoriasis and IBD. In patients with psoriasis, serum LCN2 levels were significantly higher compared to other potential markers tested, such as clusterin, soluble TNF receptor-1, IL-6, homocysteine, and uric acid (Ataseven, Kesli, Kurtipek, & Ozturk, 2014). In IBD, LCN2 is considered to be marker of disease activity marker in inflammatory bowel disease regulated by IL1-7A, IL-22, and TNFα (Stallhofer et al., 2015). In this study, serum LCN2 reflected active UC and it was demonstrated that CD patients had lower LCN2 levels, likely due to an impaired Th-17 response. In another study, LCN2 was significantly upregulated in CD patient sera (520%) and feces (783%) (Cayatte et al., 2012). Moreover, LCN2 was detected to be one of the 10 most upregulated genes in active UC and CD vs healthy controls (Østvik et al., 2013). TLR-3 was found to mediate the excessive release of LCN2 in active IBD. Animal studies further support the idea of Lcn2 as a biomarker in experimental IBD. Fecal Lcn2 by ELISA has been identified as a sensitive, dynamic, non-invasive biomarker for intestinal inflammation in mice (Chassaing et al., 2012). In a T cell transfer mouse model of IBD (anti-CD40 Rag2−/−) treated with mAb to IL23R, Lcn2 was downregulated and its transcript levels correlated with disease score and dose of mAbs used (Cayatte et al., 2012).

In summary, the involvement of LCN2 in joint manifestations (RA and OA) and in key extra-articular diseases (psoriasis and IBD) strengthens the hypothesis that LCN2 might be a potential biomarker for AS disease management.
1.8 Research Aims

The overall aim of this thesis is to assess whether lipocalin 2 is a relevant mediator for AS pathogenesis in both ank/ank mutant mice and human patients and whether it serves a reliable serological marker for treatment outcomes in AS. Specifically, the following research questions are addressed:

1. Is the ank/ank mutant mouse a relevant model for AS? Does it manifest gut inflammation and joint ankylosis, analogous to human AS?

2. Is Lcn2 a key mediator of gut-joint axis in ank/ank mutant mice? Is PPARγ involved in the aberrant Lcn2 pathway in ank/ank mice? Does sex influence these potential pathways of AS?

3. Are levels of circulating LCN2 informative in AS patients? Is there association of LCN2 with the degree of ankylosis and the presence of IBD in patients?

4. Do the HLA-B27 status and sex influence the levels of LCN2 in AS patients?

5. How do treatments with NSAIDs or TNFi affect levels of LCN2 in AS? Can LCN2 be used as an informative readout for treatment outcomes in AS, compared to CRP?

6. Do HLA-B27 status and sex affect treatment outcomes using LCN2 as a readout?
1.9 Hypothesis

As there are shared articular and serological similarities in the ank/ank mutant mouse and human AS, ank/ank mice could serve a relevant model for investigating the underlying basis of gut-joint axis in AS with better knowledge of gut involvement in these animals.

Lipocalin 2 may play a role in the gut-joint coexistence in AS. Given its pleiotropic roles in gut and joint inflammation as well bone remodeling, there could be aberrant levels of lipocalin 2 associated with joint and gut manifestations in both ank/ank mice and AS patients. Based on the known association of lipocalin 2 and PPARγ in inflammation, PPARγ would likely be involved in altered expression of lipocalin 2 in AS. In addition, there is likely an association of LCN2 (as an indicator of environmental triggers) with HLA-B27 and sex (as two key genetic factors) in AS, since AS is a complex disease reflecting dysregulation of host-microbe interactions.

Lastly, LCN2 could serve a more informative serological marker then CRP for AS treatment outcome, in light of its roles in inflammation and its association with TNFα. The HLA-B27 status and sex of the patient may affect treatment outcome using LCN2 as a readout.
Chapter 2

Gut-joint axis in an animal model of AS
ABSTRACT

Background

Little is known about the mechanism underlying clinical overlap between ankylosing spondylitis (AS) and inflammatory bowel disease (IBD). The ank/ank mouse is a murine model with both serology and joint pathology features similar to human AS. Therefore, the ank/ank mouse model was used to assess whether lipocalin 2 (Lcn2; an anti-microbial pleiotropic factor) is a key mediator of gut-joint axis in AS and whether peroxisome proliferator-activated receptor gamma (PPARγ; a nuclear receptor that regulates bone remodeling and gut inflammation) is involved in the regulation of Lcn2. Sex difference in Lcn2/PPARγ pathway was also assessed in this model since AS is a male dominant disease.

Materials and Methods

At baseline, colon pathological analysis by H & E staining was compared between 4-5 month-old ank/ank mice (n=40) and age-matched C3FeB6-A/A<sup>wj</sup> wt/wt littermates (n=21). Age-matched wildtype (n=4) and HLA-B27tg mice (n=4) on the background of C57BL/6 were used as controls. The sex of the animals was recorded. Serum and fecal levels of Lcn2 were determined by ELISA. The expression of colonic PPARγ was tested by real-time PCR. To detect whether Lcn2 was associated with PPARγ, ank/ank (n=44) and wt/wt (n=54) mice were challenged intraperitoneally with Rosiglitazone (Rosi), a PPARγ agonist, or Bisphenol A diglycidyl ether (BADGE), a PPARγ antagonist. Serum levels of Lcn2 in the Rosi/BADGE-treated mice were analyzed.


**Results**

Similar frequencies of *ank/ank* mice (40%) with ankylosis and *wt/wt* littermates (48%) had significant colon pathology, though none had clinically evident gut inflammation (e.g. diarrhea). At baseline, levels of serum Lcn2 are positively associated with the severity of gut involvement when ankylosis coexists. In mice with severe gut inflammation, *ank/ank* mice (363 ± 50 ng/ml) had higher baseline Lcn2 levels than *wt/wt* littermates (163 ± 37 ng/ml; *p*<0.01). Among *ank/ank* mice, those with severe gut involvement had higher Lcn2 levels (363 ± 50 ng/ml) compared to those with mild inflammation (218 ng/ml ± 24; *p* <0.05). In contrast to circulating Lcn2, baseline colonic PPARγ expression was negatively associated with the severity of colon inflammation regardless of the presence of ankylosis (*p*<0.01). Rosi treatment increased the serum levels of Lcn2 (313 ± 44 ng/ml) as composed to DMSO controls (177 ± 44 ng/ml; *p*<0.05) in *wt/wt* animals, but this was not detected in *ank/ank* mutants. Sex difference in the levels of serum Lcn2 at baseline is observed.

**Conclusions**

Aberrant levels of Lcn2 associated with ankylosis and gut pathology in *ank/ank* mice indicate a potential mechanism underlying the overlap of AS and IBD. The interplay of gut inflammation and ankylosis in elevating Lcn2 suggests that *ank/ank* mouse is a relevant animal model for the gut-joint interactions, as seen in AS. PPARγ plays a potential role in aberrant Lcn2 pathway in AS.
2.1 INTRODUCTION

Although it has long been recognized that there is clinical overlap between inflammatory bowel disease (IBD) and AS, the mechanism remains unknown. Ankylosing spondylitis (AS) is a chronic inflammatory disease mainly affecting the spine. It is characterized by active inflammatory spinal lesions as detected by magnetic resonance imaging (MRI) (Baraliakos et al., 2005) and chronic structural changes as demonstrated by conventional radiography (Baraliakos et al., 2007). AS is a sex-biased disease with sex ratio 3:1 in favor of men (Will et al., 1990). Over 50% of AS patients have evidence of microscopic gut inflammation, especially in the distal ileum (Ciccia, Rizzo, & Triolo, 2016), indicating the potential of shared pathways of two entities.

The ank/ank mutant mouse is an informative AS model which has features that mirror both bone and joint pathology as well as serological features seen in AS patients. ank/ank mice develop progressive ankylosis in peripheral and axial joints due to a nonsense mutation in the ank gene. Our previous studies in ank/ank mutant mice led us to the discovery of elevated NOG/SOST-IgG immune complexes (ICs) in the sera of AS patients (Tsui et al., 2014). However, little is known about the gut involvement. To study the gut-joint-axis in AS pathogenesis, investigation of gut pathology in this model is necessary.

Lipocalin 2 (Lcn2) could be one of the mediators in the gut-joint axis in AS owing to its functions in infection, inflammation and bone remodeling. Lcn2 is a secreted anti-microbial protein produced in multiple cell types in different tissues (including those in gut and joint). Both pro- and anti-inflammatory properties of Lcn2 have been reported (Abella et al., 2015; Javier Conde et al., 2016; Moschen, Adolph, Gerner, Wieser, & Tilg, 2017; Stallhofer et al., 2015; Veeriah et al., 2016). The association of Lcn2 and bone homeostasis has been demonstrated in mice over-expressing Lcn2 which have a thinner layer of cortical bone and a decreased trabecular
number (Costa et al., 2013). Furthermore, elevated serum LCN2 has been reported in patients with inflammatory bowel disease (IBD) (Stallhofer et al., 2015) and psoriasis (Ataseven et al., 2014), both of which are frequent AS comorbidities. In a disease with both inflammation and bone remodeling like AS, Lcn2 might be dysregulated.

On the other hand, the relationship of Lcn2 and peroxisome proliferator-activated receptor gamma (PPARγ) in IBD and lipid studies (Chou et al., 2017; Guo et al., 2013b; D. Jin et al., 2011a; Kundu et al., 2014a; Pereira et al., 2016b; J. Zhang, Wu, Zhang, Leroith, et al., 2008) indicates a potential role for PPARγ in the Lcn2 pathway in AS. In addition to the association with Lcn2, PPARγ is a key nuclear receptor regulating not only adipocyte/osteoblast differentiation but also gut inflammation (Barak et al., 1999; Rosen et al., 1999). For example, inhibition of PPARγ increased osteoblastogenesis and bone mass in C57BL/6 male mice (Duque et al., 2013a). Moreover, genetic variants in the gene encoding PPARγ are associated with susceptibility to Crohn’s disease (Sugawara et al., 2005). The activation of PPARγ can be regulated by the agonist (e.g. Rosiglitazone [Rosi]) and by the antagonist (e.g. Bisphenol-A-diglycidyl ether [BADGE]) (Meshkani et al., 2014; Wright et al., 2000).

In this study, gut involvement was determined in ank/ank mice compared to their wt/wt littermates. The ank/ank mouse was further used to decipher whether Lcn2 might serve as a mediator for the gut-joint axis in AS and whether PPARγ plays a role in the dysregulation of Lcn2. Sex difference in this model was also assessed.
2.2 MATERIALS AND METHODS

2.2.1 ANIMALS AND STUDY DESIGN

Heterozygous mice on a background of C3FeB6-A/Aw-j (ank+/−) were used for breeding to obtain ank/ank mice and wt/wt littermates. ank/ank mice and wt/wt littermates were cohoused until 4-5 weeks of age. To eliminate variation in ankylosis, studies were undertaken in animals at an age (>16 wk) when all had fused peripheral and axial joints.

For baseline studies, 4-5 month-old ank/ank mice (n=40; 25 males vs 15 females) and age-matched wt/wt littermates (n=21; 10 males vs 11 females) were used. Age-matched wildtype (n=4) and HLA-B27tg mice (n=4) on the background of C57BL/6 were used as controls for colon pathology studies. Whole length colon tissues were collected from ank/ank mice and wt/wt littermates. Blood samples were collected and allowed to clot at 4 °C. Samples were then centrifuged for 10 minutes at 3000g and serum samples were stored at -70°C until use. Fecal samples were also collected. 20-50 mg of fecal sample from each mouse was homogenized in PBS containing 0.1% (v/v) Tween20 (10mg fecal sample/100ul PBST) by vortexing for 20 min at room temperature. Mixtures were then centrifuged for 10 min at 12,000 rpm at 4°C. Supernatant was collected and stored at -20°C until analysis.

For PPARγ manipulation studies, 44 ank/ank mice (19 males vs 25 females) and 54 wt/wt littermates (30 males vs 24 females) were used. BADGE or Rosi (Sigma Aldrich Company) was dissolved in 5% DMSO. Mice were randomized into three treatment groups: (1) 5% DMSO; (2) 30mg/kg BADGE; (3) 10mg/kg Rosi. Intraperitoneal administration of BADGE, Rosi or DMSO was conducted daily for 4 days. Mice were sacrificed on the 6th day. Serum samples were collected and stored at -70°C.
All animals were housed in the specific pathogen-free animal facility at the Krembil Research Institute according to the guidelines of Canadian Council of Animal Care. All animal studies were reviewed and approved by the University Health Network Research Committee.

2.2.2 STAINING AND SCORING OF BASELINE GUT PATHOLOGY

Gut tissues were cut open, washed with PBS and rolled up (*Swiss roll* technique) to evaluate the gut from the proximal to the distal end. Tissues were then fixed in 4% formalin overnight and processed for hematoxylin and eosin staining.

Pathology results were scored by an experienced pathologist in a blinded fashion. Each case was scored for

1. acute inflammation (score 0-3) defined as follows: 0 - none; 1 - either rare diffuse or small focal clusters; 2 - increased numbers of neutrophils, easily identified; 3 - severe inflammation, usually associated with erosions or ulcerations.

2. chronic inflammation (score 0-3) defined as follows: 0 - none; 1 - focal and or minimally increased lymphocytes/plasma cells, particularly in the deep lamina propria; 2 - moderate inflammation; 3 - severe chronic inflammation, usually with loss of crypts, foreshortened crypts or enough chronic inflammation to push crypts apart.

3. the percentage of bowel involved (score 0-3) defined as follows: 0 - none; 1 - <20%; 2 - 20-50%; 3 - >50%.

The total score is the sum of all three scores above (0-9). Mice with a total score of ≥5 are considered to have severe gut inflammation.
To further analyze the inflammation in depth, we used a secondary scoring system with modification from an established scoring system. Briefly, tissues were scored according to:

1. Location of inflammation (0 – none; 1 – mucosal; 2 - transmural)
2. the degree of hyperplasia in colon (0 – none; 1 – 1-50% increase in height; 2 – 51-100% increase in height; 3 - >100% increase)
3. mucin depletion (0 – none; 1 – mild loss of goblet cells; 2 – moderate loss; 3 – severe loss)
4. granulomas (absent or present)
5. number of foci (0 – none; 1 - <5; 2 – 5-10; 3 - >10)
6. architecture distortion (0 –none; 1 – rare foci of arch changes (<5%); 2 – moderate with 5-50% glands showing changes; 3 - >50% of glands showing changes)
7. semi-quantification of neutrophils and plasma cells calculated on the average of cell counts per field from ten fairly random fields per mouse

2.2.3 QUANTIFICATION OF SERUM AND FECAL Lcn2 LEVELS BY ELISA

Serum samples from each mouse at baseline and following treatment were thawed and analyzed at the same time to minimize inter-assay variation. Supernatants of fecal samples from mice at baseline were treated the same way. Lcn2 levels were measured by an ELISA kit according to manufacturer’s protocol (R & D). Briefly, serum or fecal samples were diluted 1:100 (1:50) and allowed to react with the monoclonal antibody specific for human LCN2 for 2 hours, followed by another 2-hour incubation with human LCN2 conjugated to horseradish peroxidase. Recombinant human Lcn2 in a buffered protein base was used as a standard. Determination of the optical density of each well was performed using a microplate reader at 450nm with a correction at 570 nm. Total
protein of supernatant extracted from fecal samples was measured using the Pierce Coomassie Plus Protein Assay with a BSA standard. Fecal Lcn2 levels were further compared with the total protein to control for water content variable in the wet feces.

### 2.2.4 BASELINE COLONIC PPARγ EXPRESSION ANALYSIS

Total RNA was isolated from colons (whole thickness) using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The extracted RNA was purified with the RNeasy Mini Kit (Qiagen), which included an on-column DNase I treatment step, according to the manufacturer’s protocol. RNA purity was determined by spectrophotometry (A260/A280 and A260/A230). RNA was considered intact on a denaturing gel with sharp 28S and 18S rRNA bands and the 28S rRNA bands twice as intense as the 18S rRNA bands. cDNA was synthesized from 2ug of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the manufacturer's recommendation. Relative quantification of gene expression was then performed by SYBR green real-time PCR. Primers specific for PPARγ and reference gene GAPDH are listed in Table 2.1. SYBR green real-time PCR was performed in 384-well plates with a reaction volume of 10 µl using the ABI PRISM 7900HT Fast System (Applied Biosystem) and the standard cycling conditions, as per the manufacturer’s instructions. Each gene was run in duplicate. Data was normalized to the reference gene GAPDH and expressed as a fold change versus a C57/B6 mouse using the $2^{-\Delta\Delta Ct}$ method.
Table 2.1 Primers used for mRNA expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>5’ ACGTTCTGACAGGACTGTGTG 3’</td>
</tr>
<tr>
<td></td>
<td>5’ TGATGTCAAAGGAATGCGAGTG 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ TGTGTCCGTCGTGGATCT 3’</td>
</tr>
<tr>
<td></td>
<td>5’ CCTGCTTCACCACCTTCTTTGA 3’</td>
</tr>
</tbody>
</table>

2.2.5 STATISTICAL ANALYSIS

T-tests and one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison tests were carried out using GraphPad Prism 5 program. A p-value of less than 0.05 was considered significant. Data are presented as mean ± standard error.

2.3 RESULTS

2.3.1 COLON INFLAMMATION IN BOTH ank/ank AND wt/wt MICE AT BASELINE

To avoid the variation of ankylosis with age, 4-5 month-old ank/ank mice with established spinal ankylosis and age-matched wt/wt littermate controls were used. Homozygous ank/ank mice are normal at birth. Ankylosis in the ank/ank mice is first observed in distal digits at 4-5 weeks of
age. Progressive ankylosis develops from peripheral to axial skeleton, with a rigid spine evident in the homozygous mice by 12-16 weeks of age.

Given the comorbidity of AS and IBD in patients, it was speculated that ank/ank mice with ankylosis would have colon inflammation while the wt/wt mice would be normal. Surprisingly, various degrees of inflammation were observed in both ank/ank and wt/wt mice (Fig. 2.1, top panel). The pathology scores of ank/ank and wt/wt mice were not statistically different ($p=0.26$). However among animals housed in the same animal facility, age-matched C57BL/6 wildtype and HLA-B27tg mice on the same background show no or very minimal gut involvement, separately. Total pathology score consists of acute inflammation score, chronic inflammation score and the percentage of bowel involved.

Fig. 2.1 bottom panel depicts representative pathology sections. Fig. 2.1A shows an ank/ank mouse with a score 7. There are a large number of infiltrates in the crypt area with significant structural change in the colon of this mouse. Fig. 2.1C represents a wt/wt mouse with a score 5. Unlike ank/ank mice, the infiltrates in this mouse are primarily located at the lamina propria area with a small focus of mucosal chronic inflammation. Fig. 2.1B & 2.1D show ank/ank and wt/wt mouse with no colon inflammation, respectively (score 0).

Mice were then categorized into groups based on their pathology scores: (1) those with total scores $\geq 5$ were considered to have severe gut inflammation (2) those with total scores $<5$ were considered to have mild gut inflammation (Fig. 2.2A). Groups A and C represent ank/ank and wt/wt mice with severe gut involvement, respectively. Groups B and D represent ank/ank and wt/wt mice with mild gut inflammation. Similar proportions of ank/ank mice (40%) and wt/wt littermates (48%) had severe gut inflammation. None of the mice had clinically significant gut inflammation as defined by diarrhea.
In light of the different locations of infiltrates discovered in the representatives of \textit{ank/ank} and \textit{wt/wt} mouse (Fig. 2.1 A & C), further analysis was taken to detect whether there was difference in the colon inflammation of these mice in depth with a secondary scoring system. Gut inflammation in Group A vs Group C were compared (Fig. 2.2 B-H). There was a significant ($p=0.03$) difference in the degree of mucin depletion between Group A (0.06 $\pm$ 0.06) and C (0.4 $\pm$ 0.16) (Fig. 2.2B). Otherwise, there was no difference between these two groups of mice in the severity of hyperplasia, number of foci, depth of inflammation, as well as architecture distortion. There were comparable neutrophil and plasma cell counts in Group A and Group C. No granulomas were detected in any of the mice.

\textbf{2.3.2 BASELINE SERUM Lcn2 LEVELS ARE ELEVATED IN \textit{ank/ank} MICE WITH GUT INFLAMMATION}

Circulating Lcn2 levels were analyzed in both \textit{ank/ank} and \textit{wt/wt} mice. \textit{ank/ank} (276 $\pm$ 27 ng/ml [n =40]) showed significantly higher serum Lcn2 levels than \textit{wt/wt} animals (137 $\pm$ 20 ng/ml [n =21]; $p=0.0009$) (Fig. 2.3A). Mice were then categorized based on their degrees of gut involvement (Fig. 2.2A). In \textit{wt/wt} littermates, Lcn2 levels were comparable in mice with severe or mild gut inflammation (163 $\pm$ 37 ng/ml [n =10] and 114 $\pm$ 17 ng/ml [n=11] respectively) (Fig. 2.3B). However, \textit{ank/ank} mice with severe gut involvement had significantly ($p<0.05$) higher Lcn2 levels (363 ng/ml $\pm$ 50 [n=16]) compared to those with mild colon inflammation (218 ng/ml $\pm$ 24 [n=24]; Fig. 2.3B). Also, Lcn2 levels of \textit{ank/ank} mice with severe gut involvement were significantly higher than their \textit{wt/wt} littermates with same degree gut inflammation. No significant difference was found in \textit{ank/ank} mice and \textit{wt/wt} littermates with mild colon inflammation. Thus elevated serum Lcn2 levels were seen with the coexistence of gut inflammation and spinal
ankylosis, which was present only in the *ank/ank* mice. This suggested that pathways of colon inflammation in *ank/ank* mice might be Lcn2-dependent while that in *wt/wt* mice might be Lcn2-independent. However, there is no association of fecal Lcn2 levels and the severity of gut involvement before and after controlled for the total protein amount (Fig. 2.4 A & B).
Figure 2.1 Colon inflammation in \textit{ank/ank} mice and \textit{wt/wt} littermates. Top panel: Total pathology score (0-9) of 4-5 month-old \textit{ank/ank} mice (n=40) and \textit{wt/wt} mice (n=21) on the background of C3FeB6-A/A\textsuperscript{w-j} and age-matched wildtype (n=4) and HLA-B27tg mice (n=4) on the background of C57BL/6. Total scores are the sum of scores of acute inflammation, chronic inflammation and percentage of bowel involved. Bottom panel: Representative histopathological features of colons from A. \textit{ank/ank} mice with patchy colon inflammation (score 7); B. \textit{ank/ank} mice with no colon inflammation (score 0); C. \textit{wt/wt} mice with a small focus of mucosal chronic inflammation (score 5); and D. \textit{wt/wt} mice with normal colon (score 0).
**Figure 2.2 Detailed colon inflammation scores in ank/ank mice and wt/wt littermates.** A. Total scores; B. Mucin depletion; C. Hyperplasia; D. Number of foci; E. Architectural distortion; F. Depth of inflammation; G. Neutrophil count; H. Plasma cell count. Group A: *ank/ank* mice with severe gut inflammation (total scores $\geq 5$; n=16). Group B: *ank/ank* mice with mild gut inflammation (total scores < 5; n=24). Group C: *wt/wt* mice with severe gut inflammation (total scores $\geq 5$; n=10). Group D: *wt/wt* mice with mild gut inflammation (total scores < 5; n=11).
Figure 2.3 Correlation of serum Lcn2 levels and colon pathology in *ank/ank* mice versus *wt/wt* littermates. A. Detection of serum Lcn2 levels in 4-5 month-old *ank/ank* (n=40) and *wt/wt* (n=21) mice. B. Serum levels of Lcn2 in mice with severe (total scores ≥5) and mild (total scores <5) colon inflammation (*ank/ank* mice [left] versus *wt/wt* littermates [right]). Group A: *ank/ank* mice with severe gut inflammation (total scores ≥ 5; n=16). Group B: *ank/ank* mice with mild gut inflammation (total scores <5; n=24). Group C: *wt/wt* mice with severe gut inflammation (total scores ≥5; n=10). Group D: *wt/wt* mice with mild gut inflammation (total scores < 5; n=11).
Figure 2.4 No correlation of fecal Lcn2 levels and colon pathology in ank/ank mice versus wt/wt littermates. A. Fecal levels of Lcn2 in mice with severe (total scores ≥5) and mild (total scores <5) colon inflammation (ank/ank mice [left] versus wt/wt littermates [right]). B. Fecal levels of Lcn2 vs. total protein in mice with severe (total scores ≥5) and mild (total scores <5) colon inflammation (ank/ank mice [left] versus wt/wt littermates [right]). Group A: ank/ank mice with severe gut inflammation (total scores ≥5; n=15). Group B: ank/ank mice with mild gut inflammation (total scores <5; n=23). Group C: wt/wt mice with severe gut inflammation (total scores ≥5; n=10). Group D: wt/wt mice with mild gut inflammation (total scores <5; n=11).
2.3.3 BASELINE COLONIC PPARγ EXPRESSION IS SUPPRESSED IN *ank/ank* MICE WITH SEVERE COLON INFLAMMATION

To investigate whether there is an association between PPARγ and Lcn2, the expression of PPARγ at baseline was determined in the colons of mice grouped by their gut involvement (Fig. 2.2A). In *ank/ank* mice, colonic PPARγ had a higher expression in those with mild gut inflammation (Group B; 1.9 ± 0.3, n=11) than with severe colon pathology (Group A; 0.8 ± 0.09; n=13; *p*<0.01) (Fig. 2.5A). A similar trend was found in *wt/wt* animals with severe vs. mild colon inflammation, although it was not statistically significant (Group C vs. Group D).

The expression of colonic PPARγ was further linked with serum Lcn2 levels in *ank/ank* (Fig. 2.5B) and *wt/wt* mice (Fig. 2.5C). I first compared animals with the same degree of colon pathology from different genotype (Group A vs. C; Group B vs. D). In mice with mild inflammation (Group B & D), colonic PPARγ expression was higher in *ank/ank* than *wt/wt* mice but the Lcn2 levels were comparable. However, in mice with severe inflammation (Group A & C), Lcn2 levels were higher in *ank/ank* than *wt/wt* mice while the expression of PPARγ was similar. On the other hand, when comparing animals with different degree of gut inflammation within the same genotype (Group A vs. B; Group C vs. D), there was lower PPARγ expression and higher Lcn2 levels in those with severe gut inflammation (Group A & C). Nevertheless, this was reversed in animals with mild gut inflammation (Group B & D).
Figure 2.5 Baseline colonic PPARγ expression comparison with serum Lcn2. A. Colonic PPARγ transcript was determined in ank/ank and wt/wt mice. Mice were further categorized based on their degree of colon inflammation. B. The association of colonic PPARγ expression and serum Lcn2 versus gut pathology in ank/ank mice. C. The association of colonic PPARγ expression and serum Lcn2 versus gut pathology in wt/wt mice. Group A: ank/ank mice with severe gut inflammation (total scores ≥ 5; n=13). Group B: ank/ank mice with mild gut inflammation (total scores <5; n=11). Group C: wt/wt mice with severe gut inflammation (total scores ≥5; n=8). Group D: wt/wt mice with mild gut inflammation (total scores < 5; n=7). Blue circle represents mice with severe colon inflammation (total scores ≥ 5; Groups A and C); Red circle represents mice with mild colon inflammation (total scores <5; Groups B and D).
2.3.4 Lcn2 WAS UPREGULATED IN RESPONSE TO A PPARγ AGONIST IN \textit{wt/wt} MICE

To detect whether PPARγ plays a role in Lcn2 expression, intraperitoneal injections with either a PPARγ agonist (Rosi) or an antagonist (BADGE) were done in both \textit{ank/ank} and \textit{wt/wt} animals for 4 consecutive days. Animals were sacrificed on the sixth day. Serum levels of Lcn2 were analyzed. There was no DMSO effect on Lcn2 in both \textit{ank/ank} and \textit{wt/wt} mice. Neither Rosi nor BADGE treatment changed the levels of Lcn2 in \textit{ank/ank} mutants (Fig. 2.6A). In \textit{wt/wt} mice, Rosi treatment significantly augmented the levels of serum Lcn2 (313 ± 44 ng/ml) compared to DMSO controls (177 ± 44 ng/ml; \(p<0.05\)) (Fig. 2.6B). However, BADGE treated \textit{wt/wt} mice reflected similar levels of Lcn2 to DMSO controls (206 ± 21 vs. 177 ± 44 ng/ml, respectively).

\textbf{Figure 2.6 Serum Lcn2 levels after Rosi and BADGE treatment in \textit{ank/ank} mice versus \textit{wt/wt} littermates.} A. Serum Lcn2 levels of \textit{ank/ank} mice before and after Rosi/BADGE treatments. B. Serum Lcn2 levels of \textit{wt/wt} mice before and after Rosi/BADGE treatments. UN: untreated animals (baseline); DM: 5% DMSO control animals; Rosi: 10mg/kg body weight Rosiglitazone treated animals; BAD: 30mg/kg body weight BADGE treated animals.
2.3.5 SEX DIFFERENCE OF Lcn2 IN *ank/ank* MOUSE MODEL

To investigate whether there is a sex bias in the *ank/ank* mouse model, animals were further categorized based on sex.

First, the degree of colon inflammation in male *vs* female *ank/ank* mice and *wt/wt* littersmates was analyzed (Fig. 2.7). There was no significant sex difference of these mice in total scores, degree of mucin depletion, severity of hyperplasia, number of foci, depth of inflammation, as well as architectural distortion (Fig. 2.7 A-F). However, there was a trend of higher number of neutrophils and plasma cells in female *wt/wt* animals with severe and mild gut involvement compared to male *wt/wt* in the same category, respectively (Fig. 2.7 G & H). Therefore, colon inflammation in males *vs* females of the same subgroup was comparable.

Circulating Lcn2 levels were then analyzed in male *vs* female *ank/ank* and *wt/wt* mice at baseline. Serum Lcn2 levels were significantly higher in *ank/ank* (256 ± 24 ng/ml [n =25]) than *wt/wt* (106 ± 9 ng/ml [n =10]; p<0.05) in male animals (Fig. 2.8A). Such a difference was not detected in female animals, likely due to an increased level of Lcn2 in *wt/wt* females compared to males. When mice were further categorized based on their degrees of gut involvement, a relationship of higher Lcn2 levels and severe gut inflammation in *ank/ank* mice was seen only in female animals (466 ng/ml ± 90 [n=7]; Fig. 2.8B). In male *ank/ank* mutants, Lcn2 levels were comparable in those with severe *vs* mild gut inflammation (283 ± 42 ng/ml [n =9] and 240 ± 31 ng/ml [n=16] respectively), indicating less likelihood of Lcn2 pathway involved in gut inflammation in these animals. Moreover, in *wt/wt* animals with severe colon inflammation, there was a trend of higher levels of Lcn2 in females than males, which could be attributed to higher neutrophil and plasma cell counts in this female subgroup compared to their male counterparts.
Figure 2.7 Detailed colon inflammation scores of males versus female ank/ank mice and wt/wt littermates. A. Total scores; B. Mucin depletion; C. Hyperplasia; D. Number of foci; E. Architectural distortion; F. Depth of inflammation; G. Neutrophil count; H. Plasma cell count. Group A: male ank/ank mice with severe gut inflammation (total scores ≥ 5; n=9). Group B: male ank/ank mice with mild gut inflammation (total scores <5; n=16). Group C: male wt/wt mice with severe gut inflammation (total scores ≥5; n=4). Group D: male wt/wt mice with mild gut inflammation (total scores < 5; n=6). Group E: female ank/ank mice with severe gut inflammation (total scores ≥ 5; n=7). Group F: female ank/ank mice with mild gut inflammation (total scores <5; n=8). Group G: female wt/wt mice with severe gut inflammation (total scores ≥5; n=6). Group H: female wt/wt mice with mild gut inflammation (total scores < 5; n=5).
Figure 2.8 Correlation of serum Lcn2 levels and colon pathology in ank/ank mice versus wt/wt littermates of difference sexes. A. Detection of serum Lcn2 levels in 4-5 month-old male vs female ank/ank (n=25 males and n=15 females) and wt/wt (n=10 males and n=11 females) mice. B. Serum levels of Lcn2 in mice with severe (total scores ≥5) and mild (total scores <5) colon inflammation in difference sexes (male mice [left] versus female mice [right]). Group A: male ank/ank mice with severe gut inflammation (total scores ≥5; n=9). Group B: male ank/ank mice with mild gut inflammation (total scores <5; n=16). Group C: male wt/wt mice with severe gut inflammation (total scores ≥5; n=4). Group D: male wt/wt mice with mild gut inflammation (total scores <5; n=6). Group E: female ank/ank mice with severe gut inflammation (total scores ≥5; n=7). Group F: female ank/ank mice with mild gut inflammation (total scores <5; n=8). Group G: female wt/wt mice with severe gut inflammation (total scores ≥5; n=6). Group H: female wt/wt mice with mild gut inflammation (total scores <5; n=5).
To investigate whether there is sex difference in PPARγ, the colonic expression of PPARγ at baseline was determined in mice grouped by their gut involvement and sex (Fig. 2.9). Although no significance was detected, higher expression colonic PPARγ was likely related to mild gut inflammation in mice regardless of their genotype nor sex. This suggests the role of colonic PPARγ in gut inflammation is independent of sex in this model.

Lastly, the role of PPARγ agonist (Rosi) and antagonist (BADGE) in Lcn2 was analyzed in different sexes of mice to detect whether the manipulation of Lcn2 by PPARγ is sex-dependent. No sex bias of Rosi/BADGE effects on Lcn2 was detected in either ank/ank mutants or wt/wt animals (Fig. 2.10). In ank/ank mutants, neither Rosi nor BADGE treatments changed the levels of Lcn2 in either sex (Fig. 2.10 A & C). In wt/wt mice, there is a trend of increased Lcn2 levels after Rosi treatment (331 ± 70 ng/ml in males and 298 ± 60 ng/ml; Fig. 2.10 B & D) in females) compared to DMSO controls (197 ± 70 ng/ml in males and 161 ± 61 ng/ml in females). However, there was no statistical significance, likely in part due to a small number of each subgroup. Although baseline levels of Lcn2 in wt/wt animals were lower in males than females, DMSO appeared to raise the levels of male to similar levels of females.
Figure 2.9 Baseline colonic PPARγ expression in male vs. female mice. Colonic PPARγ transcript was determined in ank/ank and wt/wt mice. Mice were further categorized based on their degree of colon inflammation. Group A: male ank/ank mice with severe gut inflammation (total scores ≥ 5; n=7). Group B: male ank/ank mice with mild gut inflammation (total scores <5; n=9). Group C: male wt/wt mice with severe gut inflammation (total scores ≥5; n=3). Group D: male wt/wt mice with mild gut inflammation (total scores <5; n=6). Group E: female ank/ank mice with severe gut inflammation (total scores ≥ 5; n=8). Group F: female ank/ank mice with mild gut inflammation (total scores <5; n=4). Group G: female wt/wt mice with severe gut inflammation (total scores ≥5; n=5). Group H: female wt/wt mice with mild gut inflammation (total scores < 5; n=1).
Figure 2.10 Serum Lcn2 levels after Rosi and BADGE treatment in \textit{ank/ank} mice versus \textit{wt/wt} littermates in difference sexes. A. Serum Lcn2 levels of male \textit{ank/ank} mice before and after Rosi/BADGE treatments. B. Serum Lcn2 levels of male \textit{wt/wt} mice before and after Rosi/BADGE treatments. C. Serum Lcn2 levels of female \textit{ank/ank} mice before and after Rosi/BADGE treatments. D. Serum Lcn2 levels of female \textit{wt/wt} mice before and after Rosi/BADGE treatments. UN: untreated animals (baseline); DM: 5% DMSO control animals; Rosi: 10mg/kg body weight Rosiglitazone treated animals; BAD: 30mg/kg body weight BADGE treated animals.
2.4 DISCUSSION

While the pathogenesis of concurrent IBD and AS remains unclear, both clinical entities share common risk factors and potential pathogenic pathways. Given the functions in inflammation and bone remodeling, Lcn2/PPARγ may be one of these pathways. So far, there is no animal model which completely mimics human AS. Among several animal models commonly used for AS, the ank/ank mutant mouse is the first animal model that led us to the discovery of a distinctive serological feature (elevated serum NOG/SOST ICs) of human AS (Tsui et al., 2014). Therefore, ank/ank mouse may serve an informative model to detect whether Lcn2 and PPARγ are dysregulated and associated with the gut-joint axis in AS.

Gut inflammation was discovered in ank/ank mutant mice with ankylosis. There was colon inflammation in both ank/ank and wt/wt mice at baseline. By comparing ank/ank and age-matched wt/wt mice with severe gut involvement, no distinguishable inflammatory features were found except for higher degree of mucin depletion in the ank/ank mice. Thus the gut inflammation was independent of the presence or absence of ANK protein. The discovery of gut phenotype in wt/wt mice indicated some potential hidden defects in these animals, which remain to be discovered. It is likely due to more susceptibility of mice with C3FeB6-A/A^w^j background to potential stimuli in the facility as there is no gut phenotype in other strains of mice housed in the same environment. For example, the wildtype and HLA-B27tg mice on C57BL/B6 background revealed no gut involvement at baseline.

However, the contribution of ank gene to the pathogenesis in ank/ank mutant mice was not the main focus of these studies. These animals provided us the opportunity to interpret the gut-joint overlap in the context of aberrant pathways in AS. Since there is no clinically evident gut
inflammation (e.g. diarrhea), ank/ank mouse serves a good model for AS patients with various degree of subclinical gut inflammation.

Lcn2 is associated with ankylosis and gut inflammation in ank/ank mice. In these studies, ank/ank mice were aged to 4-5 months when all displayed full-blown ankylosis to control the phenotypic variation of ankylosis during aging. Results from ank/ank mice showed higher serum Lcn2 in those with severe gut involvement than those with mild gut inflammation, which corresponds to the elevation of LCN2 in human IBD patients (Stallhofer et al., 2015). This suggests a possible contribution of gut inflammation to the elevation of serum Lcn2. On the other hand, in animals with comparably severe gut pathology, higher Lcn2 levels were discovered in ank/ank mice compared to wt/wt littermates, possibly attributing to the functions of Lcn2 in osteoblastogenesis and osteoclastogenesis (Costa et al., 2013; Kim et al., 2015, 2016; Rucci et al., 2015b). Interestingly, in ank/ank mice with minimal gut inflammation, various levels of Lcn2 were detected. Such differences in Lcn2 could indicate various host-microbe interactions (not reflected in the degree of gut inflammation) in ank/ank mice given the anti-microbial functions of Lcn2 (Moschen et al., 2017). Although at the advanced stage of ankylosis there is no phenotypic variation in the animals, the contribution of bone to elevated Lcn2 remains to be investigated as evidence of no histological difference is lacking. On the other hand, the concurrence of ankylosis and gut inflammation in increasing serum Lcn2 implicates a possible interactive or synergistic contribution of gut and bone to circulating Lcn2 levels since neither gut inflammation alone in wt/wt mice nor ankylosis alone in ank/ank mutants was reflected in increase serum levels of Lcn2.

The role of PPARγ in gut inflammation is implicated by its association with the colon pathology in ank/ank and wt/wt mice at baseline. The levels of colonic PPARγ transcript were negatively associated with the degree of gut inflammation in ank/ank mice, which is consistent
with the findings of impaired PPARγ expression in the colon of patients with ulcerative colitis (UC) (Bouguen et al., 2015). When comparing colonic PPARγ with serum Lcn2 in our mice at baseline, the change of serum Lcn2 appeared to be inversely related to colonic PPARγ expression in the context of gut inflammation. However, the weak association of Lcn2 and PPARγ is possibly due to the different source of serum Lcn2 vs. colonic PPARγ. This might be the reason why these results are contradictory with findings from a S. typhimurium-induced colitis model, where the absence of PPARγ in intestinal epithelial cells was accompanied with lower colonic Lcn2 expression after infection (Kundu et al., 2014a).

The modulation of Lcn2 by the PPARγ agonist (Rosi) in ank/ank mouse model would indicate the involvement of PPARγ in the aberrant Lcn2 pathway in AS. Based on the relationship of PPARγ and Lcn2 discovered by other groups (Kundu et al., 2014a), it was speculated that Rosi treatment would activate PPARγ and increase the levels of Lcn2, while BADGE treatment would show the opposite effect. In our studies, a trend of elevated serum levels of Lcn2 were detected in Rosi-treated animals compared with DMSO controls in wt/wt mice, indicating a potential role of PPARγ in modulating the levels of serum Lcn2. It is known that Rosi activates NFκB pathway via the induction of PPARγ nuclear translocation (Bernier et al., 2013). As there is a binding site for NFκB within the promoter region of gene coding Lcn2 (Alessio Iannetti et al., 2008), it is hypothesized that binding of Rosi to PPARγ in cytoplasm helps the nuclear translocation of PPARγ, thus activating the expression of genes involved in NFκB pathways and the following expression and secretion of Lcn2 (Fig. 2.11). However, Rosi effect was not seen in ank/ank mice, likely due to high (saturated) baseline Lcn2 levels in these animals compared to wt/wt controls. On the contrary, serum Lcn2 levels was similar before and after BADGE treatment in both ank/ank and wt/wt mice, which could be attributed to the short BADGE treatment window in our mice. The BADGE treatment results are consistent with two experimental-induced colitis rat models (DSS-
and TNBS-) treated with BADGE with similar study design (Celinski et al., 2011; Dworzanski et al., 2010). In both studies, BADGE failed to show any effect on the expression of inflammatory cytokines in either serum or intestine. Interestingly, although there was no detectable effect of BADGE on inflammatory cytokines, the addition of BADGE to Rosi treatment in these rats neutralized the Rosi effect, suggesting a potential competition of BADGE and Rosi in binding to PPARγ. This indicates that BADGE alone may not play a role in inhibiting PPARγ activation.

Figure 2.11 A schematic offering the potential mechanism of Rosi treatment in inducing serum levels of Lcn2. Upon Rosi treatment, PPARγ is translocated from cytoplasm to nucleus and attached to the promoter regions of genes involved in NFκB pathways. Activation of NFκB pathways stimulates the expression and secretion of Lcn2.

Higher levels of Lcn2 associated with gut involvement and ankylosis were discovered only in female animals, suggesting a sex difference of Lcn2 pathway in the ank/ank mouse model. Lcn2 could be a more relevant pathway in female of ank/ank mouse model. Given similar degrees of gut inflammation and the full-blown ankylosis in male vs female animals, the difference of Lcn2 may be due to different gut bacterial composition between the sexes. Sex-biased gut microbiome is not
uncommon in both murine and human studies (Haro et al., 2016b; Kozik, Nakatsu, Chun, & Jones-Hall, 2017). Moreover, in male ank/ank and wt/wt mice, Lcn2 levels were similar of the same genotype of animals with severe vs mild gut involvement, indicating a less likelihood of Lcn2 involvement in gut inflammation in males. On the other hand, although it is not significant, there is a trend of higher Lcn2 levels of females than males in wt/wt with severe colon pathology. This is likely due to a higher number of neutrophils and plasma cell counts in female vs males in this subgroup, as both cell types are important producers of Lcn2 (Singh et al., 2016b).

However, there is no sex difference of PPARγ at baseline or in modulation of Lcn2, implying that there are factors other than PPARγ involved in the aberrant Lcn2 pathway in these mice. Those unknown modulators are likely sex-dependent, which results in a sex difference in the levels of Lcn2 at baseline of the animals. Rosi treatment increased the Lcn2 of wt/wt in both sexes to similar levels. The loss of significance in these subgrouping based on sex may be due to low number of animals, as ank/ank mutant is an outbred mouse model that larger individual variation may occur. Moreover, although at baseline, there was slightly higher Lcn2 levels in female than male wt/wt, the use of DMSO as control normalized such difference as levels of Lcn2 in males is increased by DMSO to similar levels to females.

These studies are not without drawbacks. (1) It is not known whether there is histological difference of mutants at the most advanced stage of full-blown ankylosis, although the use of 4-5 month-old mutants largely controls the phenotypic variation of ankylosis during aging. (2) A mixed group of ank/ank mice with various degrees of colon inflammation were used in the Rosi/BADGE treatments. Based on the baseline Lcn2 data, levels of serum Lcn2 are associated with the degree of gut inflammation in ank/ank mice. However, the initial colon involvement in the treatment animals is unknown. Human Lcn2 is a unique marker of neutrophil inflammation in
ulcerative colitis (Carlson et al., 2002). But there is no association between fecal Lcn2 and the severity of colon inflammation in these animals, likely due to low number of neutrophil infiltrates in these mice. Based on the semi-quantitative neutrophil and plasma cell counts in these animals, significant gut involvement of these animals is likely a chronic, plasma cell-driven inflammation rather than an acute, neutrophilic driven pattern. Whether the most commonly used fecal neutrophil-produced calprotectin would be informative in our mice remains a question (MacQueen et al., 2016b). A non-invasive method of analyzing colon inflammation before treatment has yet to be identified. (3) PPARγ translocation induced by Rosi remains to be confirmed by immunohistochemistry in the colonic tissues of mice after treatments. (4) A specific association of Lcn2 and PPARγ at the same effector sites (gut vs joint) has yet to be established. (5) Owing to the outbred nature of ank/ank mice, larger number of animals are needed to further investigate sex difference in the Lcn2/PPARγ pathway.

Nevertheless, ank/ank mice might not be an optimal option for future mechanistic study of Lcn2 and PPARγ on bone remodeling for the following reasons. (1) Modulation of Lcn2 in ank/ank mice might not be feasible since Lcn2 is intrinsically increased in these animals. To inhibit or delay the process of bone formation, negative modulation of Lcn2 in these animal is needed. As studies on bone homeostasis require a long-term design, treatments modulating Lcn2 might be limited by the short lifespan of less than 6 month of ank/ank mice. Moreover, both Lcn2<sup>Hep−/−</sup> and global Lcn2<sup>−/−</sup> mice were demonstrated to had an increased susceptibility to gastrointestinal infection with <i>K. pneumoniae</i> or <i>E. coli</i> [a138]. Suppressing the expression of Lcn2 followed by a bacterial infection may be harmful or lethal in ank/ank mice with gut inflammation. In this regard, increasing the expression of Lcn2 in wt/wt animals to stimulate bone formation seems to be more feasible. (2) Considering the relatively low likelihood of obtaining homozygous mice in our breeding, Lcn2 knockout in these mice might result in an even lower yield. On the one hand,
breeding of homozygous ank/ank mice is challenging, possibly due to decreased mobility of young homozygous mice and/or to possible defects on the reproductive system because of the ank mutation. ank/ank mice demonstrate poor fertility and the mating window is extremely short. Breeding of homozygous ank/ank mice rarely succeeds and litter size is limited to maximum 1-2 pups. Moreover, pups born from homozygous parents exhibit a more severe ankylosing phenotype with an even shorter lifespan. On the other hand, although heterozygous parents would provide a slightly bigger litter size with 2-5 pups per litter, the likelihood of obtaining ank/ank mutants is less than one per litter since there are not always ank/ank mice offspring in every litter. Therefore, considering the current breeding difficulty of getting mutant mice, knocking out an important gene (lcn2) could be troublesome. (3) The role of Lcn2 in reflecting host-microbe interactions in murine could be different from that in human. Although little is known about the gut microbiome in ank/ank mice, studies have shown different composition of gut microbiome in murine and human [a309]. Thus difference gut microbial composition in murine might fail to reflect the real relationship of LCN2 and bacterial triggers in AS patients. Collectively, ank/ank mice may not be an optimal animal model for the future mechanistic study of Lcn2 in AS.

2.5 CONCLUSIONS

Aberrant levels of Lcn2 associated with ankylosis and gut pathology in ank/ank mice indicate a potential mechanism underlying the overlap of AS and IBD. The interplay of gut inflammation and ankylosis in elevating Lcn2 suggests that ank/ank mouse is a relevant animal model for the gut-joint interactions, as seen in AS. PPARγ plays a role in aberrant Lcn2 pathway in AS.
Chapter 3

Lipocalin 2 (LCN2) is elevated in ankylosing spondylitis (AS), associated with HLA-B27 status and sex.
ABSTRACT

Background

The basis of gut-joint axis remains unknown in ankylosing spondylitis (AS) research. Lipocalin 2 (Lcn2), a host pleiotropic anti-microbial factor, is elevated in the ank/ank mouse model. Our aim is to assess whether there is a relationship of LCN2 and human AS and whether LCN2 is associated with two strong AS genetic factors, HLA-B27 and sex.

Patients and Methods

A retrospective study was performed on 519 patients with pure AS or AS with clinical IBD (AS-IBD). Cohort 1 (172 patients with pure AS) is cross-sectional with only one serum sample collected. Cohort 2 (347 patients with AS alone (n=290) or AS-IBD (n=57)) is sequential, having been followed longitudinally for 3-12 years. Concurrent clinical variables are available. Serum levels of LCN2 were measured by ELISA.

Results

AS patients (with and without concomitant IBD) had elevated LCN2 levels (220±9 and 193±5 ng/ml respectively) compared to patients with mechanical back pain (MBP; 97 ± 4; p<0.0001). AS patients with more severe ankylosis (mSASSS >10) had higher LCN2 levels (p<0.0001). The association of LCN2 and the degree of ankylosis is influenced by HLA-B27 status (p<0.05) and sex (p<0.01).
Conclusions

Serum LCN2 is elevated in AS. Levels of LCN2 are associated with coexisting IBD and with the degree of ankylosis. The relationship of HLA-B27 positivity and sex with LCN2 implicates a host-microbe interaction underlying the gut-joint axis in AS.

3.1 INTRODUCTION

Ankylosing spondylitis (AS), a heterogeneous subset of spondyloarthritis (Pal, 1987), manifests as chronic lower back pain with a propensity for progression to spinal ankylosis. The imaging hallmark of AS is neo-ossification at the site of joint inflammation (I. H. Song, Poddubnyy, Rudwaleit, & Sieper, 2008b). The severity of ankylosis in the spine is scored by modified Stoke AS Spine score [mSASSS] applied to axial X-rays. Up to 60% of AS patients have evidence of subclinical gut inflammation while 10-12% have clinical evidence of concomitant inflammatory bowel disease (IBD) (Ciccia et al., 2016; Gracey, Qaiyum, et al., 2016). Research on the gut-joint axis in AS is emerging and immune cells linking between gut and joint are being studied (Dakwar, Reddy, Vale, & Uribe, 2008). Yet the basis of this overlap in AS remains unclear.

AS is a complex disease with a strong genetic component. Aside from the robust HLA-B27 and male sex association with AS, genome-wide association studies (GWAS) have identified 48 AS non-major histocompatibility complex (MHC) immune-related loci. Yet the pathogenesis of AS is not entirely genetically determined, and environmental effects such as microbial factors, as implicated in reactive arthritis (ReA), might serve as a disease trigger (Jacques, Elewaut, &
Mielants, 2010). The role of bacterial triggers in AS development has been suggested by studies in the HLA-B27tg rat (Taurog et al., 1994b). And the presence of HLA-B27 has been shown to shape the gut bacteria in these rats, with a HLA-B27 signature of gut commensals identified in three groups of bacteria including *Firmicutes, Proteobacteria*, and *Akkermansia muciphila* (Asquith et al., 2016b; P. Lin et al., 2014). Therefore, a pleiotropic factor, produced by multiple tissues and functioning on both inflammation and bone remodeling, could potentially serve a shared pathway of gut-joint overlap in AS.

Lipocalin 2 (LCN2), originally identified in neutrophil granules (also called neutrophil gelatinase B-associated lipocalin [NGAL]) is a secreted anti-microbial protein fast released in response to microbial triggers (Singh et al., 2016a). LCN2 is produced in multiple cell types in different tissues (including gut and joint) and has both pro- and anti-inflammatory properties which are context-dependent (Abella et al., 2015; Conde et al., 2016; Moschen et al., 2017; Stallhofer et al., 2015c; Veeriah et al., 2016). Elevated serum LCN2 have been reported in patients with IBD (Stallhofer et al., 2015c) and psoriasis (Ataseven, Kesli, Kurtipek, & Ozturk, 2014b), both of which are frequent AS comorbidities. Moreover, lipocalin 2 is shown to play a role in osteoblastogenesis and osteoclastogenesis in mice (Costa et al., 2013; Rucci et al., 2015a). In Chapter 2, we identified the association of Lcn2 with ankylosis and the degree of gut inflammation in the ank/ank mouse model. Thus, LCN2 could be a mediator of gut inflammation and ankylosis in human AS.

The aim of this chapter is to explore the role of LCN2 in human AS and its association with the degree of ankylosis in the context of the HLA-B27 status and sex.
3.2 PATIENTS AND METHODS

3.2.1 HUMAN STUDY SUBJECTS

AS patients who met the modified New York classification criteria for the disease (van der Linden et al., 1984) (with unilateral SIJ scores of 3 or 4) and mechanical back pain (MBP) patients were recruited from Toronto Western Hospital AS clinic. MBP patients had no clinical evidence of inflammatory back pain and no radiographic evidence of sacroiliitis. 158 healthy controls (HC) and 85 IBD without AS patients were also available. Two AS patient cohorts were used in this study: Cohort 1 (172 patients) is cross-sectional with only one serum sample collected. Cohort 2 (347 patients) is sequential, having been followed longitudinally for 3-12 years. Among patients in cohort 2, there are 57 AS patients with clinical IBD. Sequential serum samples were obtained at each visit, usually at one year intervals. The latest modified Stoke AS Spine score [mSASSS] of each patient is available. Sex and HLA-B27 status are available. Demographic features of both cohorts are summarized in Table 3.1. The study was approved by UHN ethics committee and all participating patients provided written informed consent.

3.2.2 DETERMINATION OF SERUM LCN2 LEVELS

Aliquots of serum samples were stored at -70°C until use. The sequential samples from each patient were thawed and analyzed at the same time to minimize assessment variabilities. LCN2 levels were measured by an ELISA kit according to manufacturer’s protocol (R & D).
3.2.3 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test was carried out using GraphPad Prism 5 program. A $p$-value of less than 0.05 is considered significant. Data is presented as mean ± standard error.
Table 3.1 Demographic features of AS patients

(Cohort 1, Cross-sectional)

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS alone</td>
<td>76% (131/172)</td>
<td>24% (41/172)</td>
<td>172</td>
</tr>
<tr>
<td>HLA-B27-positive</td>
<td>75% (95/126)</td>
<td>63% (25/40)</td>
<td>72% (120/166)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>3 - 62</td>
<td>2 - 42</td>
<td>2 - 62</td>
</tr>
</tbody>
</table>

(Cohort 2, Longitudinal)

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS alone</td>
<td>76% (219/290)</td>
<td>24% (71/290)</td>
<td>290</td>
</tr>
<tr>
<td>AS-IBD</td>
<td>77% (44/57)</td>
<td>23% (13/57)</td>
<td>57</td>
</tr>
<tr>
<td>Total (AS + AS-IBD)</td>
<td>76% (263/347)</td>
<td>24% (84/347)</td>
<td>347</td>
</tr>
<tr>
<td>HLA-B27 positive in AS alone</td>
<td>83% (182/219)</td>
<td>77% (55/71)</td>
<td>82% (237/290)</td>
</tr>
<tr>
<td>HLA-B27 positive in AS-IBD</td>
<td>50% (22/44)</td>
<td>77% (10/13)</td>
<td>56% (32/57)</td>
</tr>
<tr>
<td>Disease duration (yr) for AS alone</td>
<td>4 - 53</td>
<td>2 - 44</td>
<td>2 - 53</td>
</tr>
<tr>
<td>Disease duration (yr) for AS-IBD</td>
<td>2 - 53</td>
<td>10 - 47</td>
<td>2 - 53</td>
</tr>
</tbody>
</table>
3.3 RESULTS

3.3.1 SERUM LEVELS OF LCN2 ARE ELEVATED IN AS PATIENTS

We first assessed whether LCN2 levels are elevated in patients with AS alone (Cohort 1: 172 patients) compared to healthy controls (HC; n=158), IBD (n=85) and MBP patients (n=52). Consistent with results from the ank/ank mouse model (Chapter 2), AS patients had significantly higher serum levels of LCN2 (154 ± 7 ng/ml) than HC (88 ± 2 ng/ml), IBD (91 ± 10 ng/ml) or MBP patients (97 ± 4 ng/ml; p <0.0001) (Fig. 3.1).

3.3.2 SERUM LEVELS OF LCN2 ARE ASSOCIATED WITH COEXISTING IBD AND WITH THE DEGREE OF ANKYLOSIS IN AS

In light of the involvement of Lcn2 in the gut inflammation and presence of ankylosis in the ank/ank mouse model (Chapter 2), the role of LCN2 in the gut-joint axis of human AS were analyzed.

To investigate whether LCN2 is related to gut involvement in human AS, LCN2 levels were measured in sequential samples in Cohort 2 consisting of patients with AS alone (n=290) and AS-IBD (n=57). The peak level from each patient in the respective groups was used for calculations. Both AS and AS-IBD patients had higher levels of serum LCN2 (193 ± 5 and 220 ±9 ng/ml respectively; Fig. 3.2) compared to HC, and to disease controls (IBD alone or MBP patients). Levels of LCN2 are higher in AS-IBD than AS patients (p=0.01). The positive predictive value of LCN2 for distinguishing AS from MBP was 94%, while the sensitivity was 93% and specificity was 67% (Table 3.2).
Unlike the uniform degree of axial ankylosis in \textit{ank/ank} mutants, the availability of mSASSS in the patients enabled us to analyze whether an association of LCN2 and severity of ankylosis exists. The degrees of ankylosis were arbitrarily defined as more severe ankylosis (mSASSS>10) and less severe ankylosis (mSASSS\leq10). Patients with AS alone was the focus of this investigation. In both Cohort 1 and Cohort 2, AS patients with more severe ankylosis (mSASSS>10) had higher LCN2 levels (Fig. 3.3A; 174 ± 15 ng/ml [n=60]; \(p<0.001\); & Fig. 3.3B; 216 ± 8 ng/ml [n=91]; \(p<0.05\)) than those with mSASSS\leq10, suggesting a relationship between ankylosis and circulating LCN2. Moreover, AS patients from both cohorts had higher LCN2 levels than MBP controls (97 ± 4 ng/ml; \(p<0.0001\)) irrespective of their mSASSS status.
Figure 3.1 Serum LCN2 levels in AS patients (Cohort 1). Detection of serum LCN2 levels in patients with AS (n = 172), MBP (n = 52), IBD (n = 85) and HC (n = 158). One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test was used. A $p$-value of less than 0.05 is considered significant.

![Figure 3.1](image1)

Figure 3.2 Serum LCN2 levels in AS patients with and without IBD (Cohort 2). Detection of serum LCN2 levels in patients with AS (n = 290), MBP (n = 52), IBD (n = 85), AS-IBD (n=57) and HC (n = 158). One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test was used. A $p$-value of less than 0.05 is considered significant.

![Figure 3.2](image2)
Table 3.2 Predictive values of subgroups from Cohort 2

<table>
<thead>
<tr>
<th>Cohort 2</th>
<th>AS</th>
<th>AS</th>
<th>AS</th>
<th>AS-IBD</th>
<th>AS-IBD</th>
<th>AS-IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vs MBP</td>
<td>vs HC</td>
<td>vs IBD</td>
<td>vs MBP</td>
<td>vs HC</td>
<td>vs IBD</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>94</td>
<td>87</td>
<td>91</td>
<td>76</td>
<td>58</td>
<td>68</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>63</td>
<td>85</td>
<td>74</td>
<td>95</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93</td>
<td>93</td>
<td>93</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Specificity</td>
<td>67</td>
<td>75</td>
<td>69</td>
<td>67</td>
<td>75</td>
<td>69</td>
</tr>
</tbody>
</table>
Figure 3.3 Serum LCN2 levels in AS patients with different degree of ankylosis. A. Detection of serum LCN2 levels in patients with mSASSS>10 (n=60) and with mSASSS<10 (n=107) vs patients with mechanical back pain (MBP; n=52)) (Cohort 1). A. Detection of serum LCN2 levels in patients with mSASSS>10 (n=91) and with mSASSS<10 (n=109) compared with MBP patients (n=52)) (Cohort 2). One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison tests were used. A $p$-value of less than 0.05 is considered significant.

3.3.3 THE ASSOCIATION OF LCN2 WITH HLA-B27 STATUS AND SEX

Since AS is a complex disease affected by both genetic and environment factors, we asked the whether there was a relationship of LCN2, which may reflect gut microbiome changes, and two important genetic factors of AS, HLA-B27 and sex. Patients with AS will be the focus of this section because HLA-B27 and sex are not genetic risk factors for IBD, the comorbidity of IBD in AS patients may skew the relationship. Data from Cohort 1 and Cohort 2 (as defined above) are combined for this section.
The association of LCN2 and ankylosis was first assessed in the context of HLA-B27 status. HLA-B27-positive AS patients with more severe ankylosis (mSASSS >10) had higher LCN2 levels (Figure 3.4A; 200 ± 9 ng/ml [n=123]) than those with mSASSS≤10 (174 ± 5 ng/ml [n=216]; p<0.05). However, in the absence of HLA-B27, the levels of LCN2 in patients were comparable with different degrees of ankylosis (186 ± 15 ng/ml [n=30] and 161 ± 7 ng/ml [n=65] for patients with mSASSS>10 and mSASSS≤10, respectively). This implies that HLA-B27 status in AS affects the association of circulating LCN2 and the degree of ankylosis.

When patients were categorized by sex regardless of their HLA-B27 status, male patients with more severe ankylosis had higher LCN2 levels (205 ± 9 ng/ml [n=156], p<0.01) compared to males with less severe ankylosis (Fig. 3.4B). However, levels of LCN2 in female patients with different degrees of ankylosis are similar (172 ± 26 ng/ml [n=15] vs. 168 ± 8 ng/ml [n=88]), likely due to limited number of female patients with mSASSS>10 (n=15).

When both HLA-B27 status and sex are considered, the association of LCN2 with the degree of ankylosis was only present in HLA-B27-positive male patients (Fig. 3.4C Top panel). In this subgroup, there are higher levels of LCN2 in patients with more severe ankylosis (212 ± 10 ng/ml [n=104], p<0.01) than those with less severe ankylosis (172 ± 6 ng/ml [n=165]).

Taken together, LCN2 is influenced by both sex and HLA-B27 in AS.
Figure 3.4 Serum LCN2 levels in AS patients with different HLA-B27 status and sex (Cohort 1 & 2). A. Detection of serum LCN2 levels in HLA-B27-positive vs HLA-B27-negative patients with different degree of ankylosis (mSASSS>10 vs. mSASSS≤10). B. Detection of serum LCN2 levels in male vs female patients with different degree of ankylosis (mSASSS>10 and mSASSS≤10). C. Top panel: Detection of serum LCN2 levels in HLA-B27-positive vs HLA-B27-negative male patients with different degree of ankylosis (mSASSS>10 and mSASSS≤10). Bottom panel: Detection of serum LCN2 levels in HLA-B27-positive vs HLA-B27-negative female patients with different degree of ankylosis (mSASSS>10 and mSASSS≤10). One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test was used. A $p$-value of less than 0.05 is considered significant.
3.4 DISCUSSION

The upregulation of LCN2 in AS patients confirms the findings in ank/ank mice in Chapter 2. Both gut-initiated and inflammation-perpetuated upregulation of LCN2 levels are likely present in AS patients. However, it remains unclear at present what tissues are the primary source of high systemic LCN2 levels in AS patients. In mice, most systemic Lcn2 is derived from IEC, hepatocytes (Singh et al., 2016a), and neutrophils to a lesser extent. The results from the progressive ankylosis ank/ank mice in Chapter 2 implicates that both gut and joints are the source of circulating Lcn2 in these animals. However, it is unclear whether similar situation occurs in our AS cohort as our current protocol does not include colonoscopy in AS patients without gastrointestinal symptoms. It has been reported that in SpA patients, elevated serum calprotectin and CRP are independently associated with subclinical gut inflammation (Cypers et al., 2016). Although potential PPARγ contribution to aberrant Lcn2 has been established in ank/ank mouse model (Chapter 2), in view of the complexities of LCN2 actions (both pro- and anti-inflammatory and context-dependent) at many sites, further studies are needed to elucidate mechanistically the dysregulation of LCN2 in human AS.

The likely role of LCN2 in providing a link between chronic inflammation and ankylosis in AS patients is supported by its pleiotropic functions (Abella et al., 2015; Ataseven et al., 2014b; Javier Conde et al., 2016; Moschen et al., 2017; Singh et al., 2016a; Stallhofer et al., 2015c; Veeriah et al., 2016). Our data showed that AS-IBD patients had higher LCN2 levels than patients with AS alone, suggesting that circulating LCN2 in part reflects gut inflammation. Importantly, patients with mechanical back pain have normal levels of LCN2. 31% of patients with IBD without AS had elevated LCN2 levels, further supporting a gut-derived stimulus for elevated LCN2 levels. We also showed that AS patients with more severe ankylosis (mSASSS>10) had higher LCN2
levels, suggesting a relationship between ankylosis and circulating LCN2. LCN2 has been shown to be mechano-responsive in osteoblasts and chondrocytes (Shen et al., 2005; Veeriah et al., 2016) and thus could contribute to ankylosis. Transgenic mice over-expressing LCN2 in bone have a thinner layer of cortical bone (Costa et al., 2013) which we have shown to be a feature of AS (Nigil Haroon et al., 2015).

As elevated LCN2 levels implicate ongoing gut inflammation and relate to progressive spinal ankylosis, our findings suggest that normalization of LCN2 could be viewed as a treatment target in AS. Further long term validation studies are required before this concept can be translated into modification of treatment recommendations.

Since AS is a complex disease influenced by the interactions of genetic and environmental factors, analyzing genetic susceptibility or environmental risks alone is not sufficient to unravel the puzzle of this disease. For example, although the structure and functions of HLA-B27 molecule have been intensively studied over decades, its role in AS is still unclear. A similar situation applies to the male dominant nature of the disease. However, it is challenging for direct measure of gut microbiome quantitatively and qualitatively to unravel its association with HLA-B27 and sex. The complexity of the gut microbiome analysis is due to the fact that gut microbiome can be regulated by a number of host and environmental factors and it can change over time. Large cohorts with multiple-point sampling, carrying high cost, would be required for the exploration of AS-related gut microbiome signatures. Moreover, complex bioinformatics analysis is needed for linkage between the host genetic risks with AS gut microbiome signatures, although there are advantages of the most advanced high-throughput sequencing techniques in microbiology studies (Ignyś, Szachta, Gałęcka, Schmidt, & Pazgrat-Patan, 2014; Turpin et al., 2016).
Molecules like LCN2 which are sensitive and responsive to changes of the gut microbiome may serve as better indicators for indirect measure of abnormality of host-microbe interaction than direct measure of gut microbiome in patients. Since the comorbidity of IBD may affect the interaction of HLA-B27 and sex with gut microbiome, patients with only AS were selected and categorized according to their HLA-B27 status and sex. LCN2 levels were positively associated with the severity of ankylosis in HLA-B27 positive group, which likely reflects the contribution of interaction between environmental factors (e.g. gut bacteria) and HLA-B27 to AS progression. Nevertheless, there was no such finding in HLA-B27 negative patients, possibly indicating a lesser role of LCN2 in these patients. Similarly, the discovery of higher LCN2 levels associated with more severe ankylosis in HLA-B27 positive males suggests an aberrant interactions of host and microbe in this subgroup. The fact that male patients are more susceptible to infection in general (Libert et al., 2010; Washburn, Medearis, & Childs, 1965a) may link this sex bias to the shifted gut microbiome in AS. Although the sex difference of gut microbiome in AS is not clear, such difference was already found in patients with obesity (Haro et al., 2016b). As there is close relationship between host immunity and gut bacteria, the microbial effect on sex-biased immune response may be another explanation.

We are aware that there are some limitations in these studies: (1) As mentioned above, patients with pure AS is a mixed group owing to the lack of colonoscopy data on subclinical gut inflammation. (2) As levels of LCN2 are affected by the changing level of inflammation in patients, single point analysis in Cohort 1 may omit the highest LCN2 level through disease development, thus compromising the difference of patients vs. controls. (3) Although the above results show the association of LCN2 levels and AS genetic risk factors (HLA-B27 and sex), the role of LCN2 in reflecting the interactions between these factors and gut microbiome has yet to be confirmed. Animal models, however, fall short of accurately mirroring AS in this regard because
of important differences between human and murine gut microbiota at the level of specific genus/species. The differences in gut microbiome might be caused by intrinsic differences between these two mammalian systems, and also by various confounding factors ranging from diet to exposure to pathogens. Moreover, although human gut microbiota can be transferred to germ-free mice to establish a humanized gnotobiotic animal model, the host-microbe interaction might be different especially during course of diseases. To solve this problem, further studies will be needed on the exploration of additional pleiotropic factors in addition to LCN2 in both the gut and joint of AS patients. Moreover, it might also be essential to discover the association between LCN2 and other AS susceptible genes possibly interacting with the gut microbiome.

3.5 CONCLUSIONS

Serum LCN2 levels are elevated in AS. Levels of LCN2 are associated with the comorbidity of IBD and the degree of ankylosis. The relationship of HLA-B27 positivity and sex with LCN2 implicates a host-microbe interaction underlying the gut-joint axis in AS.
Chapter 4

Lipocalin 2 (LCN2) is a novel biomarker for treatment outcomes in ankylosing spondylitis (AS)
ABSTRACT

Background

In ankylosing spondylitis (AS), reliable serum biomarkers for monitoring treatment responses are lacking. Since elevated Lipocalin 2 (LCN2) levels implicate gut dysbiosis, gut inflammation and relate to progressive spinal ankylosis, our findings in Chapter 3 suggest that normalization of LCN2 could be viewed as a treatment target in AS. LCN2 was assessed whether it served a better serological marker in reflecting treatment outcomes in AS.

Patients and Methods

A retrospective study was performed on 519 patients with pure AS or AS with clinical IBD (AS-IBD). Cohort 1 (172 patients with pure AS) is cross-sectional with only one serum sample collected. Cohort 2 (347 patients with AS alone (n=290) or AS-IBD (n=57)) is sequential, having been followed longitudinally for 3-12 years. Concurrent clinical variables are available. Serum levels of LCN2 were measured by ELISA.

Results

LCN2 was more informative than CRP in our AS cohorts as: (1) In Cohort 1, 72% had elevated LCN2 vs 34% having elevated CRP. (2) In Cohort 2, sequential LCN2 profiles post-treatment showed 20% were normalized. In the 69% with fluctuating LCN2 levels, LCN2 was predictive of subsequent relapse. Only 11% of patients had high BASDAI (>4) but consistently normal LCN2 levels, implicating a different mechanistic pathway in this subgroup of AS patients. (3) Over 50% of patients had a normal CRP at the time of a high BASDAI. (4) A sequential study of patients
treated with infliximab showed that LCN2 was more informative than CRP (in 81% of patients) and ESR (in 75% of patients).

In AS, the HLA-B27 status and sex affect LCN2 levels after treatment outcome as reflected by LCN2. Sequential ESR profiles were more informative than CRP, most clearly in HLA-B27 positive female patients.

**Conclusions**

Normalization of LCN2 could be viewed as a treatment target in AS. Serum LCN2 serves a more sensitive biomarker for treatment outcome in 89% of AS patients. HLA-B27 status and sex affect treatment outcome using LCN2 as a readout.

### 4.1 INTRODUCTION

Ankylosing spondylitis (AS), a heterogeneous subset of spondyloarthitis (Pal, 1987), manifests as chronic lower back pain with a propensity for progression to spinal ankylosis. The imaging hallmark of AS is neo-ossification at the site of joint inflammation (Dakwar et al., 2008). Nonsteroidal anti-inflammatory drugs (NSAIDs) are the first-line drug treatment for AS patients with symptomatic disease (I. H. Song et al., 2008a). Patients with an inadequate response to NSAIDs are candidates to receive biologics such as TNF-α inhibitors (TNFi). TNFi therapy is highly effective in controlling symptoms and improving quality of life of many AS patients. However, this treatment is not curative as symptoms often recur when biologics are discontinued. Additionally, about 30% of AS patients are not responsive to, or are intolerant of TNFi (Glintborg
et al., 2013b). To date, there are 5 TNFi agents approved in Europe, USA and most other parts of the world: infliximab, etanercept, adalimumab and golimumab and certolizumab.

There has been a continued challenge in AS management as there is a lack of informative serological markers. In AS, the current standard to evaluate disease activity and treatment effectiveness is the AS disease activity index (ASDAS), incorporating some core elements of Bath AS Disease Activity Index (BASDAI- a patient-reported pain score) with C-reactive protein (CRP- a serological marker) (Machado et al., 2011). CRP is a nonspecific indication of inflammation and is elevated in approximately 30% of AS patients with active disease (Benhamou, Gossec, & Dougados, 2010). Thus, a normal CRP does not comprehensively rule out the possibility of active disease. In addition, CRP is poorly correlated with clinical response following biologic treatments (de Vries et al., 2009). Therefore, the utility of an outcome measure consisting of a single serological marker (CRP), such as ASDAS, in evaluating treatment response may be compromised. Novel biomarkers which more accurately reflect treatment response could aid clinicians in better disease management.

Lipocalin 2 (LCN2), produced by multiple tissues and functioning on both inflammation and bone remodeling, could potentially serve a better marker for AS. AS is a complex disease not only determined by genetic components but environmental factors like gut bacteria. LCN2 is a secreted anti-microbial protein released in response to microbial triggers (Singh et al., 2016a). The likely role of LCN2 in providing a link between chronic inflammation and ankylosis in AS patients is supported by its pleiotropic functions (Abella et al., 2015; Ataseven et al., 2014b; Conde et al., 2016; Moschen et al., 2017; Singh et al., 2016a; Stallhofer et al., 2015c; Veeriah et al., 2016). Serum LCN2 is elevated in patients with AS and AS-IBD, indicating a possible role for LCN2 in the gut-joint axis in AS (Chapter 3). Since elevated LCN2 levels implicates gut dysbiosis with
ongoing gut inflammation and likely relates to progressive spinal ankylosis, the findings in Chapter 3 suggest that normalization of LCN2 could be viewed as a treatment target in AS.

Therefore, we aim to explore the role of LCN2 compared with CRP as a biomarker for treatment outcome in AS patients in this chapter. The influence of HLA-B27 status and sex on treatment outcome will be further investigated using LCN2 as a read-out.

4.2 PATIENTS AND METHODS

4.2.1 HUMAN STUDY SUBJECTS

AS patients who met the modified New York classification criteria for the disease\textsuperscript{21} (with unilateral SIJ scores 3 or 4) were recruited from Toronto Western Hospital AS clinic. Two AS patient cohorts were used in this study: Cohort 1 (172 patients) is cross-sectional with only one serum sample collected. Cohort 2 (347 patients) is sequential, having been followed longitudinally for 3-12 years. Among patients in cohort 2, there are 57 AS patients with clinical IBD. Sequential serum samples were obtained at each visit usually at one year intervals. Sex, HLA-B27 status, BASDAI, ESR, CRP, and treatments are available. Sequential serum samples were banked post-infliximab every 2-6 weeks as part of an observational study of infliximab from 16 patients in Cohort 2. Demographic features of both cohorts are summarized Table 4.1. The study was approved by UHN ethics committee and all participating patients provided written informed consent.
4.2.2 DETERMINATION OF SERUM LCN2 LEVELS

Aliquots of serum samples were stored at -70°C until use. The sequential samples from each patient were thawed and analyzed at the same time to minimize inter-assay variabilities. LCN2 levels were measured by an ELISA kit according to manufacturer’s protocol (R & D).

4.2.3 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test was carried out using GraphPad Prism 5 program. Chi-square tests were calculated using an online calculator (socscistatistics.com). A $p$-value of less than 0.05 is considered significant. Data is presented as mean ± standard error.
Table 4.1 Demographic features of AS patients

*(Cohort 1, Cross-sectional)*

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS alone</td>
<td>76% (131/172)</td>
<td>24% (41/172)</td>
<td>172</td>
</tr>
<tr>
<td>HLA-B27-positive</td>
<td>75% (95/126)</td>
<td>63% (25/40)</td>
<td>72% (120/166)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>3 - 62</td>
<td>2 - 42</td>
<td>2 - 62</td>
</tr>
</tbody>
</table>

*(Cohort 2, Longitudinal)*

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS alone</td>
<td>76% (219/290)</td>
<td>24% (71/290)</td>
<td>290</td>
</tr>
<tr>
<td>AS-IBD</td>
<td>77% (44/57)</td>
<td>23% (13/57)</td>
<td>57</td>
</tr>
<tr>
<td>Total (AS + AS-IBD)</td>
<td>76% (263/347)</td>
<td>24% (84/347)</td>
<td>347</td>
</tr>
<tr>
<td>HLA-B27 positive in AS alone</td>
<td>83% (182/219)</td>
<td>77% (55/71)</td>
<td>82% (237/290)</td>
</tr>
<tr>
<td>HLA-B27 positive in AS-IBD</td>
<td>50% (22/44)</td>
<td>77% (10/13)</td>
<td>56% (32/57)</td>
</tr>
<tr>
<td>Disease duration (yr) for AS alone</td>
<td>4 - 53</td>
<td>2 - 44</td>
<td>2 - 53</td>
</tr>
<tr>
<td>Disease duration (yr) for AS-IBD</td>
<td>2 - 53</td>
<td>10 - 47</td>
<td>2 - 53</td>
</tr>
</tbody>
</table>
4.3 RESULTS

4.3.1 LCN2 IS MORE INFORMATIVE THAN CRP IN AS (COHORT 1)

To evaluate LCN2 as a biomarker in AS, LCN2 levels were compared with CRP of patients with single sampling in Cohort 1. The upper limit of normal for serum LCN2, 94ng/ml, is established as by the mean of HC with the addition of 3 times standard deviation. CRP levels above 11mg/L are considered elevated. 72% (124/172) of AS patients in cohort 1 had elevated LCN2 levels, while only 34% (58/172) had elevated levels of CRP (Chi-square: 50.8, p<0.0001; Table 4.2). The concurrent LCN2 vs CRP from the same serum sample were then analyzed. 47% (81/172) of these patients had elevated LCN2 at a time when the concomitant CRP level was normal (Table 4.3). Only a small subset (14/172: 8%) had concurrent elevated CRP but normal levels of LCN2 (Table 4.3). Thus, LCN2 is a more sensitive biomarker than CRP. A combination of LCN2 and CRP is informative in more than 80% of AS patients in cohort 1 (Table 4.3).

4.3.2 SERUM LEVELS OF LCN2 PREDICT SUBSEQUENT RELAPSE POST-TREATMENT (COHORT 2)

In AS, disease development is a dynamic process, varying during the course of disease owing to treatment as well as individual variability. Single point analysis may not truly reflect the actual disease process. This is also of importance when comparing serological markers potentially functioning in different pathways. In cohort 2, the analysis of sequential samples enabled us to assess whether LCN2 vs CRP profiles reflect treatment outcomes. Fig. 4.1A shows a representative case in which patient 202 (B27+ve, SIJ score: 4/4 with disease duration 6 yr) was assessed for LCN2, ESR and CRP levels after infliximab treatment. All three serum markers were elevated
before TNFi and normalized 6 wk post-treatment. However, at 12 wk post-treatment, an elevation in LCN2 level was observed and this preceded elevation of ESR and CRP which occurred 10 wks later. This example indicates that LCN2 may serve as an early and predictive biomarker for subsequent flare. Additional examples of comparison of treatment outcome during the course of disease using LCN2 vs CRP as read-outs are illustrated in Fig. 4.1B. In all three cases shown, elevation of LCN2 preceded elevation of CRP. This temporal difference in the elevation of these two markers demonstrated the dynamic nature of the disease process.

Table 4.2 Comparison of the percentage of AS patients with elevated LCN2 vs elevated CRP (Cohort 1).

<table>
<thead>
<tr>
<th></th>
<th>LCN2</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>elevated</td>
<td>124 (72%)</td>
<td>58 (34%)</td>
</tr>
<tr>
<td>normal</td>
<td>48 (28%)</td>
<td>114 (66%)</td>
</tr>
</tbody>
</table>

Table 4.3 A two-by-two comparison of LCN2 vs CRP levels in AS patients (Cohort 1).

<table>
<thead>
<tr>
<th></th>
<th>elevated CRP</th>
<th>normal CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>elevated LCN2</td>
<td>44 (26%)</td>
<td>81 (47%)</td>
</tr>
<tr>
<td>normal LCN2</td>
<td>14 (8%)</td>
<td>33 (19%)</td>
</tr>
</tbody>
</table>
Figure 4.1 Sequential serum LCN2 levels in AS patients (Cohort 2). A. Tracking of treatment outcome post-TNFi in patient 202 (B27positive, 4/4 SIJ scores and 6yr disease duration) using three serological read-outs (LCN2, ESR and CRP). B. Tracking of treatment outcome post-TNFi in patient 318, 373 and 247. Black arrow denotes NSAID treatments. Color arrows denote different Bg treatments. Each arrow indicates the initial use of a particular Bg.
4.3.3  THE USE OF LCN2 vs CRP LEVELS TO STRATIFY TREATMENT OUTCOME IN AS PATIENTS

Consistent with the examples in Fig. 4.1, the profiles of LCN2 and CRP are independent in most cases in cohort 2. The optimal way of analyzing these markers for treatment outcome would be, instead of single point analysis, comparing the profiles of LCN2 and CRP throughout the course of disease. Analyzing concomitant LCN2 vs. CRP from a single point of a patient may omit possible elevation before or after the point of analysis. Moreover, it is difficult to make such comparison due to the missing data of concurrent LCN2 vs. CRP in some of our cohort 2 patients.

We carried out a longitudinal study on AS cohort 2 in which patients were stratified according to TNFi biologics (Bg) treatment as follows. Group 0Bg consists of those receiving NSAIDs/DMARDs and no Bg; Group 1Bg included those patients who had received only one Bg; Group 2Bg consisted of patients treated with more than one Bg.

Three patterns of LCN2/CRP profiles reflecting treatment outcome emerged in these three groups of patients. Fig. 4.2 and Fig. 4.3 show two representative patient profiles for each pattern of LCN2 and CRP, respectively. Three graphs are shown for each patient profile: L denotes LCN2 profile; B denotes BASDAI and C denotes CRP. Pattern 1 represents Normalization (pN): patients with initial elevated LCN2/CRP which returned to normal levels after treatment (Figure 4.2A & 4.3A). Pattern 2 represents Fluctuations (pFl): patients with fluctuating LCN2/CRP levels during the disease course (Figure 4.2B & 4.3B). Pattern 3 (pL; Figure 4.2C & 4.3C) represents patients with consistently Low (normal) LCN2/CRP levels throughout the pre- and post-treatment periods.

Similar number of patients were evaluated for each treatment group (Group 0Bg: n=104; Group 1Bg: n=116; Group 2Bg: n=127). There is a significant difference in the LCN2 pattern
amongst the three treatment groups, indicating LCN2 reflected treatment outcome \((p\text{-value}: 0.02;\ \text{Table}\ 4.4)\). Group 1Bg had a higher percentage of Pattern 1 \((pN: 29\%\)\) compared to Groups 0Bg and 2Bg \((19\%\ \text{and}\ 13\%\ \text{respectively})\). For all three groups, the majority of patients showed Pattern 2 of LCN2 \((pFl: 68\%,\ 66\%\ \text{and}\ 73\%\ \text{for}\ \text{Groups}\ 0Bg,\ 1Bg,\ \text{and}\ 2Bg\ \text{respectively})\). Unlike LCN2, there is no significant difference in the CRP pattern among the three treatment groups \((p\text{-value}: 0.06;\ \text{Table}\ 4.5)\). Strikingly, the majority of patients demonstrated Pattern 3 of CRP \((pL: 53\%,\ 60\%\ \text{and}\ 47\%\ \text{for}\ \text{Groups}\ 0Bg,\ 1Bg,\ \text{and}\ 2Bg\ \text{respectively})\), suggesting CRP is not informative at any point during the course of disease regardless of treatment in about half of our patients.

Overall, by comparing three patterns of LCN2 and CRP as readout for all three treatment strategies, there is higher proportion of LCN2 \((89\%)\) than CRP \((47\%)\) demonstrating both Pattern 1 \((pN)\) and 2 \((pFl)\) \((\text{Chi-square}: 41.3,\ p\text{-value}< 0.00001;\ \text{Table}\ 4.6)\). Compared to over 50\% of the patients with Pattern 3 for CRP, only 11\% \((37/347)\) of them exhibited Pattern 3 \((pL)\) for LCN2, suggesting LCN2 is more sensitive to change than CRP. All of these pL patients with low levels of LCN2, however, showed elevated BASDAI scores, suggesting that in a small subset of AS patients, LCN2 may play a minor role in disease pathogenesis. It is noted that 27\% \((10/37)\) of the patients with low levels of LCN2 demonstrated Pattern 1 or 2 of CRP, indicating that in a small subset of AS patients CRP can be an additional marker when LCN2 is not elevated. In summary, LCN2 as a biomarker for AS treatment outcome is informative in 89\% of our patients.
Figure 4.2 The use of LCN2 levels to stratify treatment outcomes in AS patients (Cohort 2). Based on the use of biologics, patients were stratified into 3 groups: group 0Bg, 1Bg and 2Bg. Three patterns of LCN2 profiles were shown: pN (top panel); pFl (middle panel) and pL (bottom panel). Each panel contains two representative patient profiles. L denotes LCN2 and B denotes BASDAI. Black arrow denotes NSAID treatments. Color arrows denote different Bg treatments. Each arrow indicates the initial use of a particular Bg.
Figure 4.3 The use of CRP levels to stratify treatment outcomes in AS patients (Cohort 2). Categories defined as in Figure 4.2. Each panel contains two representative patient profiles. C denotes CRP and B denotes BASDAI. Black arrow denotes NSAID treatments. Color arrows denote different Bg treatments. Each color arrow indicates the initial use of a particular Bg.
Table 4.4 LCN2 patterns among the three treatment groups

<table>
<thead>
<tr>
<th>Response (Cohort 2)</th>
<th>0Bg %</th>
<th>1Bg %</th>
<th>2Bg %</th>
<th>3x3 Chi² (p-value)</th>
<th>All treatments %</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN</td>
<td>19 (20/104)</td>
<td>29 (33/116)</td>
<td>13 (16/127)</td>
<td>20 (69/347)</td>
<td></td>
</tr>
<tr>
<td>pFl</td>
<td>68 (71/104)</td>
<td>66 (77/116)</td>
<td>73 (93/127)</td>
<td>69 (241/347)</td>
<td></td>
</tr>
<tr>
<td>pL</td>
<td>13 (13/104)</td>
<td>5 (05/116)</td>
<td>14 (18/127)</td>
<td>11 (37/347)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.4 (0.02*)</td>
</tr>
</tbody>
</table>
### Table 4.5 CRP patterns among the three treatment groups

<table>
<thead>
<tr>
<th>Response</th>
<th>0Bg</th>
<th>1Bg</th>
<th>2Bg</th>
<th>3x3 Chi² (p-value)</th>
<th>All treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cohort 2) %</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>(p-value)</td>
<td>%</td>
</tr>
<tr>
<td>pN</td>
<td>10 (10/104)</td>
<td>18 (21/116)</td>
<td>13 (17/127)</td>
<td></td>
<td>14 (48/347)</td>
</tr>
<tr>
<td>pFl</td>
<td>37 (39/104)</td>
<td>22 (26/116)</td>
<td>39 (50/127)</td>
<td></td>
<td>33 (115/347)</td>
</tr>
<tr>
<td>pL</td>
<td>53 (55/104)</td>
<td>60 (69/116)</td>
<td>47 (60/127)</td>
<td></td>
<td>53 (184/347)</td>
</tr>
</tbody>
</table>

9.2 (0.06)
Table 4.6 Comparison of LCN2 vs. CRP patterns in all treatments

<table>
<thead>
<tr>
<th>Response (Cohort 2)</th>
<th>LCN2</th>
<th>CRP</th>
<th>3x3 Chi² (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN</td>
<td>20</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(69/347)</td>
<td>(48/347)</td>
<td></td>
</tr>
<tr>
<td>pFl</td>
<td>69</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(241/347)</td>
<td>(115/347)</td>
<td></td>
</tr>
<tr>
<td>pL</td>
<td>11</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(37/347)</td>
<td>(184/347)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41.3 (&lt;0.00001)</td>
</tr>
</tbody>
</table>
4.3.4 COMPARISON OF PROFILES OF LCN2 vs. ESR vs. CRP IN AN INFlixIMAB TRIAL

To further confirm the role of LCN2 in reflecting treatment outcome, 16 patients from Cohort 2 in an infliximab trial were analyzed. Profiles of LCN2, ESR and CRP post-infliximab were compared based on three patterns defined in Fig. 4.2 & 4.3 (Pattern 1 (pN), Pattern 2 (pFl) and Pattern 3 (pL)). Fig. 4.4 shows examples of patients with different patterns for three markers. A biomarker is considered to be more informative than the others when: (1) The biomarker showing pFl is considered to be most informative while the biomarker with pL is the least informative. For example in p215 (Fig. 4.4A), LCN2 (pFl) is more informative than ESR (pN) and CRP (pN). Similarly in p240 (Fig. 4.4B), LCN2 (pFl) is more informative than CRP (pL). (2) If the patterns of different biomarkers are the same category (pN or pFl), the elevation/resolution time point of each marker will be compared. If the elevation and/or resolution of LCN2 precedes that of ESR/CRP, LCN2 is considered to be more informative. For example, LCN2 is more informative than ESR/CRP in Fig. 4.4C, while LCN2 is less informative than ESR in Fig. 4.4B. Lastly, when all the markers show pattern 3 (pL), none of them is informative (e.g. Fig. 4.4D). Among these 16 patients on infliximab, LCN2 is more informative than ESR in 75% and more informative than CRP in 81% of them. Therefore, LCN2 reflects infliximab treatment outcome in AS better than ESR and CRP.
Figure 4.4 Patterns comparison of sequential LCN2, ESR and CRP in AS patients receiving infliximab treatment. Black arrow denotes the start of infliximab (Bg14) treatments and purple arrow denotes the final treatment.
4.3.5 HLA-B27 status and sex difference affect treatment outcome using LCN2 as a readout (Cohort 2)

AS is a complex disease affected by both genetic and environment factors. In chapter 3, the relationship of LCN2 and ankylosis is shown to be affected by two important genetic factors of AS, HLA-B27 and sex. The influence of HLA-B27 positivity and sex on treatment outcome using LCN2 as a readout will be assessed. Patients with AS only will be the focus of this section because HLA-B27 and sex are not genetic risks for IBD and the comorbidity of IBD in AS patients may skew the relationship.

For this section, patients treated with TNFi biologic (Bg) are the focus and stratified according to TNFi treatment as defined above: Group 1Bg included patients who have received only one Bg, while Group 2Bg are TNFi switchers who have received at least two Bgs.

As demonstrated in Fig. 4.2, three patterns of LCN2 profiles after treatment were shown in three groups of patients: Pattern 1= Normalization (pN), Pattern 2= Fluctuations (pFl) and Pattern 3= persistently low (pL). However, the stratification of pN and pFl is influenced by the window of observation and the length of assessment period (Fig. 4.5A). Therefore, in this section, Pattern 1 and Pattern 2 are combined as the LCN2 informative group (pNFl) since LCN2 levels were elevated at some point in the disease course. The pL group is retained and regarded as the LCN2 not informative group as patients in this group had high BASDAI scores (Fig. 4.5B). Abnormal BASDAI indicated high disease activity, but the LCN2 levels failed to reflect the abnormality throughout pre- and post-treatment in this subset of patients.
Figure 4.5 LCN2 profiles following treatment in AS patients with different HLA-B27 status.

A. The stratification of Pattern 1 (pN) and Pattern 2 (pFl) is arbitrarily defined depending on the window and the length of the assessment period. B. Two representative patient profiles of Pattern 3 (pL). L. Denotes LCN2; B denotes BASDAI and C denotes CRP. Black arrow denotes NSAID treatment. Colour arrows denotes different Bg treatments. Each arrow indicates the initial use of a particular Bg.
Patients were first grouped by their HLA-B27 status. In Cohort 2, similar number of patients were evaluated for each treatment group as follows: Group 1Bg, n=86 and Group 2Bg, n= 75 for HLA-B27-positive patients; Group 1Bg, n=15 and Group 2Bg, n=25 for HLA-B27-negative patients. There was a significant difference in the LCN2 pattern between HLA-B27 positive and negative patients in 1Bg group (p-value: 0.009; Table 4.7). In Group 1Bg, there was higher percentage of patients showing pattern 3 (pL) in HLA-B27-negative group (13%) than the HLA-B27-positive group (3%), indicating LCN2 plays a minor role in this subset of HLA-B27-negative patients. Therefore, the presence of HLA-B27 likely affects treatment outcome using LCN2 as a readout. It indicates that LCN2 is less informative in HLA-B27 negative patients. However, with limited number of HLA-B27 negative patients, further studies on HLA-B27 effect on treatment outcome need to be done.
Table 4.7 LCN2 patterns in the two treatment groups in patient with different HLA-B27 status

<table>
<thead>
<tr>
<th>Response (Cohort 2)</th>
<th>1Bg</th>
<th>2Bg</th>
<th>All Bg treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>pNFl</td>
<td></td>
<td></td>
<td>pNFl</td>
</tr>
<tr>
<td>pL</td>
<td></td>
<td></td>
<td>pL</td>
</tr>
<tr>
<td>HLA-B27+</td>
<td>97</td>
<td>84</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>(83/86)</td>
<td>(63/75)</td>
<td>(146/161)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(3/86)</td>
<td>(12/75)</td>
<td>(15/161)</td>
</tr>
<tr>
<td>HLA-B27-</td>
<td>87</td>
<td>80</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>(13/15)</td>
<td>(20/25)</td>
<td>(33/40)</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>(2/15)</td>
<td>(5/25)</td>
<td>(7/40)</td>
</tr>
<tr>
<td>2x2 Chi² (p-value)</td>
<td>6.79</td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>(0.009*)</td>
<td>(0.5)</td>
<td>(0.09)</td>
</tr>
</tbody>
</table>

Due to the limited number of HLA-B27 negative patients, only HLA-B27 positive patients were further categorized based on their sex. Using the LCN2 profile as readout for treatment outcome, there were significantly higher proportion of female patients showing pattern 3 (pL) in Group 1Bg (9%) and Group 2Bg (31%) than male patients ($p=0.003$; Table 4.8). Overall, LCN2 is informative in higher percentage of male (93%) than in female (83%) patients. This suggests LCN2 plays a minor role in female AS.
Taken together, HLA-B27 status and sex dimorphism influence treatment outcome in AS patients.

**Table 4.8 LCN2 patterns in the two treatment groups in HLA-B27 positive patients of different sexes**

<table>
<thead>
<tr>
<th>Response (Cohort 2)</th>
<th>1Bg</th>
<th>2Bg</th>
<th>All treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>pNfl</td>
<td>pL</td>
<td>pNfl</td>
</tr>
<tr>
<td>males</td>
<td>98</td>
<td>2</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>(63/64)</td>
<td>(1/64)</td>
<td>(54/62)</td>
</tr>
<tr>
<td>females</td>
<td>91</td>
<td>9</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>(20/22)</td>
<td>(2/22)</td>
<td>(9/13)</td>
</tr>
<tr>
<td>2x2 Chi² (p-value)</td>
<td>14.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.003*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All treatments</td>
<td>93</td>
<td>7</td>
<td>(117/126)</td>
</tr>
<tr>
<td></td>
<td>(29/35)</td>
<td>(6/35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.03*)</td>
</tr>
</tbody>
</table>
4.3.6 Sex difference in LCN2 vs. ESR vs. CRP in HLA-B27 positive AS

In addition to LCN2, sex difference in CRP and ESR was further assessed in HLA-B27 positive patients in cohort 2. Initially, patients were categorized based on the patterns of LCN2 (pNFl and pL). As demonstrated in last section, pNFl is the LCN2-informative group while pL is LCN2 non-informative group. Based on the patterns of ESR vs CRP, patients were further grouped into 3 different categories: (1) Concordant (+/+): ESR and CRP were elevated with the same patterns of change; (2) Concordant (-/-): ESR and CRP were both normal throughout; (3) Discordant (+/-): ESR is elevated at some points, while CRP remained normal throughout. There were few cases showing an elevation of CRP but normal ESR. Fig. 4.6 and Fig. 4.7 showed several examples of these three patterns of ESR/CRP when LCN2 is informative or non-informative, respectively. Patients were further segregated by sex.

When LCN2 is informative (Fig. 4.6), a much higher proportion of discordant (+/-) group in females (48%) than males (18%) is observed, indicating ESR was more sensitive than CRP in females (Chi-square 20.8; \( p=0.00003 \)). In the Concordant (-/-) group 29% were female 52% were male. Thus LCN2 was elevated in this group of patients when neither ESR nor CRP was informative.

When LCN2 is non-informative (Fig. 4.7), there was no significant difference in male versus female patients when comparing the three patterns of ESR vs. CRP. Despite no sex differences being observed, ESR was elevated in 50% of females and 40% of males comprising Concordant (+/+)) and Discordant (+/-) groups. However, CRP was elevated in fewer patients (33% of females and 30% of males in Concordant (+\( \ensuremath{\wedge} \)+)). Thus, in the small subset of patients in whom LCN2 is not informative, ESR and CRP can be informative.
Table 4.9 summarized the proportion of each patterns comparing ESR vs. CRP. Regardless of LCN2 levels, ESR was more informative than CRP as there was higher proportion of patients with elevated ESR (48% males and 67% females) than those with elevated CRP (30% males and 26% females). Moreover, by comparing the percentage of sex differences in the patients, ESR was more informative in females than males. Yet there was still a small subgroup of patients when none of the three markers was informative. Thus, more sensitive and informative markers are needed.
Figure 4.6 Three patterns of ESR vs. CRP when LCN2 is informative. (1) Concordant (+/+): ESR and CRP were elevated with the same patterns of change; (2) Concordant (-/-): ESR and CRP were both normal throughout; (3) Discordant (+/-): ESR is elevated at some points, while CRP remained normal throughout.
Figure 4.7 Three patterns of ESR vs. CRP when LCN2 is non-informative. (1) Concordant (+/+): ESR and CRP were elevated with the same patterns of change; (2) Concordant (-/-): ESR and CRP were both normal throughout; (3) Discordant (+/-): ESR is elevated at some points, while CRP remained normal throughout.
Table 4.9 LCN2 vs. ESR vs. CRP patterns in HLA-B27 positive AS

<table>
<thead>
<tr>
<th>%</th>
<th>LCN2 informative</th>
<th>LCN2 non-informative</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Concordant (+/-)</td>
<td>30</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>(36/121)</td>
<td>(5/21)</td>
<td>(3/10)</td>
<td>(2/6)</td>
</tr>
<tr>
<td>Concordant (-/-)</td>
<td>52</td>
<td>29</td>
<td>60</td>
</tr>
<tr>
<td>(63/121)</td>
<td>(6/21)</td>
<td>(6/10)</td>
<td>(3/6)</td>
</tr>
<tr>
<td>Discordant (+/-)</td>
<td>18</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td>(22/121)</td>
<td>(10/21)</td>
<td>(1/10)</td>
<td>(1/6)</td>
</tr>
<tr>
<td>2x3 Chi² (p-value)</td>
<td>20.8</td>
<td>2.9</td>
<td>13.9</td>
</tr>
</tbody>
</table>


4.4 DISCUSSION

Management of AS patients has been hampered by the lack of reliable and objective measures. In this study, instead of using ASDAS (Machado et al., 2011) (a combined index) as a read-out of treatment outcome, we evaluated the inflammatory domain (CRP, a nonspecific marker) separately from the symptomatic domain (BASDAI, a patient-reported outcome). Comparing BASDAI with serological markers is not as straightforward due to the self-assessment nature of BASDAI. BASDAI scores can be very subjective and likely influenced by other factors such as psycho- or neuro-status and pain tolerance. Here we chose to compare LCN2, a novel serological marker, to the current serological markers CRP and ESR.

We showed that LCN2 is a better serological marker than CRP (the current standard) as:

(1) Using a single point assessment, 47% of patients from cohort 1 had elevated LCN2 at a time when the CRP was normal and thus LCN2 is more informative; (2) By comparing profiles of sequential samples from the same patient in cohort 2, 89% of the patients showed elevated LCN2 profiles while only 47% demonstrated high CRP; (3) LCN2 is relevant to AS etiology and pathogenesis since it implicates gut-derived bacterial triggers and gut-joint inflammation. Fig. 4.8 shows a schematic based on published literatures relating mainly to gut inflammation. LCN2 is produced by intestinal epithelial cells (IEC) upon an entero-bacterial pathogen trigger. Alterations of iron homeostasis leads to gut dysbiosis\textsuperscript{13} and chronic gut inflammation with upregulation of pro-inflammatory cytokines. Thus, LCN2 elevation likely represents an early event (bacterial triggers) in the disease process though it can be upregulated further secondarily by the ongoing inflammation. CRP is likely elevated by ongoing inflammation which represents a later event compared to the initial LCN2 elevation. This could explain our observation that sequential LCN2 assessment can predict clinical relapse earlier than CRP (Fig. 4.1B). So far, there is no single
inflammatory molecule identified that is restricted to AS. Biomarkers which are sensitive to change and truly reflect the inflammatory process of AS are the ideal goal. LCN2 appears to meet these requirements. So a combination of LCN2 and CRP can be more informative than CRP alone.

The traditional single point analysis of biomarkers may not truly reflect the changing status of patients. It should be noted that inflammation and bone remodeling are dynamic processes, which at any point in time can be worsening or improving or static. Data collected over time with sequential analysis of markers from the same patients offers a better opportunity to analyze and validate markers. Moreover, as serological marker levels are affected by treatment, the interpretation of these markers needs to take treatment into account, which is seldom done in conventional assessment of a potential markers for AS. This may explain a recent publication reporting normal LCN2 levels in a single point assessment of 21 AS patients (Turina et al., 2017).

Although conventionally, patients are described as NSAID-responders (those treated with only NSAIDs/DMARDs), biologic-responders (those received only one Bg) and biologic-switchers (those treated with more than one Bg), this categorization may not truly reflect treatment responsiveness because: (1) The treatment decision is now largely based on BASDAI, which can be very subjective and likely influenced by other factors such as psycho- or neuro-status and pain tolerance; (2) Confounding factors like insurance policy would also affect the use/switch of biologic treatments.

The stratification into Pattern 1 (pN) and Pattern 2 (pFl) is arbitrarily defined. As illustrated in Fig. 4.5A, LCN2 levels commonly fluctuate during the course of disease. Depending on the window and the length of the assessment period, those deemed to show Pattern 1 (pN) could actually be Pattern 2 (pFl) had the patient been assessed for a longer period of time and/or at a different window of the disease course. This highlights that the AS disease process is dynamic.
About 70% of AS patients, irrespective of treatments, showed a fluctuating pattern of LCN2. Most AS patients have a course of waxing and waning activity as we have described previously (Stone et al., 2008). Our findings highlight the main shortcoming of treatment with TNFi since (a) These biologics are not universally effective in AS; and do not predictably prevent radiographic damage in AS; (b) Disease flares still occur while on biologics in many patients. The fluctuating pattern of LCN2 profile may offer a biological basis for why TNFi therapy is not curative (Fig. 4.8). While TNFi blocks TNFα, other cytokines may persist to maintain the inflammatory process. IL17A synergizes with IL22 and TNFα to induce LCN2 expression in the colonic epithelium (Stallhofer et al., 2015b) as well as in osteoblasts (Shen et al., 2005). Based on this model, anti-IL-17 therapy in AS may have better results than TNFi therapy as suggested by a recent report of less structural changes in AS following treatment (Braun et al., 2017). If the inciting event (a bacterial trigger) which induces the elevation of LCN2 remains unchecked, the disease continues to progress, albeit in a more controlled manner in most of the patients receiving or anti-IL-17 therapy. There is a need for better therapeutic agents and targeting upstream mediators contributing to both inflammation and ankylosis, such as LCN2, could be a step towards remittive and even curative therapies.

For patients treated with more than one TNFi, 14% had consistently low LCN2 levels, but high BASDAI (>4) throughout the course of disease. This suggests that there are other pathways not involving LCN2 which might be contributing to the disease pathogenesis in a subset of patients; possibly those with no subclinical gut inflammation (Ciccia et al., 2016; Gracey, Qaiyum, et al., 2016). Thus in this subset of AS patients, a combination of therapies may have superior effect in controlling disease activity. Currently, most patients on TNFi receive concomitant NSAIDs as well. Identification of this category of patients (pL) would help discovery of other
pathways/targets involved in their AS pathogenesis, leading to novel therapeutics for these patients.

The role of HLA-B27 and sex in affecting treatment outcome as reflected by LCN2 suggests various host-microbe interactions may have an effect on treatment effectiveness. The persistently low LCN2 profile seen more frequently in HLA-B27 negative patients as well as in HLA-B27 positive female patients indicates that LCN2 might play a minor role in these subgroups of patients. Moreover, since TNFα is a strong inducer of LCN2, normal levels of LCN2 might be a surrogate marker for lower TNFα levels, thus predicting non-responsiveness of TNFi treatments of these patients. This is consistent with results in women with poorer response rate to biologic treatments (Gran & Husby, 1990; van der Horst-Bruinsma, Zack, Szumski, & Koenig, 2013b), further supporting the role of LCN2 in predicting treatment outcome. Additional biomarkers are needed for these subgroups in future studies. The small number of HLA-B27 negative patients is the limitation of this study and a larger cohort is needed for further analysis.
Figure 4.8 A schematics offering an explanation for the ability of TNFi therapy to control but not eradicate the inflammatory process in AS patients. Following an entero-bacterial pathogen trigger, LCN2 is produced by intestinal epithelial cells (IEC). Alterations of iron homeostasis lead to dysbiosis, resulting in chronic gut inflammation and induction of pro-inflammatory cytokines such as TNFα, IL17A and IL22. While TNFi blocks TNFα, other cytokines may persist to maintain the inflammatory process. IL17A synergizes with IL22 and TNFα to further induce LCN2 expression in the colonic epithelium.

Lastly, the comparison of ESR and CRP reveals another sex difference in AS, suggesting ESR might likely be a more informative marker than CRP for female patients. There was a much higher proportion of female than male patients with elevated ESR at some points of analysis with persistently normal CRP throughout, regardless of LCN2 levels. Discordance of CRP and ESR is not uncommon in various clinical settings such as RA, systemic lupus erythematosus and infectious diseases (Costenbader, Chibnik, & Schur, 2007; Feldman et al., 2013). Although CRP has been shown to be higher males vs females with AS (van der Horst-Bruinsma et al., 2013b),
most of the past studies only focused on single point analysis. In our cohort among patients with elevation in serum LCN2 levels, about 50% of patients had normal ESR and CRP independent of sex. When LCN2 was not elevated, ESR was elevated in 50% of females and 40% of males. Comparable figures for CRP were 15% of females and 17% of males. In the past, elevated ESR has been found in fewer patients when compared with concurrent elevated CRP (Susanne Juhl Pedersen et al., 2011). Here for the first time, ESR is shown to be likely more informative than CRP in AS.

We are aware the retrospective nature of our studies which have limitations that can only be overcome by a prospective design. However, in AS, any structural change is accumulative over a long period of time and the ongoing process of inflammation might change temporarily. Hence, short-term prospective designs (less than 5 years) will not reveal the true nature of the disease and it is difficult to assess whether a biomarker is informative for the disease process. Since discontinuance of TNFi treatment can induce recurrence of symptoms, it would be considered unethical to randomize patients, who are currently on a biologic treatment, to a placebo group for a long-term study. Thus, a prospective study with a long follow-up period may not be practical or feasible.

4.5 CONCLUSIONS

Our results showed that LCN2 is an informative biomarker following TNFi treatment in 89% of AS patients. HLA-B27 status and sex affect LCN2 levels following treatment. Increased LCN2 implicates a bacterial trigger in AS, provides a link between gut inflammation and ankylosis, and suggests an upstream target for novel therapeutic interventions.
Chapter 5

General Discussion, Future Directions and Conclusions
5.1 General Discussion

The coexistence of AS and IBD is not uncommon but the basis for this interaction is unknown. Gaining insight into the mechanisms underlying the overlap of AS and IBD would lay the groundwork for more effective therapeutics in these diseases.

My thesis work has made a significant contribution to the understanding of AS, as summarized below:

I. It provides insights and grounds for a working model of AS gut-joint axis and offers potential novel therapeutic targets.

The findings in the *ank/ank* mouse model and in human patients have provided important insights and allowed me to propose a working model on the gut-joint axis in AS pathogenesis (Fig. 5.1). Generally, in healthy subjects, there are balanced and dynamic host-microbe interactions in the gut. Upon enterobacterial pathogen triggering, this balance is disrupted and changes on the gut commensals is induced temporarily. Protective mechanisms by the host to defend against gut pathogens involve an increase of LCN2 production and secretion by IEC. In the first instance, augmentation of LCN2 limits iron availability which impacts the survival of pathogens, thus restoring the balance between host and gut microbiome. However, in disease-predisposed subjects such as HLA-B27 positive males, the normalization of LCN2 after acute infection may fail. Persistently high levels of LCN2 in the gut leads to alteration of iron homeostasis which further affects the composition of the gut microbiome (Singh et al., 2016b). Gut dysbiosis can lead to chronic gut inflammation followed by induction of pro-inflammatory cytokines such as TNFα, IL-17A, and IL-22. In the presence of AS susceptible factors like HLA-B27 or male sex, such immune responses could persist and eventually affect extra-enteric sites such as axial joints. At such sites
LCN2 may also play a role in bone remodeling as well. Therefore, the prolonged dysregulated host-microbe interactions may eventually contribute to ankylosis, reflecting the coexistence of chronic gut inflammation and AS.

Based on this working model, treatments targeting upstream mediators in inflammation and ankylosis processes such as LCN2 or targeting early events such as dysbiosis, could be a step towards remittive or curative therapies. Insights into novel treatments will be discussed later in the section of future studies. This working model also offers an explanation of the limitations for the current TNFi treatments.
Figure 5.1 A schematics offering a working model of gut-joint axis in AS pathogenesis. Upon entero-bacterial pathogen triggering, LCN2 is produced by IEC. In AS-predisposed individuals (by factors such as HLA-B27 and male sex), the alterations of iron homeostasis lead to dysbiosis, resulting in chronic gut inflammation and induction of pro-inflammatory cytokines such as TNFα, IL-17A and IL-22. Such dysregulated host-microbe interactions could underlie the gut-joint coexistence in AS. While TNFi blocks TNFα, other cytokines may persist to maintain the inflammatory process. If the inciting bacterial trigger which induces the elevation of LCN2 remains unchecked and dysbiosis persists, the disease continues to progress, albeit in a more controlled manner in most of the patients receiving TNFi or anti-IL-17 therapy. Potential treatments targeting dysbiosis or LCN2 could serve better therapies than the current TNFi.
II. Animal study: It provides additional and important insights into *ank/ank* mutant mice being a relevant model for AS pathogenesis studies:

A. **My thesis is the first report of subclinical gut involvement in the *ank/ank* mice**

Although there are no animal models completely mirroring the human disease, gut-joint manifestations in these models should provide insight of the gut-joint axis in AS. However, among these animal models, there are no mouse models with coexistence of axial ankylosis and gut involvement (Table 5.1). The HLA-B27/hβ2m tg rat is an informative animal model with both gut and joint manifestations. A high copy number of the B27 transgene in this model is associated with spontaneous peripheral and axial inflammation, as well as extra-articular features including enterocolitis. Two other animal models of AS are hTNFtg and TNF<sup>AARE</sup> mice, both with enhanced TNFα production. Overexpression of TNFα- is associated with Crohn’s-like ileitis and AS features including sacroiliitis, spondylitis and enthesitis. Another inflammatory AS model with gut involvement is the SKG mouse. The SKG mouse was originally a mouse model for RA, in part due to the detection of rheumatoid factor (RF). With the introduction of curdlan, a β-1,3-glucan derived from fungal and bacterial cell walls, the SKG mouse develops peripheral and axial arthritis similar to human AS. Articular and extra-articular manifestations found in the SKG mouse reflect an aberrant IL-23/IL-17 axis related to an impaired TCR signaling induced by a spontaneous mutation in ZAP-70. However, no ankylosis is observed in either the TNFα overexpression models or the SKG mouse.
Table 5.1 Animal models commonly used in AS studies

<table>
<thead>
<tr>
<th>Model</th>
<th>Peripheral arthritis /joint ankylosis</th>
<th>Spondylitis /Spinal ankylosis</th>
<th>Extra-articular manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B27 hβ2m tg rat</td>
<td>Yes/Yes</td>
<td>Yes/Yes</td>
<td>IBD, psoriasis</td>
</tr>
<tr>
<td>hTNFtg and TNFΔARE mouse</td>
<td>Yes/No</td>
<td>Yes/No</td>
<td>CD-like IBD</td>
</tr>
<tr>
<td>SKG mouse</td>
<td>Yes/No</td>
<td>Yes/No</td>
<td>Ileitis, psoriasis</td>
</tr>
<tr>
<td>ank/ank mouse</td>
<td>Yes/Yes</td>
<td>Yes/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>Aging DBA/1 mouse</td>
<td>Yes/Yes</td>
<td>No/No</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>HLA-B27 β2m⁻/⁻ tg mouse</td>
<td>Yes/Yes</td>
<td>No/No</td>
<td>NA</td>
</tr>
</tbody>
</table>

Little has been known about the gut involvement in the ank/ank mouse, but it has proved to be an informative model for ankylosis. Homozygous mutant mice exhibit peripheral and joint bone proliferation resembling human AS due to a nonsense mutation in the ank gene locus. Another feature in these mice similar to human AS is altered Wnt/β-catenin signaling in the joints. ank/ank mice have long been recognized as a non-inflammatory model for AS since there is no signs of inflammation observed in these animals. Recently, a study with NLRP3⁻/⁻ in ank/ank mice
showed a partial rescue of the joint phenotypes, suggesting the involvement of inflammasome in the ankylosis of this model. Besides the progressive ankylosis phenotype, \textit{ank/ank} mice was the animal model that led our lab to discover a novel serological feature in human AS, which is the elevation of serum NOG and SOST-IgG-ICs. Therefore, better knowledge of gut involvement in the \textit{ank/ank} mouse would provide us the opportunity to investigate gut-joint axis of AS in this model.

My work is the first to discover the subclinical gut inflammation in the \textit{ank/ank} mutants. This has broadened the application of \textit{ank/ank} mice in studies from purely bone dysregulation to the coexistence of gut-joint axis in AS. Although none of the animal had clinical gut inflammation (e.g. diarrhea), histology results uncovered various degrees of subclinical colon involvement in \textit{ank/ank} and \textit{wt/wt}. Thus, this model proves useful as a parallel of those 60\% of AS patients having subclinical gut inflammation (Altomonte et al., 1994; De Vos et al., 1989; Leirisalo-Repo et al., 1994; Mielants, Veys, Cuvelier, & de Vos, 1988). Furthermore, distinctive gut and joint contribution to disease progression could be dissected out by comparing groups of animals with various degrees of gut and joint involvement.

These findings establishes the \textit{ank/ank} mouse as an informative model for gut-joint overlap in the pathogenesis of AS, considering the similarity of bone phenotype and serological abnormality (SOST/NOG-ICs) to AS, as well as the subclinical gut involvement.

B. **Serum Lcn2 reveals a link underlying gut-joint axis in \textit{ank/ank} mice**

Dysregulated Lcn2 in \textit{ank/ank} mutant mice sheds light on a novel pathway of gut-joint axis applicable to AS. Here for the first time, circulating levels of Lcn2 are associated with gut inflammation in an ankylosis model. Among \textit{ank/ank} mutant mice, higher serum Lcn2 levels are detected in those with more severe gut inflammation, indicating gut involvement with the
abnormal Lcn2 levels. This is also the first report to show the association of Lcn2 with ankylosis in animals controlled for degree of gut inflammation. The association of both ankylosis and gut inflammation with increased serum Lcn2 implicates a possible interactive or synergistic contribution of gut and bone to circulating Lcn2 levels since neither gut inflammation alone in \textit{wt/wt} mice nor ankylosis alone in \textit{ank/and} mutants was reflected in increased serum levels of Lcn2. While increased Lcn2 could play a role in the subsequent inflammatory response, the downstream inflammatory cytokines (such as IL-17 and TNFα) would enhance Lcn2 expression by a feedforward loop (Kjeldsen, Bainton, Sengeløv, & Borregaard, 1994; Raffatellu et al., 2009b). Over-expression of Lcn2 has been shown to modulate bone homeostasis in animals (Costa et al., 2013). Thus, it is conceivable that the secondary upregulation of Lcn2 by the ongoing inflammation may induce a persistent increase in Lcn2, which may have an effect on chronic bone inflammation and remodeling in AS.

Elevated serum Lcn2 in \textit{ank/ank} mutants with subclinical gut inflammation also implicates an involvement of gut microbiome in the gut-joint axis of AS. Lcn2 is an anti-microbial factor as reflected in its iron binding function during acute infection. Therefore, levels of Lcn2 could potentially reflect changes of microbiome in the gut. AS is a complex disease, the pathogenesis of which is attributed to various combinations of genetic risks and environmental factors. Current concepts postulate that that without environmental triggers, genetic elements alone are not sufficient to cause disease, indicating the importance of environment in pathogenesis. In light of the coexistence of clinical/subclinical gut inflammation and ankylosis in AS, it is hypothesized that a dysregulated gut microbiome may serve as a trigger for AS onset. Given the known association of Lcn2 and gut dysbiosis, the findings of high levels of Lcn2 in \textit{ank/ank} mice with severe subclinical gut inflammation provide indirect support for the theory of aberrant gut microbial composition as a contributor to AS initiation and perpetuation. It is speculated that an
initial infection-induced gut dysbiosis is likely an early event in AS. Lcn2 is a pivotal part of host defense against such infections. However, in the resolving phase following the acute response, individuals carrying AS susceptibility genes may fail to reduce Lcn2, resulting in sustained high levels and subsequent induction of a cascade of a chronic inflammatory response leading to structural damage in target joints. Moreover, during the disease development, there is constant and dynamic interaction between the host and gut microbes which is likely to underlie the fluctuating levels of Lcn2 throughout the disease.

Lastly, the difference of Lcn2 in males versus females in ank/ank mouse model implies sex dimorphisms of this pathway in AS. Because significant differences were only detected in female animals, Lcn2 might be a more relevant pathway for females in this model. This is in contrast to findings in the human LCN2 study, likely due to intrinsic difference between murine and human immune systems. Both male and female mice used for the baseline were at the advanced stage of ankylosis and no significant difference of colon inflammation were observed. As sex difference on the composition of gut microbiome is not uncommon (Kozik, Nakatsu, Chun, & Jones-Hall, 2017a), based on the relationship between Lcn2 and microbes, difference of Lcn2 levels may indicate different compositions of gut microbiome in male and female animals. This hypothesis needs further study.

C. **The role of PPARγ in aberrant regulation of Lcn2 reveals a relevant molecular pathway in AS and provides insights on potential novel therapies.**

My discovery of PPARγ modulation of Lcn2 in the ank/ank mouse model reveals a relevant molecular pathway in AS pathogenesis. According to the effect of PPARγ agonists on Lcn2, high basal levels of Lcn2 in these animals could be due to increased/activated PPARγ during disease
development. As nuclear translocation of PPARγ would activate NFκB pathway, binding of NFκB to the Lcn2 promoter likely leads to an upregulation of Lcn2. Therefore, PPARγ acts as a positive modulator of Lcn2 in the ank/ank mouse model. Although at baseline, the change of serum Lcn2 appeared to be inversely related to colonic PPARγ expression in the context of gut inflammation, this opposite finding could be possibly due to the comparison of serum Lcn2 and colonic PPARγ. A more direct relationship of systemic Lcn2 and PPARγ is illustrated by the following PPARγ agonist/antagonist administration experiments. Activation of PPARγ by Rosi has shown a trend of increase in serum levels of Lcn2, which should indicate a positive modulation of PPARγ on Lcn2 in this mouse model. Therefore, PPARγ is likely a relevant pathway to aberrant Lcn2 in AS. Moreover, there was no detectable sex bias of PPARγ at baseline nor on the modulation of Lcn2. This indicates that, in addition to PPARγ, there are likely other molecules involved in the Lcn2 pathway which account for the sex differences in Lcn2 profiles seen in these mice.

In addition to the regulation on Lcn2, the contributing role of PPARγ to gut inflammation and MSC differentiation highlights its potential relevance to AS pathogenesis. In the ank/ank mice at baseline, colonic PPARγ expression was associated with gut inflammation. The levels of colonic PPARγ transcript were negatively associated with the degree of gut inflammation in ank/ank mice, indicating a potential modulating effect of PPARγ on the gut inflammation in AS. Yet, the function of PPARγ on bone homeostasis remains unclear in the ank/ank mouse model. Characterizing genomic or proteomic signatures of PPARγ in bone presents significant methodological challenges but could be addressed in future studies.

The discovery of PPARγ modulation on Lcn2 sheds light on potential novel therapies for AS which might target PPARγ. In the ank/ank mouse model, higher levels of Lcn2 are associated with ankylosis and subclinical gut inflammation, suggesting that normalization of Lcn2 could be
viewed as a treatment goal in AS. Since activation of PPARγ increased serum levels of Lcn2 in \textit{wt/wt} animals, treatments suppressing PPARγ could potentially normalize Lcn2.

Since PPARγ antagonists as therapies would appear to conflict with recognized adipogenic effect of PPARγ on bone marrow mesenchymal stem cell differentiation, focus on cell-specific functions of PPARγ on bone remodeling have to be considered. A study by Wang et al. has identified that the use of a PPARγ inhibitor GW9662 displayed opposite effects on the expression of tissue non-specific alkaline phosphatase (TNAP) in human chondrocytes compared to that in vascular smooth muscle cells (Lencel et al., 2011). TNAP is an enzyme hydrolyzing pyrophosphate ions, which are potent mineralization inhibitors, thus playing a role in bone homeostasis. Moreover, although the adipogenic function of PPARγ has been discovered in both physiological and pathological contexts, its differential role on adipogenesis vs osteoblastogenesis in AS remains to be discovered.

PPARγ-targeted treatments are likely more effective than the current AS treatments as they not only have an effect on the upstream molecule Lcn2, but they may also play a role in bone remodeling, thus controlling ankylosis in AS (Beresford, Bennett, Devlin, Leboy, & Owen, 1992; Dorheim et al., 1993). Taken together, treatments targeting PPARγ may prove superior to current TNFi therapies which are not curative.

In summary, the ank/ank mouse model is a relevant animal model for dissecting mechanisms underlying the gut-joint linkage in AS. Although the \textit{ank/ank} mouse model does not completely recapitulate AS in humans, the similarities of these mice to AS indicates that the \textit{ank/ank} mouse provides an informative animal model for mechanistic analysis. Such similarities include structural changes, comorbidity of gut inflammation and serological abnormalities as summarized in Table 5.2.
Table 5.2 Comparison of the *ank/ank* mouse model and human AS

<table>
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<tr>
<th></th>
<th><em>ank/ank</em> mouse</th>
<th>Human AS</th>
</tr>
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<tr>
<td><strong>Articular features</strong></td>
<td></td>
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<tr>
<td>Peripheral arthritis/ankylosis</td>
<td>Yes/Yes</td>
<td>Uncommon/Uncommon</td>
</tr>
<tr>
<td>Spondylitis/ankylosis</td>
<td>Yes/Yes</td>
<td>Yes/Yes</td>
</tr>
<tr>
<td>Sacroiliitis</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sex difference of ankylosis</td>
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<td>Male</td>
</tr>
<tr>
<td><strong>Extra-articular manifestations</strong></td>
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<td></td>
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<tr>
<td>Gut involvement</td>
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<td>Psoriasis</td>
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<td><strong>Serological features</strong></td>
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</tr>
<tr>
<td>Elevated SOST/NOG IgG ICs</td>
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<td>Yes</td>
</tr>
<tr>
<td>Elevated Lipocalin 2</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Sex bias in Lipocalin 2</td>
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III. Human study: discovery of a serological feature (aberrant LCN2) in human AS

A. Elevated serum LCN2 levels in AS patients: impact of sex and HLA-B27 status

This is the first report showing increased serum levels of LCN2 as a novel serological feature in AS patients, which is consistent with the findings in ank/ank mouse. AS has been considered to be a seronegative disease as there are no unique autoantibodies found in these patients. The previous discovery of SOST/NOG-ICs in our lab has changed that concept. LCN2 is now established as a second serological feature of AS. Although an increase in serum LCN2 is not limited to AS patients but also discovered in some IBD patients, a high sensitivity of LCN2 to AS is determined. The findings in IBD underscore the shared pathogenic pathways in AS and IBD.

The involvement of LCN2 pathways in the gut-joint axis of AS is indicated by its association with the coexistence of IBD and its correlation with the degree of ankylosis in AS patients, respectively. On the one hand, AS patients with clinical IBD showed significantly higher serum LCN2 levels than those with AS alone, implicating a key contribution of gut inflammation to high levels of LCN2. Although the information of subclinical gut inflammation in patients with pure AS is unknown, the elevation of LCN2 in these patient could also be attributed to the involvement of gut. On the other hand, the positive association of LCN2 with mSASSS indicates a possible contribution of LCN2 to radiographic changes in AS.

The role of LCN2 in AS pathogenesis is further supported by its relationship with two crucial genetic factors, HLA-B27 and sex. The association of LCN2 with the severity of AS progression was observed only in HLA-B27-positive male patients. This is congruent with current clinical concepts of AS in which HLA-B27 status and male sex define the primary clinical profile of the disease. LCN2 pathway seems to be more relevant to this classical profile of AS. Although ank/ank mouse model is an informative model of ankylosis, the model is independent of HLA-
B27 and of male dominance, likely due to the inherent differences of animals and humans, serving as a cautionary note against over simplistic inferences from animal models.

Given the association of LCN2 and the gut microbiome, the aberrant levels of LCN2 in HLA-B27 positive males could well reflect the dysregulated interactions of gut dysbiosis with host sex and HLA-B27 status. Indeed, HLA-B27 may modulate gut microbiome, as has been discovered in the rat model of AS (P. Lin et al., 2014). Although a sex-biased gut microbiome has not been clearly defined in AS, such difference have been observed in patients with obesity (Haro et al., 2016a) and mice (Kozik et al., 2017a). Further studies are needed to clarify this issue.

However, the current working model is based on the relevance of LCN2 to gut inflammation and dysbiosis. Thus LCN2 might not be informative in AS patients without gut involvement. Since among AS patients, up to 60% demonstrate subclinical gut inflammation, LCN2 may remain normal for the remaining 40% of these patients. Therefore, additional serological features are needed for this subgroup.

B. **LCN2 is superior to CRP or ESR as a biomarker for treatment outcomes in AS**

Currently, AS is lacking sensitive and specific biomarkers for monitoring treatment outcomes. The current standards for assessing treatment response have been BASDAI and CRP/ESR and more recently ASDAS. BASDAI is a patient self-reported outcome measure which is subjective and can be influenced by the psycho- and neuro-status as well as pain tolerance of a patient. Treatment decisions by rheumatologists have been largely dependent on BASDAI scores and current treatment guidelines define a threshold BASDAI score as indication for biologic therapy. Owing to the subjective nature of BASDAI, it is unclear how accurately BASDAI reflects disease activity and progression. Other parameters are needed to provide more objective indicators
in addition to the subjective BASDAI scores. The serological markers CRP and ESR have been used in this regard but both suffer from low specificity and sensitivity in AS. Although ASDAS shows higher discriminatory effect than BASDAI in differentiating patients with different levels of disease activity and patients with different levels of structural changes (van der Heijde, Lie, Kvien, Sieper, Van den Bosch, Listing, Braun, Landewé, et al., 2009), it remains controversial since in some cohorts of AS patients, BASDAI shows better criterion validity than ASDAS both for the patient acceptable symptom state (PASS) and for the physician PASS (Sellas i Fernandez et al., 2017). This is likely due to the difference of cohorts from disparate regions of the world with various genetic backgrounds and living environments and cultures. Moreover, by combining the symptomatic domain (BASDAI) with the inflammatory domain (CRP/ESR), it may obscure the true nature of disease as a normal value may cancel out another abnormal score. It is of noted that subjectivity in the major components of BASDAI and ASDAS is unavoidable. Thus there is some urgency in identifying more informative and objective markers in assessing treatment responsiveness. The discovery of such biomarkers for treatment response relies on better understanding of AS.

Our findings on aberrant LCN2 in patients indicate that normalization of LCN2 could constitute one of the meaningful treatment targets in AS for patients with clinical/subclinical gut involvement. Elevated LCN2 levels implicate aberrant interactions between gut bacteria, gut inflammation and host response. A normal LCN2 value likely indicates resolution of gut inflammation or normalization of gut dysbiosis, or both. Furthermore, as higher levels of LCN2 may contribute to progressive spinal ankylosis, normalizing LCN2 may aid in resolving inflammation and interrupting the bone proliferation process in AS.
Our finding is the first to show that LCN2 levels better reflect treatment outcome in AS than ESR or CRP. TNFα is a strong inducer of LCN, and the effectiveness of TNFi would be expected to be reflected in the levels of LCN2. In this study, LCN2 has been shown to be more sensitive and informative than CRP in both single point analysis (Cohort 1) and longitudinal study with treatment taken into account (Cohort 2). In addition, the longitudinal studies on patients treated with infliximab demonstrated that the elevation of LCN2 preceded elevation of ESR and CRP, indicating that LCN2 is an earlier and more sensitive marker in reflecting treatment outcomes. However, in a complex disease like AS, a single factor may not be sufficient to capture the whole spectrum of the disease. Therefore, a combination of markers at both up- and down-stream of disease development would be more powerful in disease management. The influence of HLA-B27 and sex on treatment outcomes is also detected in post-treatment profiles of LCN2. Moreover, the application of LCN2 to measure TNFi treatment outcomes has implications for LCN2 being a treatment target not only in AS but also in other diseases using TNFi as a therapy, such as IBD. It is noted that in some rare cases when LCN2 are constantly normal, CRP and/or ESR are elevated, indicating biomarkers in addition to LCN2 are needed as CRP and ESR are not sensitive enough for AS. Therefore in this subgroup of patients, consistently normal LCN2 levels but aberrant CRP/ESR/BASDAI indicate non-responsiveness of TNFi treatments that another treatment strategy or a combination of therapies would be recommended. Moreover, as stated above, aberrant LCN2 pathway in AS could be associated with the subgroup of patients with clinical or subclinical gut pathology. Thus, additional serological markers which reflect treatment outcomes in subgroups with no gut involvement are to be discovered.

The longitudinal design of our study allows tracking of disease progression of each patient with sequential samples collected for up to 12 years. It should be noted that inflammation in AS is a dynamic process, that a single point analysis may not truly reflect the real disease progression.
Inflammation can be worsening, improving or quiescent at various points during the course of disease. Biomarkers related to inflammation may also have a delayed response time to stimuli depending on their pathways. Ideally, biomarkers should be sensitive to change and truly reflective of the inflammatory process of disease. However, cross-sectional analysis of values of different serological markers does not provide the opportunity to track changes over time and compare the reaction time of each marker. Longitudinal data collection and sequential analysis of markers from each patient offers a better strategy to validate novel biomarkers and to track disease evolution.

In addition, this is one of the first few longitudinal studies which takes treatment into account. Serological markers functioning in inflammation may be significantly affected by the use of anti-inflammatory agents including NSAIDs and TNFi. Pre- and post-treatment levels of serological markers (LCN2 vs ESR/CRP) were compared over a long study period, which is rarely done in other AS biomarker studies. This may explain a recent European study reporting normal LCN2 levels in a single point assessment of AS patients (Turina et al., 2017). Most serological studies in AS focus on a single-point analysis (S. Arends et al., 2011b; Choi et al., 2008; Maksymowych et al., 2007; Mattey et al., 2012a). A pre-treatment determination of a candidate marker is examined for its predictive value for treatment responsiveness determined by post-treatment BASDAI or ASDAS reading. Post-treatment readings of potential markers are, however, seldom considered.

While LCN2 was proved to be an informative serological marker, CRP was found to be less informative than ESR in AS. Discordance of CRP and ESR is not uncommon in various clinical settings such as RA, systemic lupus erythematosus and infectious diseases (Costenbader et al., 2007; Feldman et al., 2013). In most AS clinical studies, ESR is less used than CRP due to its time-sensitive nature and its difficulty in transporting for single source testing. I have
demonstrated for the first time an elevated of ESR in 60% of females and 45% of males when LCN2 is informative in contrast to only 18% of females and 30% of males having increased CRP. Similar findings were observed in patients with normal LCN2 levels. This suggests that CRP is less informative in AS compared to ESR. Moreover, sequential analysis on profiles of serological markers revealed the importance of ESR in female patients compared to CRP which reflects a sex difference in the comparison between ESR and CRP. A higher percentage of females than males had informative sequential ESR profiles despite normal CRP values regardless the status of LCN2. This suggests that ESR may be a more relevant marker to female than male AS. As females with AS have been under-diagnosed or recognized late, the less sensitive CRP may be contributing factor to this. With LCN2 being an informative serological marker, the use of the three serological markers together would inform better patient management in AS.

C. LCN2 as a marker of treatment response highlights limitations of TNFi therapy

The relationship of LCN2 and TNFα offers a possible insight into shortcomings of TNFi therapy in AS. LCN2 profiles of post-TNFi treatment showed that TNFi is not always responsive to treatment nor are post-treatment changes stable over time. As shown in Fig. 5.1, although TNFi block TNFα, other cytokines (e.g. IL17, IL22 and IFNγ) may persist to sustain the inflammatory process. In circumstances in which TNFi do not achieve complete inhibition of TNFα, the remaining TNFα could work synergistically with IL17 to further increase the levels of LCN2.

Moreover, as hypothesized in our working model (Fig. 5.1), the early events of AS could involve dysbiosis following gut infection, followed by aberrant innate (e.g. LCN2) and adaptive (e.g. TNFα, IL17, IL22 and IFNγ) immune response. Blocking a downstream molecule (TNFα) would not completely resolve the upstream abnormalities (elevated LCN2 and dysbiosis). In
addition, over the course of disease, there are likely ongoing bacterial triggers which constantly influence dynamic interactions between host and microbes. If an initial bacterial trigger inducing the increase in LCN2 remains unchecked, the disease may continue to progress, albeit in a more controlled manner following TNFi or anti-IL17 therapy. Furthermore, once the initial events of disease start, the resulting signaling cascades may vary among patients and different effectors may have a feed forward effect on the upstream molecules. Thus, treatments focusing on the downstream master inflammatory molecules such as TNFα may not be able to control the disease as effectively compared to those targeting an upstream target.

5.2 Future Directions

There is a need for novel and better therapeutic agents as close to 40% of AS patients do not respond adequately to current biologic treatments. TNFi is not curative for AS. The latest therapeutic goal for AS, proposed by an international task force in 2017, is clinical remission of inactive disease of musculoskeletal and extra-articular manifestations (Smolen et al., 2018).

The working model constructed based on the novel findings and insights from my thesis work provides the potential for new therapeutic targets (Fig. 5.1). (a) Modulation of LCN2. Since abnormal LCN2 levels imply the aberrant host-microbe interaction as well as the ongoing inflammation in AS, novel treatments targeting LCN2 or its upstream modulator PPARγ could be therapeutic targets (section 5.2.1). B. Modulation of gut commensals. Considering gut dysbiosis as an early of AS onset, another potential therapy would be treatments targeting gut commensals which could resolve dysbiosis in patients. Given the comorbidity and shared pathways of AS and IBD, novel treatments for IBD targeting dysbiosis might be applicable to AS disease. One of these
therapies targets is inflammasome NLRP12 which will promote protective gut commensals while inhibit inflammation (section 5.2.2).

5.2.1 Novel treatment strategies targeting LCN2

5.2.1.1 Antibodies to LCN2

Since elevated levels of LCN2 in AS indicate ongoing inflammation, normalization of LCN2 with monoclonal antibodies could be an alternative treatment strategy to TNFi. Considering the role of LCN2 on TNFα modulation, the application of LCN2 antibodies may control disease better than TNFi as it targets the upstream molecule LCN2. Moreover, as aberrant LCN2 may play a role in gut dysbiosis, normalization of LCN2 could not only resolve gut dysbiosis but also some inflammatory cytokine induced by dysbiosis such as TNFα, IL-18 and IL-23, IL-17 and IL-22 (Kjeldsen et al., 1994; Raffatellu et al., 2009b).

However, there are some potential side effects of anti-LCN2 treatments. First, owing to the protective role of LCN2 in infection, LCN2 inhibitors may put patients at risk for opportunistic infections with certain bacterial, mycobacterial, or fungal pathogens. Secondly, inhibition of LCN2 may also cause unexpected consequences as LCN2 is a pleiotropic factor involved in a large number of physiological processes. Lowering the levels of LCN2 systemically might have adverse effects on other organs. Lastly, the efficacy of LCN2 antibody treatment may be compromised overtime owing to the development of anti-drug antibodies.
5.2.1.2 PPARγ antagonists

The studies in the ank/ank mice have revealed a potential treatment for AS with PPARγ antagonists based on the relationship between PPARγ and LCN2. Although the application of PPARγ as a treatment target has been used in several diseases, clinical data are scarce with the application of PPARγ antagonists. Owing to its regulation on glucose and lipid metabolism and inflammation, activation of PPARγ has already been the treatment target in obesity, type II diabetes and IBD (Bertin, Dubuquoy, Colombel, & Desreumaux, 2013; Lea, Sura, & Desbordes, 2004; Scatena, Bottoni, & Giardina, 2008; Tachibana, Yamasaki, Ishimoto, & Doi, 2008). Thiazolidinediones (TZDs) are examples of PPARγ agonists used for type II diabetes and obesity. A recent study of PPARγ indicated the administration of pioglitazone (a type of TZD) decreased arthritis severity in an adjuvant-induced arthritis model (Koufany, Jouzeau, & Moulin, 2014). However, functions of PPARγ in RA might be different from that of AS given the differing pathogenesis and clinical outcome of these two manifestations.

While the majority of PPARγ-targeted therapies focus on the activation of PPARγ, natural products which inhibit PPARγ expression or transcriptional activity have become popular recently. Therapies with these natural products would be potential AS treatments. The majority of current natural products discovered are phytochemicals extracted from dietary and medical plants, which are relatively safe and easily accessed sources. Examples include Genistein, Retinoic acid, Piperine, Berberine, etc as reviewed by Feng et al. (Feng, Reuss, & Wang, 2016). These natural products and certain active compounds have been partly associated with the regulation of PPARγ or its upstream modulators in expression as well as activation (Feng et al., 2016).

Possible pathways have been demonstrated for the natural products to be involved in regulation of PPARγ expression. For instance, treatment with a plant sterol guggulsterone
extracted from the resin of guggul tree was demonstrated to downregulate PPARγ2 in 3T3-L1 cells (J.-Y. Yang, Della-Fera, & Baile, 2008). In addition to expression modulation, regulation of PPARγ transcriptional activity is another role played by the natural products. For example, a new thiophene-acetylene type of derivative called 7-chloroarctinone-b (CAB), which is isolated from Rhaponticum uniflorum’s root, was found to be a specific PPARγ antagonist (Y. Li et al., 2009). The inhibitory effects of CAB on PPARγ relies on its high binding affinity for the PPARγ ligand-biding domain, therefore preventing the rosiglitazone-stimulated PPARγ activation. Interestingly, comprehensive regulation of PPARγ via mitogen-activated protein kinases (MAPKs) has been discovered in many natural compounds (T. Wang et al., 2008). It has been demonstrated that MARK–mediated phosphorylation of PPARγ contributes to the reduction of PPARγ transcriptional activity and thereby inhibits the downstream gene expression (Hu, Kim, Sarraf, & Spiegelman, 1996). A major alkaloidal compound in the fruit of Evodia fructus was found to increase the activation of MARK and thereby to reduce the expression of PPARγ (T. Wang et al., 2008). With better understanding of AS and the potential role of PPARγ in AS, investigation into the functional mechanism of PPARγ along with the effective regulators of PPARγ would benefit AS treatments.

A natural product could be an effective agent in AS if it not only targets an upstream molecule like PPARγ but also targets other known pathways modulating PPARγ expression and or transcriptional activity. Genistein, for example, has been illustrated to repress PPARγ expression by inhibiting E/EBPβ and activating the Wnt/ β-catenin pathway, whereas it also controls PPARγ transcriptional activity through activation of AMPK (Feng et al., 2016). Moreover, although not yet confirmed, combined treatment with different natural products could provide other therapeutic options for AS. More investigations are needed, with well-designed clinical trials, for appropriate evaluation of natural products.
By inhibiting PPARγ, natural products could indirectly reduce the levels of LCN2. However, there are some concerns regarding the use of PPARγ-targeting natural products in AS treatments. First, the safety of these natural products remains to be validated. Although the application of medicinal plants has a long history in Asia, the active molecules of these plants may not be clearly identified. Secondly, since the involvement of PPARγ in metabolism and bone remodeling could be context-dependent, unexpected adverse effects of modulation of PPARγ should be investigated. Moreover, given the relationship of PPARγ and cancer, caution should be exercised with treatments targeting PPARγ. A meta-analysis showed that use of pioglitazone is associated with slightly higher risk of bladder cancer compared to the general population (Ferwana et al., 2013).

5.2.2 Novel treatment strategies targeting gut commensals

Manipulation of gut microbiome could be another potential treatment strategy due to the aberrant interactions of gut bacteria and the host immune response in AS. Although there is no single bacteria identified to be the cause of AS, there is emerging interest in novel therapies targeting the gut microbiome. Given the shared pathways of AS and IBD, novel treatments targeting inflammasome NLRP12 might also be applicable to AS as well as IBD.

Molecules like NLRP12 which influences both inflammation and gut microbes could be a potent treatment strategy for AS. NLRP12 is an intracellular pyrin-containing Nod-like receptor (NLR) protein which plays a vital role in various processes including infection and inflammation (Williams, Taxman, Linhoff, Reed, & Ting, 2003; Wu, Chen, & Hsieh, 2013; Ye et al., 2008). On the one hand, NLRP12 is a negative regulator of innate immunity by inactivating NFκB as a
damper of the inflammatory response (L. Wang et al., 2002). Recent studies highlighted the interaction of NLRP12 not only with NIK, which is associated with the degradation of canonical NFκB, but also with TRAF3, which is directly involved in non-canonical NFκB degradation (I. C. Allen et al., 2012; Lich et al., 2007). On the other hand, a recent study showed that NLRP12 attenuated colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacteria (Liang Chen et al., 2017). In this study, mice deficient in NLRP12 showed increased colonic basal inflammation, a less diverse and loss of protective gut commensal strains and increased colitogenic strains. Last but not least, a beneficial role for treatments targeting NLRP12 is attributed to the role of NLRP12 as a checkpoint for osteoclast differentiation via inhibition of NFκB (Wu et al., 2013). Therefore, treatments targeting NLRP12 may serve a novel therapy for AS.

The first step is to establish whether the expression of NLRP12 is down-regulated in AS in view of the lowered expression of NLRP12 in human UC (Liang Chen et al., 2017). Since NLRP12 is expressed predominantly in the myeloid cell lineage, the expression of NLRP12 in peripheral blood mononuclear cells (PBMC) of AS patients should be compared to that of healthy control as well as MBP patients. Association analysis of NLRP12 with HLA-B27 positivity, sex as well as LCN2 levels could be done. If the above associations of NLRP12 and AS established, NLRP12 as treatment target would be an important next step for research.

Potential treatment targeting gut commensals include: (1) Pidotimod targeting NLRP12. Pidotimod is a synthetic dipeptide with immunomodulatory properties largely used for treatment and prevention of infections in pediatric patients. Recently, pidotimod is shown to induce the expression of NLRP12 (both mRNA and protein levels) and attenuates TLR-associated inflammation (Fogli et al., 2014). The modulation of NLRP12 by pidotimod paves the road for
development of innovative therapeutic approaches for AS. Treatments targeting NLRP12 may likely serve a better therapy than the current biologic treatments. This is likely because of the reasons that the TNFi only controls the subsequent inflammatory response, while the modulation of NLRP12 would play a direct role in regulating the imbalanced gut microbe as well as subsequent inflammation. There are likely some limitations in using Pidotimod as a treatment for AS. The efficacy of pidotimod on adult patients remains to be investigated as most of clinical reports on pidotimod largely focus on pediatrics. Since skin rash is the common side effect of pidotimod, caution must be taken when applying this medication considering the comorbidity of psoriasis in some AS patients. (2) Probiotics (*Lachnospiraceae*) inhibiting pathogen growth. In mice deficient of Nlrp12, there is a decrease of *Lachnospiraceae* and an increase of *Erysipelotrichaceae* (Liang Chen et al., 2017). Administration of *Lachnospiraceae* has shown to suppress colitis in these nlrp12−/− animals. Several possible impacts of these protective strains on the host immune response have been proposed: (a) The mucosal folds are the main habitat of *Lachnospiraceae*, which may enable the interaction of bacteria with lamina propria residual immune cells, indicating their potential functions in immune regulation during enteric pathogen invasion. (b) The short chain fatty acid (SCFA) propionate produced by *Lachnospiraceae* favors the generation of peripheral regulatory T cells (Arpaia et al., 2013b; Reichardt et al., 2014; P. M. Smith et al., 2013). (3) Oral administration of *Lachnospiraceae* is shown to suppress the growth of *Erysipelotrichaceae*. *Erysipelotrichaceae* have been linked with more NFκB and STAT3 activation in Nlrp12−/− mice (Liang Chen et al., 2017). Thus, *Lachnospiraceae* may play a protective role in part by inhibiting over-growth of intestinal *Erysipelotrichaceae*. In view of role of *Lachnospiraceae* in the absence of NLRP12, *Lachnospiraceae* administration may be another potential therapy compensating the lowered NLRP12 expression in patients with AS. However, there are inevitably concerns about
probiotics treatments, such as the efficacy of oral probiotic administration and the safety of fecal transplants. More study is needed before the application of this treatment proposal.

5.2.3 Research on dysbiosis in AS

To better understand AS pathogenesis, it is essential to unravel the early event of the disease, which is most likely gut microbiome dysbiosis. However, it has proved challenging to detect directly the change of gut microbiome. It will also be challenging to dissect out the aberrant interactions from host to microbes.

First and foremost, it is challenging to analyze the gut microbiome in AS patients compared with healthy controls, although the modern quantitative and qualitative analysis of the gut commensals composition may provide some clues for us. Studies on the gut microbiome signature in AS require disease cohorts with large number of patients from all around the world with all the confounding factors analyzed. High cost, huge bioinformatics data, and high requirement on technical support are major challenges for these studies. The gut microbiome is a complex and dynamic system which can be influenced by the host and the environment. Compositions of a balanced gut microbiome in healthy subjects can be very diverse. In addition, gut microbiome composition may differ in AS patients with different genetic backgrounds. Gut bacteria can vary among patients with the same genetic risks but living in different geographic areas with different life styles, which is also true for the same patient having different lifestyles, diet and living environments at different stages of life. The interactions among commensals and between commensals and pathogens further complicate the analysis on gut microbiome.
Secondly, studies with a single analyzed point on gut microbiome signature in patients alone will not be enough, because the onset and development of complex diseases is attributed to the interaction between the host and gut bacteria. Although attempts are being made to connect the gut microbiota with the host gene signature recently (unpublished data by Matt Brown), it is still not sufficient to thoroughly understand pathogenesis of AS since the interaction between the host and microbe is dynamic which may change from time to time. Similarly the expression of the host genes, immune response, as well as the gut microbiome ecosystem changes over time. Thus, a single point or even multiple-pointed analysis on the host genes and microbial signature may not be sufficient to reflect the real interactions, but it will create a massive dataset for bioinformatics analysis since both human and microbiome genomes are huge.

Finally, some may propose first to unravel such interaction between host and microbes in animal models. However, it remains questionable how relevant and informative the mouse gut microbiota is to that of human. For one reason, given the similarity in physiology and anatomical structures in mouse and human intestinal tract, there are still prominent differences such as the different distribution of Paneth cells, goblet cells and transverse folds, likely due to their diverging diets, feeding patterns, body sizes and metabolic requirements (Nguyen, Vieira-Silva, Liston, & Raes, 2015). Secondly, although in a healthy state the gut microbiota of human and mice are dominated by the same two major phyla, Bacteroidetes and Firmicutes, 85% of bacterial genera found in the mouse gut microbiota are not present in human (Ley et al., 2005; Ruth E Ley et al., 2006). The difference of gut microbiome in murine and human can be influenced by environmental factors including housing conditions (SPF vs. conventional conditions) and diets (mouse chow vs. human diet). Most importantly, genetically modified models cannot capture the whole genetic heterogeneity of human owing to the inherent genetic differences between the two species. Studies on gene-microbes interaction in complex disease settings will be hampered by the intrinsic
difference between humans and mice. Genetically modified murine models may reveal the contribution of a gene to disease development to a certain extent. However gut microbes are not only influenced by the target genes but also by a multiplicity of murine genes, which can be significantly different from the human. Furthermore, the inbred nature of mice eliminates the genetic heterogeneity. A good example is the failure of IL-10 treatment in IBD clinical trials, although IL-10 was suggested an important cytokines in IL-10 knock-out mouse model (Ley et al., 2005; Ley et al., 2006). In humans, the efficiency of IL-10 in dampening inflammation might be affected by genetic heterogeneity. Lastly, humanized gnotobiotic mice (germ-free animals inoculated with human microbiota) does resembles the majority of the human gut microbiota composition. Yet, there have been limitations in these models (Nguyen et al., 2015). For example, there was lack of human-specific gut bacteria and the proportion of bacterial phyla was different compared to human. Essentially, the immune responses as well as other responses from physiological processes in experiments using humanized gnotobiotic mice will likely not faithfully recapitulate the conditions present in humans.

Although animals do not completely mirror AS, insights from these experimental models could help unravel cellular and molecular pathways underlying the human disease. Moreover, animals could help investigate the mechanisms and safety of novel treatments before undertaking clinical trials in patients.
5.2.4 Controversial findings in AS research

In a complex disease like AS, controversial findings are not uncommon. For instance, in contrast to our results of the elevated LCN2 levels in AS patients, a recent European study reported normal LCN2 levels in their cohort (Turina et al., 2017). Another example is the analysis of calprotectin in AS. Some studies found an increase of serum calprotectin in AS patients compared to healthy controls, while others failed to identify such an increase (Klingberg et al., 2012; Turina, Yeremenko, et al., 2014).

Several possible reasons could lead to the controversy: (1) Patient cohorts may vary in different studies owing to a lack of specific diagnostic criteria. Diagnosis of AS largely relies on two classification criteria (modified New York criteria and the ASAS classification criteria). The choice of reference could result in various patient groups because the ASAS criteria include not only patients with advanced AS identified by modified New York criteria, but also patients at an early stage of AS as well as those with USpA and nr-axSpA. Therefore, the use of ASAS criteria may introduce a more heterogeneous patient population not limited to AS (van Tubergen, 2015). More precise diagnostic criteria are needed. (2) Given the complexity of the disease, patients with similar clinical features may have different underlying etiologies reflecting various combinations of genetic and environmental factors. For example, although HLA-B27 has been identified to be the strongest genetic association with AS, distinctive associations of HLA-B27 subtypes with specific genetic background of patients (e.g. Caucasians, Middle-eastern, North African, and Asian, etc.) have been identified. Thus, subgrouping patients based on genetic background, environmental factors and clinical features is needed. (3) The size of study cohort is another factor contributing to the inconsistent results given the heterogeneity of AS. A large size of cohort is necessary for stratifying patients into more homogeneous subsets for analysis. (4) While AS is a
dynamic disease, most AS studies focus on a single-point analysis (S. Arends et al., 2011b; Choi et al., 2008; Maksymowych et al., 2007; Mattey et al., 2012a). Therefore, an abnormality could be missed at time of assessment. Data collected over time and sequential analyses from the same patients may offer better opportunity to profile disease progression. (5) Evaluation of relevant mechanisms could be skewed by the use of specific treatments. For instance, the use of NSAIDs and TNFi has been shown to be associated with higher and lower fecal calprotectin, respectively (Klingberg et al., 2017). Missing treatment information of patients from different studies could be an explanation of conflicting results. Therefore, treatments should always be taken into account.

5.3 Conclusions

The ank/ank mouse model is a relevant model for AS pathogenesis. Elevation of LCN2 reveals a gut-joint axis and aberrant host-microbe interaction in AS. The association of PPARγ with LCN2 indicates a novel molecular pathway of AS. With normalization of LCN2 as a treatment target, LCN2 is a novel serological marker for monitoring treatment outcome in AS.
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