DEVELOPMENT OF TRANSLATIONAL MODELS FOR INTERVERTEBRAL DISC DEGENERATION USING A COMPARATIVE APPROACH FOR CANINE AND HUMAN PATIENTS

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DEDICATION

아껴주시고 응원해주신 전주 이씨 (全州 李氏)와 한산 이씨 (韓山 李氏) 가족 분들에게 바칩니다.

Dedicated to the ever-loving and supportive members of the Lee family of Jeonju and the Lee family of Hansan.

And to my dogs, Dochi and Odin.

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LIST OF ABBREVIATIONS

IVD: Intervertebral disc

IVDD: Intervertebral disc disease

CD: Chondrodystrophic

NCD: Non-chondrodystrophic

DMEM: Dulbecco's Modified Eagle's Medium

IL: Interleukin

TL: Thoracolumbar

LS: Lumbosacral

MTN: Midwest Transplant Network

TLRO: Thompson Laboratory for Regenerative Orthopaedics

CAD: Cadaveric donor spine

BH: Benjamini-Hochberg

ASD: Adjacent segment disease

IACUC: Institutional Animal Care and Use Committee

IRB: Institutional Review Board

CSM: Cervical spondylomylopathy

DLSS: Degenerative lumbosacral stenosis

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ABSTRACT

Intervertebral discs (IVDs) are unique musculoskeletal tissues within functional spinal unit organs comprising the spinal column that distribute loads and allow complex movements for vertebrates. IVD degeneration has been closely associated with manifestations of symptomatic IVD disease (IVDD). IVDD spontaneously occurs in canine and human populations. As such, dogs can serve as highly relevant and ethical preclinical models for both human and canine IVDD. Chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds of dogs show different phenotypes of IVDD, each of which mimic phenotypes described for human patients. The main goal for this PhD research was to develop and validate canine models for human IVDD with a focus on distinguishing molecular characteristics of key disease phenotypes. Using biomarkers associated with inflammation and degradation, IVD health and disease were characterized for the two species. Taken together, this body of work suggests that CD and NCD dogs demonstrate distinctly different biomarker profiles in both health and disease that represent key human IVDD phenotypes such that they can be used as effective models for translational research towards clinical diagnosis, prevention, and treatment strategies for canine and human degenerative disc disorders.

Chapter 1: Introduction

Intervertebral disc (IVD) is a unique combination of tissues that connect vertebrae in the vertebral column, forming a dynamic organ termed a functional spinal unit (FSU). Each IVD comprises a fibrocartilaginous joint that allows movements and absorbs and disperses loads associated with activities. All vertebrates including humans and dogs heavily rely on IVDs for physical structure, support of the torso, and functional movement. Abnormal disc structure and/or function can cause significant musculoskeletal and neurologic abnormalities that lead to disability and decreased quality of life. Low back and neck pain affects a wide range of individuals across ages, occupations, and socioeconomic spectrums. In 2016, low back and neck pain were associated with the highest annual health care spending in the US, estimated at $134.5 billion. [1] Low back pain has been identified as one of five leading healthcare disorders globally and the leading cause of years-lived-with-disability in all 195 countries and territories surveyed. [2]

While there are multiple etiologies for back and neck pain, degeneration of the IVD is one of the most commonly implicated causes. [3] While disc degeneration can remain asymptomatic for years, it progresses with aging often to a degree resulting in protrusion or extrusion with associated pain and neurologic deficits. Disc degeneration also significantly alters the structural anatomy and biomechanics of the spinal column, affecting associated muscles, ligaments, nerves, and facet joints and leading to more pain and disability. [4]

Each IVD consists of fibrotic annular outer layers called annulus fibrosus (AF) and a mostly clear, jelly-like structure in the core called nucleus pulposus (NP). Cartilaginous end-plates (CEP) transport nutrients and waste products in and out of the disc by diffusion between the vertebral bones where rich vascular networks are found[5]. These three primary components of the IVD are distinct from each other structurally and biochemically, and must be maintained in this way for optimal disc health. AF is a firm annular structure that maintains the shape of IVD and it largely consists of laminae of collagen type I and II fibers. Collagen type I is mainly distributed in the edge of the fibrotic ring and supports the shape of IVD. NP is a gel-like structure with high water content and mostly populated by notochordal cells (NC), which are lost relatively early in the human IVD development in contrast to dogs that have persistent NC population into adulthood. Healthy adult IVDs have very few blood vessels which are found in the outer layer of the AF, resulting in a largely avascular structure[7]. There are nerves, however, they are also limited to the outer layer of AF as well.

All components of IVDs are susceptible to degeneration and degenerative pathology in any component can initiate and exacerbate degeneration in other components. While the roles for CEP in IVD degeneration (IVDD) are still being elucidated, alterations in CEP structure or function that prevent proper nutrient and waste exchange are thought to be contributing factors in the early stages of degeneration[6]. Degeneration at cellular or molecular levels eventually leads to structural alterations which result in pain and neurologic deficits.

In terms of studying IVD degeneration in humans, most of the focus has centered on *in vitro* and induced animal models as there are few valid spontaneous-disease animal models that recapitulate disease pathobiology and progression. Valid spontaneous-disease animal models include sand rats, dogs, baboons, and macaques[8].

Based on disease similarities and our laboratory's focus on comparative medicine, dogs were selected as the preferred large animal spontaneous-disease model for IVDD. Symptomatic cervical and lumbar IVD disorders occur commonly in both dogs and humans. In dogs, these degenerative disc disorders often result in profound neurologic deficits that can be cause for euthanasia of otherwise healthy animals. As such, research aimed at elucidating disease mechanisms that identify key targets for effective diagnostic, preventative, and therapeutic strategies for IVDD can directly benefit clinical patients of both species.

Dog breeds can be divided into two groups that show distinctive differences with respect to IVDD. Chondrodystrophic (CD) breeds are characterized by short legs and long torsos. Some of the more popular CD breeds are Dachshund, Beagle, American Cocker Spaniel, French Bulldog, and Cardigan Welsh Corgi. Young adult to middle-aged CD dogs often suffer from an acute manifestation of IVDD characterized by explosive herniation of degenerative NP and/or AF that directly damages the spinal cord, incites inflammation, and causes sustained compression. This occurs most commonly in the thoracolumbar junction segments. Depending on the degree of herniation and spinal cord damage, clinical signs vary from pain to mild paresis to complete paralysis. Dogs with normal leg and torso proportions fall into the non-chondrodystrophic (NCD)

breeds. In NCD dogs, IVDD tends to manifest as more chronic conditions that affect older dogs or highly active dogs. Military, working, and sporting dogs often undergo repetitive high-load, high-strain motions in their backs while performing their activities.[9] These activities in conjunction with aging are thought to contribute to the chronic degenerative changes in NP and AF that result in disc protrusion, inflammation and sustained compression that are characteristic of IVDD in NCD breeds. While less common, NCD dogs can also experience acute disc herniation similar to that seen in CD breeds.

Chondrodystrophic dogs have been the main focus of IVDD research as a large pre-clinical model for human IVDD. Onset of IVD degeneration is typically noted in the third decade of life in humans and approximately translates to onset in CD breeds, which occurs around 2-3 years of age[10]. There has been less focus on IVDD in NCD breeds, in part due to the later onset of disease. However, NCD dogs have high potential to serve as a powerful tool to study a chronic lumbar degeneration that manifests as low back pain in millions of individuals.

Currently, there are no known regenerative therapies that restore structural or functional integrity to degenerative IVDs. Current medical treatments target associated pain and inflammation, while surgical interventions remove offending portions of disc and then fuse the affected segment(s) if indicated. Importantly, none of these treatments directly address degenerative mechanisms of disease for any of the affected structures, and adjacent segment degeneration or disease (ASD) is common in both species[11]. Along with limited therapeutic options, diagnostic and prognostic indicators,

or biomarkers, for IVD have not yet been validated for clinical application. Biomarkers are typically considered measurable data that can be obtained from tissue, blood, urine, or other bodily fluids[15]. Biomarkers reflect indicators of normal and pathologic processes or responses to therapeutic interventions. Biomarkers in orthopedics, especially for common bone and joint disorders, have received tremendous attention for their potential for early diagnosis, screening, staging, treatment monitoring and prognostication for these conditions[12–14]. While there is increasing interest in characterizing valid biomarkers for spine disorders, these research efforts are in early stages of development. Programmatic research in this area is needed and a translational approach using canine models appears to have high potential for addressing current limitations in effectively managing IVDD.

Given these important unmet needs in human and veterinary medicine, the research included in this PhD dissertation aimed to characterize mechanistic biomarker responses and establish comparative IVDD biomarker profiles for NCD and CD dogs with relevant human counterparts. To achieve these aims, the following studies were performed. 1) IVD biomarkers from normal NCD and CD dogs were compared to understand the differences in these two models, 2) normal canine IVD biomarkers were compared to the degenerative canine IVDs to delineate potential molecular changes associated with degeneration, 3) normal and degenerative canine IVD biomarkers were then compared to normal and degenerative human IVD biomarkers, and 4) the human IVD biomarkers were further analyzed by identifying differences between non-symptomatic and symptomatic IVD degeneration. These studies together will define and characterize

biomarker production profiles of both normal and degenerative IVDs from canine and human subjects and allow development of effective and ethical translational canine IVDD models.

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Chapter 2: Literature Review

Disorders of the spine comprise a major global healthcare concern in terms of pain, disability, and associated costs. While there have been tremendous efforts and funding to further understand pathogenesis of intervertebral disc disease (IVDD) and basic and clinical breakthroughs have been made, translational animal models that effectively connect the gap from the benchtop to bedside appear to be underutilized. Domesticated dogs can be used for large animal preclinical models that are very effective in providing translational evidence to address this unmet need. Because IVDD affects canine patients with similar clinical and economic impacts to those reported for human patients, canine models produce data with high translational impact that can be directly applied to veterinary patients as well. Therefore, the objectives of the present review are to outline the applicable similarities in the key features of spine disorders between dogs and humans, describe relevant canine models, and highlight the applicability of these models for advancing understanding in mechanisms of disease, diagnosis, prognosis, prevention, and treatment of spine pathology.

Intervertebral Disc Disease in Humans and Dogs

In humans, symptomatic spine disorders are typically classified into 1 of 4 categories: axial back/neck pain syndromes, stenosis, instability, and deformities. Axial pain syndromes have had several etiologies implicated including paraspinal muscle dysfunction[1], facet joint arthrosis[2], inflammatory arthritides[3], and intervertebral disc degeneration[4]. Similar spinal pathologies have been reported for canine patients. The

primary sources of pathology and/or pain generators, including disc, endplate, facet joint, and muscle-tendon are the main categories. Disc pathologies leading to IVDD are the most prevalent and most investigated, and endplate-driven, facet-driven, and muscle-driven disorders of the spine have been reported as well. Endplate abnormalities in dogs include discospondylitis, fatty infiltration, dysplasia/remodeling, osteochondrosis, and Schmorl's nodes. The lumbosacral (LS; L7-S1 in dog) region has a predilection for endplate pathology based on imaging studies. Endplate dysplasia, sclerosis, remodeling, and/or degeneration are associated with vertebral instability in the canine LS region (LS instability) and caudal cervical region (caudal cervical spondylomyelopathy (CCSM) or Wobblers syndrome), both of which typically include some degree of IVDD.[5–8] CCSM is most common in Great Danes and Doberman Pinschers, while LS instability occurs most frequently in German Shepherd Dogs, Border Collies, Australian Shepherds, Labrador Retrievers, Rottweilers, Bernese Mountain Dogs, Boxers, Dalmatians, and Irish Setters. LS instability appears to have genetic[9,10] and biomechanical (activity-related)[11–19] components, and is being diagnosed more commonly in dogs with the growth in number of working, service, and performance dogs worldwide, as well as availability and use of advanced diagnostic imaging in veterinary medicine. [6,20–25] Many dogs affected with LS instability have larger, less sagittally oriented facet joints at L7-S1, which are associated with increased LS flexion and extension[26], and both LS instability and CCSM can also be considered in the facet-driven category based on concurrent dysplasia, remodeling, and degeneration of affected facet joints.[5,7–10,27] Other facet-driven disorders in dogs include

hypoplasia/aplasia[28] and osteoarthritis.[29] In terms of muscle-driven disorders of the canine spine, muscular dystrophy in Golden Retrievers[30] and spinal muscular atrophy in Brittany Spaniels[31] have been reported. Spondylosis deformans, diffuse idiopathic skeletal hyperostosis (DISH), and scoliosis may also involve muscle-driven mechanisms.

It is likely that there is a large degree of crossover with respect to the anatomic "drivers" of spine disorders in both canine and human patients. As such, it is important to consider the whole organ or functional spinal unit (FSU) and whole body when treating patients and modeling disease. Since the majority of clinical disease and related research have centered on the intervertebral disc (IVD) and endplate-driven, facet-driven, and muscle-driven disorders typical involved or affect the disc, the present review focuses on IVD disease and degeneration.

IVDD is often characterized by loss of water from the nucleus pulposus (NP) with associated alterations in disc composition and structure, reducing its ability to function as a hydraulic cushion in vertebral column loading bearing and motion. [32–34] As degeneration progresses in dogs and humans, NP cells form large clusters and shift from collagen II to collagen I synthesis further compromising the critical biomechanical balance that determines its material properties and function.[35,36] Annulus fibrosus (AF) cells and matrix also undergo degenerative changes that result in unstable or weak regions of the disc. There is evidence that inflammatory and degradative processes drive IVDD in both dogs and humans.[36–39] As IVDD progresses, significant changes in articular facets, vertebral endplates and bodies, ligaments, and musculature ensue.[40,41] As with any animal model, there are associated limitations that should be considered when

using and translating data from canine studies towards understanding human disease. Anatomically, dogs have seven lumbar IVDs while humans have only five (Table 1). However, clinical, compositional, histologic, and biomechanical similarities between canine and human IVDD have allowed for comparative research to evaluate aspects of degeneration that may translate to either species, making canine models of IVDD extremely useful research tools.

Ex Vivo Models

Models using cells, single tissues, or whole organs provide a controlled method for investigating mechanisms of disc degeneration.[42] Cell culture models allow for control of certain variables and are typically less complex and expensive to employ than other options.[43] However, extracellular matrix (ECM) is altered or absent, which commonly results in cell dedifferentiation and does not allow for valid assessment of biomechanics or morphological integrity.[44] Tissue cultures of IVDs without adjacent endplates allow for better maintenance of cell distribution and differentiation, ECM integrity, and material properties, but biologic and biomechanical influences of endplate cartilage and vertebral bone are lost, and the NP is allowed to freely swell in culture.[45–47] Based on these limitations, whole organ IVD explant models have been developed in several species and used to study biologic and biomechanical components of the functional spinal unit in health and disease. Canine ex vivo models have been used effectively to address questions regarding nutrient and oxygen supply, osmolarity, cell phenotype, gene expression, cell signaling pathways, and biomarkers for diagnosis, staging, and therapeutic targets.[22,48–51] Used in these ways, these models can serve as excellent

screening tools for focused, efficient, and ethical use of animal models for translational studies towards clinical application.

Canine Models

Numerous animal models have been developed to investigate specific questions about IVDD across the spectrum of disease mechanisms, diagnosis, staging, prevention, treatment, and prognostication. Animal models range from rodents to primates, induced to spontaneous, and acute to chronic with spontaneous disc degeneration in non-human primates, age-related disc degeneration in mice, and genetically-engineered spontaneous disc degeneration in mice having attractive modeling characteristics. When considering all of the factors involved in selecting an animal model including availability, ethics, cost, and translational applicability, canine models can also be considered strong candidates.[52–54] Spontaneous and induced canine IVDD models have been used to investigate a wide spectrum of biologic, biomechanical, and clinical components of spine disorders in their human counterparts.

Induced Models

Induced models provide a method for creating standardized pathology to consistently initiate desired disease processes while mitigating confounding variables and associated variability. The primary types of induced models in dogs include surgical or chemical focal annular injury, removal of disc material, or a combination of these insults.

Annular injury is the most common induced IVDD model across species, having been used for nearly a century to consistently initiate degeneration of intervertebral discs in

dogs.[55,56] Annular injuries are induced by incision, puncture, or direct disruption of the AF and/or its attachment to the endplate. These models are intended to mimic IVDD resulting from annular tears in humans by introducing a small AF injury that leads to the known sequelae that result in symptomatic disc disease. These sequelae include structural compromise of the annulus, loss of resistance to hydrostatic forces within the disc, abnormal loading, apoptosis, necrosis, and cell phenotype shifts, NP protrusion/extrusion, extradiscal exposure of NP causing impingement and/or inflammatory responses, loss and remodeling of extracellular matrix, and ultimately, IVD failure.[57] As such, annular injury models can allow for assessments of biochemical, histologic, and biomechanical perturbations that lead to the clinical manifestations of symptomatic IVDD. The primary limitations involve artificial ways in which the pathology is created, the relative severity of the injury and resultant timing and progression of disease, and the otherwise-normal condition of the spine in the research dogs.

In an attempt to address these limitations, endplate models,[58,59] biomechanical injury models,[60] and discectomy models[39,52] have been developed and implemented in dogs. Endplate models employ a mechanical disruption or physical barrier at the cartilaginous endplate with the goal of inhibiting IVD imbibition. The resulting endplate perfusion perturbations are thought to cause nutritional deficits in the disc, inducing degeneration. Initial data from this model showed extracellular matrix alterations and histopathology consistent with some components of degenerative disc disease in people.[61]

A biomechanical-induced IVDD model has also been attempted in dogs by attaching coil springs to vertebral bodies to facilitate compressive overloading of discs.[60] However, the investigators reported no macroscopic or radiographic indications of degeneration and only minimal histologic changes, suggesting that this biomechanical method may not have translational validity and highlighting the difficulty of using biomechanical insults in vivo.

In order to induce more expedient and severe inflammatory and degradative changes, surgically and chemically induced partial discectomy models have been employed in dogs. Surgical excision of a portion of the disc (subtotal discectomy) is the most common means of creating this models and has been used to study mechanisms and timing for disease processes, correlations among diagnostic and staging modalities, and safety and efficacy of potential therapies.[52,62,63] Recently, percutaneous laser discectomies have become more widely performed to create discectomy models in order to avoid confounding variables associated with more invasive surgical models and be more directly translational in nature.[53,54,57] However, direct comparisons among these models have not been reported to the authors' knowledge.

Chemically induced discectomy, or chemonucleolysis, uses enzymes to degrade the NP, effectively reducing its viability, volume, and material properties.[64] Agents commonly used for IVD chemonucleolysis include chondroitinase or papain.[65] To initiate disc degeneration, chemonucleolysis is dose-dependent such that chemically induced models frequently require high concentrations of these agents to effectively result in relevant degenerative changes, which may limit translational potential.[66] The benefits of

chemically induced models include their capabilities for targeted damage without associated fibrosis and other confounding variables associated with surgically induced models.[67] These models may also have therapeutic relevance in that chemonucleolysis has been successfully used as a treatment option in select human and veterinary patients.[64,65,67–69] Surgically and chemically induced models have also been used in chondrodystrophic breeds of dogs in order to include the spontaneous-disease components to methods for initiating and perpetuating insults.[39,41,70,71]

Spontaneous Models

In dogs, selective breeding has resulted in wide phenotypic diversity varying from teacup Yorkshire Terrier to Great Dane.[72] Selective breeding and artificial selection created breeds with varying characteristics that have resulted in disorders that are similar to those in humans. As such, dogs are often used as valid translational models of human disorders.[73] IVDD is a prime example of a disorder that is shared between the two species and for which dogs can serve as robust preclinical animal models.

Dog breeds can be categorized as either chondrodystrophic (CD) or non-chondrodystrophic (NCD) dogs with IVDD affecting both categories in different ways. The lifetime prevalence of IVDD in dogs has been estimated at between 2% to 5%[32–34] with highest prevalence in in older dogs and in CD breeds such as Dachshund, Cocker Spaniels, and Beagle.[20,33,74,75] In Dachshunds, relative risk for IVDD is 10 to 12 times higher than other breeds with between 19% to 24% of Dachshunds showing clinical signs of IVDD during their lifetimes.[29] Other CD breeds at higher risk for IVDD include

Beagle, Cocker Spaniel, Cavalier King Charles Spaniel, Tibetan Spaniel, and Shih Tzu, while high-risk NCD breeds include Doberman Pinscher, Papillon, Rottweiler, Dalmatian, German Shepherd Dog, Miniature Schnauzer, and Bernese Mountain.[20] Furthermore, IVDD has been reported to be more pervasive in the purebred population compared to mixed-breed dogs.[34,76]

Spontaneous canine models of IVDD rely on naturally occurring degeneration to closely mimic the cumulative structural and metabolic changes that occur in association with human IVDD. Large population studies have estimated that between 71% to 77% of humans harbor degenerative discs before 50 years of age.[77] Spontaneous or naturally occurring IVD degeneration appears to progress in similar ways clinically, macroscopically, histologically, and biochemically for both species,[39] and disc herniation occurs at similar rates (approximately 2%) in humans and dogs.[33,78]

In dogs, IVDD is categorized as Hansen Type I (calcified NP extrusion out of IVD through AF) or Hansen Type II (weakened AF protrudes outward into the vertebral canal).[20,23,24,39,79,80] CD breed dogs mostly present with Hansen Type I and NCD breed dogs often present with Hansen Type II. Chondrodystrophic dogs encompass smaller breeds that are known to experience IVD degeneration at an earlier age than their NCD counterparts.[22] Based on the earlier onset and typical clinical presentation, CD dogs, most often Beagles and Dachshunds, are used for spontaneous models of acute traumatic or overuse IVDD in younger patients,[22,39] whereas NCD dogs more closely model chronic IVDD in older patients. While disc degeneration and herniation are diagnosed less commonly in NCD dogs compared to CD dogs, degenerative changes do

occur commonly in NCD dogs on a histologic level.[24] Importantly, CD and NCD dogs have many characteristics that are similar to various clinical manifestations seen in human IVDD patients. Clinical signs, imaging findings, histology, treatments, biomechanics, and molecular markers of IVDD between the two species share numerous similarities. As such, degenerative and healthy discs from CD and NCD dogs can be assessed over the lifespan to study changes in cell phenotype (notochordal cells, NP cells), gene expression, signaling pathways, and extracellular matrix alterations in degenerative IVDs in order to better understand disease mechanisms, develop and validate biomarkers, and advance early diagnosis, prevention, treatment, and development of prognostic indicators for IVDD in dogs and humans.[49,50,79]

Mechanisms of IVDD

Based on shared features of development and progression, spontaneous IVDD in dogs is a powerful model to study mechanisms of disease for human IVDD.[39,81–84] Recognized biologic mechanisms of IVD degeneration in both species include calcification of cartilage end plates reducing nutrient supply to the NP, increased cell death,[25,85,86] loss of the notochordal cell population and replacement with chondrocyte-like cells of the NP,[81] transition of the gel-like NP to a more fibrous and/or chondroid tissue, [87] weakening of the AF through degeneration of the extracellular matrix and development of fissures and cracks,[82] increased intrinsic and extrinsic tissue inflammation,[88] and increased degradative enzyme production and activity.[37,89] However, the precise roles, interactions, links, and correlations among these mechanistic components of disease

and their contributions to the various forms of symptomatic IVDD have not been fully characterized.

The IVDs of chondrodystrophic dogs undergo many of the changes that occur in human IVDs at an early age.[82] Calcification of cartilage endplates and NP can occur as early as 5 months of age in CD dogs, and is observed in 31.2% of cervical and 43% of lumbar discs by 1 year of age.[82] Relative within-animal differences in degree and timing of calcification and associated pathology can be used to characterize drivers of IVD calcification and related clinical disease while reducing the number of animals needed for valid study.

In the NP, the notochordal cell population is lost in humans and CD dogs and replaced with a chondrocyte-like cell population. This transition in cell population is associated with a shift in biochemical composition of the NP from a gel-like tissue with a high proteoglycan-to-collagen ratio to a more fibrous and/or chondroid tissue with reduced proteoglycan and water content. Calcification of the cartilage endplates and a resultant reduction in nutrient delivery to and waste removal from the NP is believed to be a primary contributor to these degenerative changes in the NP. In NCD dogs, these changes in the NP occur less consistently and later in life compared to CD dogs. Therefore, comparative studies that use CD and NCD dogs can be designed to elucidate factors driving the age- and disease-related changes that occur in the NP of dogs and humans.

Another key mechanism in IVD degeneration is cell death due to apoptosis and autophagy.[86] The cells of the NP and AF are required to maintain the complex extracellular matrix of the IVD. Progressive loss of NP and AF cell content is a common feature with age and degeneration in both human and canine patients. Loss of cells is associated with extracellular matrix alterations and decreased integrity of both tissues, however, the order and sequence of events in this degenerative pathway and its effects on likelihood and timing of disc herniation are unknown. Herniation of the IVD occurs by one of two general mechanisms in human patient cohorts as well as in CD versus NCD dogs.[82] Complete extrusion of the NP through the AF is common in traumatic disc ruptures in relatively younger human patients and is the Hansen type I herniation most commonly seen in CD dogs. IVD protrusion is most commonly noted in association with aging and/or chronic degenerative disc disease noted in relatively older human patients and NCD dogs. Spontaneous IVDD in CD and NCD dogs can provide novel insight into cell loss and matrix alterations in AF versus NP in contributing to distinct pathways for IVD herniation.

Inflammation and degradative enzyme activity have been observed in degenerative IVDs in human and canine patients.[25,37,88,89] While the inflammatory cytokines IL-1β and TNF-α have been implicated in increased cell death, increased degradative enzyme production, and decreased production of extracellular matrix proteins, the dynamics of cytokine involvement with age and degeneration are still incompletely characterized. in addition, the precise roles and effects of inflammatory cytokines and the dynamics of related matrix metalloproteinase, aggrecanase, and TIMP production in normal and

degenerative IVDs tissues are not well delineated. Because CD and NCD dogs develop IVD degeneration at different rates and often at different ages, these spontaneous IVDD models can provide clinically relevant information on the roles of inflammatory and degradative mediators in acute and chronic degenerative disc diseases.

While the biologic and biomechanical components of the spine are inextricably linked in IVD health and disease, they are often approached in separate, parallel pathways with respect to experimental design, outcome measures, and application. Primary biomechanical mechanisms for IVDD include deficiencies or failures to maintain hydrostatic pressure transduction, to transmit load, and/or to allow functional movements. For each of these disease mechanisms to be avoided, the composition, structure, and integrity of all components of the functional spinal unit including the NP, AF, endplates, vertebral bodies, facet joints, ligaments, and paraspinal muscles and tendons must be maintained in balance, relationship, and function.[87] In the IVD, alterations in the critical balance of water, proteoglycan, and collagen composition and structure of the NP can cause rapid and profound loss of material properties that govern compressive load distribution,[90] nutrient and waste transport,[91] cell signaling, and mechanotransduction.[49,91] Similarly, physical and/or biochemical disruptions of the concentric rings of the AF and/or its attachments to the endplates negatively affect its ability to contain the NP and to effectively resist the omnidirectional hydrostatic pressures, load transmission, and stability requirements for posture and activity. These alterations and disruptions directly affect nutrient and waste diffusion, loading and movement of the functional spinal unit, and disc integrity.[90,91] Because the IVD relies on

these biomechanical processes to maintain its health and function, loss of these inevitably propagates a vicious cycle of compensatory tissue remodeling, inflammation, degradation, dysmetabolism, degeneration, pain, and dysfunction.[87,90–93]

Causes of IVDD in Dogs

IVDD in humans and dogs is considered to be a complex multifactorial spectrum of disease influenced by genetics, aging, overuse, and/or trauma. Specific genes have been implicated in both species and many types of IVDD are considered familial.[1,7–10,12–19,29,35,94–96] Aging has significant effects on canine IVDs with strong evidence for progressive degenerative changes in discs and associated increased likelihood for symptomatic IVDD in older dogs.[20,23,39,82,97,98] Facet joints also have significant alterations with increasing age,[9] which further drive disc degeneration and associated morbidities.[41] Environmental and lifestyle factors that increase biomechanical loading of IVDs, especially repetitively, are associated with IVDD and are more pronounced with increasing age.[16,99] While NCD dogs are relatively protected against IVDD in general, the incidence of IVDD increases in performance and working NCD dogs consistently experiencing repetitive movements of the spine under load.[11–16,18,19,39,100–102] Overt trauma to the spine can also occur in these working and performance dogs, and a traumatic event (e.g., jumping off the couch) is often reported in association with acutely symptomatic IVDD in CD dogs. Anatomical and biomechanical factors including spinal canal diameter, associated ligaments, epaxial and hypaxial musculature, flexion, extension, rotation, and loading moments on the spine likely influence overuse and traumatic causes of IVDD as well.

While obesity is accepted as a significant risk factor for symptomatic disc disease in humans,[103–109] this association is less clear in canine patients. In general, obesity is considered a relative risk factor for IVDD in dogs,[74,110] however, in CD breeds, specifically Dachshunds, body condition score has not been reported to have a strong correlation with prevalence of IVDD.[111,112] This may be a true lack of higher risk or it may be that other risk factors for IVDD—such as disc calcification and spine biomechanics—predominate in CD dogs.

To the author's knowledge, there are no data reporting the effects of cigarette smoking (second-hand smoke) on IVDD in dogs. However, other animal models report that exposure to components of tobacco is associated with decreased nutrient transport, altered cell morphology and function, increased oxidative stress and cell death, decreased ECM content and synthesis, and structural changes in IVDs.[113–124] Tobacco use is clearly implicated in symptomatic disc disease in human patients.[107,108,125] Similarly to nicotine, caffeine also has been indicated in dose dependent IVD degeneration.[126] As such, research aimed at the effects of second-hand smoke on canine companions could provide important insight into mechanisms for IVDD associated with tobacco use, as well as disc degeneration pathways, in general.

Diabetes mellitus (DM) is a chronic metabolic disorder that has been indicated as a risk factor for accelerating IVD degeneration in human patients.[125,127–135] DM is thought to accelerate IVD degeneration by increasing advanced glycation end-product (AGE) accumulation in discs.[136–139] Studies examining the degenerative effects of AGEs on IVDs have been performed in murine models primarily. Dogs are affected by DM and require

monitoring and insulin therapies such that diabetic dogs could serve as a valid large animal model for study of DM-associated disc degeneration.

Diagnosis of IVDD in Dogs

Symptoms associated with IVDD in dogs closely mimic those seen in human patients. Evidence of pain and a “hunched” or “roaching” appearance are common complaints for owners of dogs with IVDD. Other early signs include difficulty rising, getting into a car, or going up stairs and/or weakness during recreational, performance, or work-related activities or even those of daily living. These signs may be episodic, may resolve, and/or may progress to ataxia or even paralysis. For CD dogs with acute disc herniation, ataxia or paralysis are often the first symptoms noticed by owners.

After taking a complete history and performing a general physical examination, complete neurologic examination is the foundation of diagnosis for IVDD. The comprehensive, systematic neurologic examination allows the clinician to localize the lesion to forebrain, brainstem, cerebellar, vestibular, cranial nerve, peripheral nerve/neuromuscular, C1-5, C6-T2, T3-L3, L4-S3, caudal, or multifocal. It also provides at least an initial assessment of severity of disease and prognosis. This knowledge allows the clinician and the owner to make informed decisions and directs diagnostic imaging.

After neurologic assessment and localization, diagnostic imaging is indicated to provide further detail regarding location, extent and severity of the lesion(s) and to determine treatment options and prognosis. For dogs with spine disorders, radiographic assessment is a mainstay of diagnostic imaging in order to provide a comprehensive

assessment of the patient, and radiographs alone may be sufficient for diagnosis of some disorders. When plain radiographic studies are insufficient for definitive diagnosis, advanced imaging should be performed. Magnetic resonance imaging (MRI) is considered to be the preferred diagnostic imaging modality for IVDD in dogs, when available.

To the authors' knowledge, the only diagnostic imaging grading system used for canine intervertebral disc disease to date is the Pfirrmann system based on MRI. This system uses a grading scale from 1 to 5. Grade 1 is the normal, homogenous, hyperintense disc on T2 spin-echo weighted sequences, while grade 5 is an inhomogenous, hypointense disc signal with no distinction between the nucleus and annulus and collapse of the disc space.[31] There was high correlation between the Thompson system of disc degeneration and the Pfirrmann scoring system using low-field MRI in both small and large breed dogs, although there was a group of dogs that were scored higher when the presence of spondylosis was seen. Spondylosis can be seen in dogs with mild disc degeneration and even normal discs on MRI. There are several other factors that may influence the correlation of these two systems. There is variation in the size, shape, and age of the dogs; the resolution in small breed dogs is lower than in large breed dogs; and the coil effect of the MRI has brighter signal of the discs within the focus area of the MRI and decreasing signal of the disc outside of this area. This may falsely affect the grade of the disc at the periphery of the MRI focus.[31]

Treatment of IVDD in Dogs

Treatment of IVDD in dogs also mimics standard-of-care therapeutic algorithms for human patients. Symptoms of pain, stiffness, and muscle spasm without significant neurologic deficits are typically treated with oral non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids, analgesics, muscle relaxants, and/or gabapentin along with activity modification and physical therapy. Corticosteroid, opioid, and/or local anesthetic epidural, sacroiliac, and facet joint injections have also been performed with success. Acupuncture and chiropractic treatments have been advocated by some, but evidence for safety and efficacy is currently lacking in veterinary medicine.

When nonsurgical treatment has failed, significant neurologic deficits are present, and/or the pathology necessitates, surgical treatment for symptomatic IVDD is indicated. The most common indication for surgical treatment of canine IVDD is acute disc extrusion in CD dogs. These cases are treated by surgical decompression and partial discectomy via partial corpectomy ("ventral slot"), laminectomy, hemilaminectomy, facetectomy, or foraminotomy of the affected disc space(s) depending on anatomic location and severity. LS stenosis and LS instability are surgically treated by laminectomy with facetectomy, partial discectomy, and/or dorsal (posterior) fusion as indicated. Importantly, adjacent segment disease is a common sequela to IVDD and associated surgical treatments in dogs in a similar manner to that encountered in human patients.[6,7,98,140–145]

Outcome Measures for Canine Models of IVDD

Clinical-Functional

Based on the common and frequent management and care of IVD disorders in veterinary medicine, all of the clinical diagnostics described above can be employed in translational studies using spontaneous or induced models. Importantly, these can be performed using standard-of-care technology before and after treatments that are nearly identical to those performed in human patients.

For preclinical studies using canine IVDD models, inclusion of repeated neurologic exams, diagnostic imaging, and assessment of pain is recommended. In addition, activity monitoring, kinetic, and/or kinematic assessments may be additive to specific experimental designs.[146–153]

Biomechanics

The material properties of the intervertebral disc are a key measure of health and function of the spine[49,87,90–92,154–156] and should be included as an outcome measure when possible. The validity of any quadrupedal model of spine disease has been called into question based on the perception of fundamental differences in axial loading dynamics. However, biomechanical studies on human and canine spines have revealed that a significant amount of IVD compression can be attributed to the paraspinal musculature, suggesting that spine biomechanics are comparable between the two species.[71,157] Together with the knowledge that IVDD occurs with similar frequency, mechanisms and causes in dogs, best current evidence indicates that disc degeneration is not solely a product of human bipedal spine biomechanics[158] and supports the use of canine models for study of the biomechanical components of IVDD as well. Ideally, the

biomechanical properties of the FSUs should be evaluated in bending, compression, and rotation for pivotal preclinical studies using canine models.[92,159] Each of these tests mimics natural movements of the spine that have been validated in canine and human FSUs. These biomechanical data can then be correlated to clinical, diagnostic imaging, biomarker, macroscopic, and histologic data in order to characterize the effects of degenerative changes on function and differentiate tissue involvement, roles and mechanisms in disc health, and disease.

Incremental loading tests are designed to measure material responses to forces applied in an increasing or decreasing stepwise manner.[160] For IVD testing, incremental loading can be applied in compression to a single disc or FSU or in compression, bending, and/or rotation to a spinal segment. Incremental loading tests are used to create force-response profiles to characterize tissue properties.[161] Compression tests are commonly performed on IVDs, FSUs, and spinal segments. As IVD compression is essentially constant due to muscle forces and gravity, and resistance to compression is a key feature of disc health, various forms of compression testing can be used to assess the compressive modulus, elasticity, creep, stress-relaxation, and permeability of the IVD in order to characterize its functional composition, integrity, and viscoelasticity.[47,58,160–165] In theory, compressive, bending, rotational, biaxial, and multiaxial biomechanical tests can be incremental, single or cyclic or both, and non-destructive or destructive. If loading stays within physiologic ranges and the tissues retain their properties following testing, it can be considered non-destructive such that other assessments can be

performed on the same tissues. Destructive, or load-to-failure, testing may be necessary based on experimental design or intended purpose of the study.

Biomarkers

The National Institutes of Health's Biomarker Definitions Working Group defines a biomarker as "a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes or pharmacologic responses to therapeutic intervention."[51] Currently, there are no biomarkers that have been validated for clinical use to diagnose, stage, prognosticate, or assess outcomes for any component of IVDD in dogs or humans. However, intensive research in this arena is being pursued using in vitro, translational, and clinical studies, and preclinical canine models provide a powerful tool in this effort.

The ideal biomarker(s) for IVDD would provide precise, accurate, and early information for diagnosing and categorizing likelihood, type and severity of disease, for deciding timing and type of intervention, for evaluating response to treatment, and for determining prognosis using an easy-to-obtain and minimally invasive sample, such as oral swabs, blood, or urine. For example, a panel of molecular biomarkers from an oral swab for polymerase chain reaction analyses could determine relative risk for symptomatic and progressive scoliosis in pediatric patients, or a panel of serum protein biomarkers could determine likelihood for response to nonsurgical management of low-back pain in men over 60 years of age. For each of these examples, the appropriate intervention could then be determined and implemented with higher likelihood for

compliance and success. Similarly, veterinarians could use a panel of urine protein biomarkers to monitor Dachshunds at annual wellness appointments for progression of disc degeneration associated with extrusion, or they could use a panel of molecular biomarkers from puppies bred for military service to ascertain relative risk for lumbosacral instability prior to assignment to time- and cost-intensive training. Both of these veterinary medical examples would also effectively inform breeding decisions.

In humans and dogs, IVDD is associated with inflammation, altered matrix synthesis, catabolic metabolism, cell death, and neural and vascular ingrowth in the disc and surrounding tissues.[166] As such, MMPs, ADAMTs, cytokines, chemokines, and ECM proteins have been the main focus of biomarker studies.[167–169] Proteomics[170–176] and metabolomics[177] are the tools often used to identify and develop biomarkers of IVDD. In dogs, 15F2t isoprostane in urine of IVDD patients,[178] Runx2 Runt-related transcription factor 2 (Runx2) expression in calcified IVDs in Beagle dogs,[179] NG2 proteoglycan expression in degenerative Dachshund IVDs,[180] and Link-N that interacts with proteoglycan aggregates[181] in CD and NCD dogs have been reported as having potential to serve as clinically relevant biomarkers. There are other biomarker studies in dogs that focus on molecular signatures of inflammation such as IL-6, IL-8, and other cytokines and chemokines in degenerative IVDs.[38,168,182]

Based on the breadth of similarities between human and canine IVDD and the currently unmet need for clinically relevant biomarkers in human and veterinary medicine, the candidate's PhD focused on programmatic research aimed at identifying protein biomarker panels for spine disorders. This ongoing characterization of biomarker

production patterns will help delineate potential clinical applications for both species. If conserved protein expression or metabolite signatures of IVDD can be identified in both species, the fundamental process that lead to early degeneration of IVDs could be explored as diagnostic, prognostic, preventative, and therapeutic targets.

Conclusions

Dogs provide powerful models for disorders of the spine. Pathogenesis, causes, clinical presentations, treatment options, and diagnostic tools for IVDD are highly similar between human and canine patients. In particular, spontaneously occurring IVD degeneration in chondrodystrophic and non-chondrodystrophic breeds of dogs provide highly translatable preclinical data for symptomatic disc degeneration disorders seen across the spectrum of age-, cause-, and pathology-associated patient cohorts. Measurable data obtained through scientific studies in dogs provide insights into histopathology, biomechanics, and various biomarkers with high clinical relevance, but that cannot be practically or ethically obtained from human patients.

When choosing a preclinical model for spine research, it is critical to remember that biologic and biomechanical components of IVD health and disease are inextricably linked. Furthermore, it is important to acknowledge and address limitations of canine models including subtle differences in vertebral column anatomy, biomechanics, genetics, physiology, and lifestyles. As such, comprehensive outcome assessments with correlations among metrics are important for validity and translatability. Spontaneously-occurring canine models are amenable to this comprehensive and correlative approach

while also corresponding directly to in vitro, ex vivo, and induced-disease canine models. Taken together, preclinical studies using the full breadth of canine models can guide targeted research towards developing valid and effective tools for early diagnosis, prevention, and treatments both for human and canine patients.

	Human	Canine (NCD)	Canine (CD)
Vertebral formula	C 7; T12; L5; S5	C 7; T13; L 7; S 3; Cd variable	
Most commonly affected IVDs	C5-C7 L4-S1	C5-T1 L6-S1	C2-C3 T12-L1

Table 1: Comparative vertebral anatomy and IVDD

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Chapter 3: Development of a Translational Model for Intervertebral Disc Disease Using Canine Explants

Introduction

Intervertebral disc disorders (IVDD) have been implicated in diseases of the spine that result in decreased function and quality of life.[1] Most often, IVDD manifests as acute or chronic spontaneously occurring neck or back pain with sensory and/or motor dysfunctions associated with affected spinal segments. Mechanisms for development and progression of IVDD are not fully delineated although aging, injury, inflammation, genetics, diabetes, trauma, overuse, body habitus, and mechanical stress have been suggested as contributing factors.[2–8] IVDD is also a common and significant health concern in dogs, affecting about 2% of the canine population. In certain breeds (chondrodystrophic (CD) dogs), incidence of IVDD is much higher.[9,10] For example, IVDD has been reported to affect approximately 25% of Dachshunds.[11] Canine IVDD occurs most commonly in the thoracolumbar (TL) region, especially for CD breeds, however, cervical and lumbosacral (LS) IVDD are also noted.[12] Non-chondrodystrophic (NCD) dogs, most often suffer from chronic back pain associated with caudal lumbar and/or LS IVDD that typically presents later in life.[13,14] These differences in regional predilections have been speculated to be due to conformational differences between NCD and CD breeds[15,16], however, precise biomechanical mechanisms for these regional differences have not been elucidated. In general, canine IVDD leads to functional impairment, nociception, and if not successfully managed, may ultimately necessitate premature

euthanasia due to decreased quality of life.[17,18] Thus, it is important to understand the biology and pathology of IVDs to predict and prevent the development and progression of IVD degeneration.

The current treatment options for canine IVDD are similar to strategies used in the human counterpart. Importantly, either conservative/medical approaches (e.g., cage rest, modified physical activities and/or rehabilitation methods with analgesic and anti-inflammatory medications) nor surgical interventions (e.g., decompression, discectomy, fusion)[19] fully restore the function or structural integrity of degenerative IVDs. Furthermore, current diagnostic modalities are not able to elucidate disease mechanisms or predict progression of IVDD and recovery, leaving many veterinarians and owners frustrated and ill-equipped to optimally manage these canine patients. This lack of correlation for imaging findings with disease mechanisms and clinical progression of IVDD also holds true for human patients. [20–22] A major reason for this disconnect appears to be related to timing and nature of the metabolic processes that drive IVD degeneration, which begin at an early age in most canine and human patients but do not result in symptoms of neck or back pain until middle to older age.[23] Based on these challenges, it is vital to develop and validate canine ex vivo models that are capable of characterizing the earliest changes involved in IVD degeneration for translational application to symptomatic IVDD in both species.

By developing in vitro culture models, investigating mechanisms of disc degeneration can be performed in a controlled manner.[24] Monolayer cell culture models are useful for isolating cellular mechanisms of disease, but lack capabilities for delineating important

cell phenotype and extracellular matrix (ECM) components for IVDD. By using tissue culture, cell distribution and differentiation and ECM composition and structure can be maintained and assessed.[25] Canine tissue culture models have not yet been fully explored to understand basal production of key signaling mechanisms and responses to clinically relevant stimuli.[26,27] As such, there is significant need to develop and validate ex vivo canine IVD tissue culture models in order to comprehensively characterize roles for biomarkers released from IVD tissues in contributing to autocrine, paracrine, and endocrine mechanisms of IVD health and disease.[28] With a validated culture model, IVD degeneration pathogenesis and evaluation of treatments to address IVD degeneration can be characterized and translated to clinical application in dogs and humans.

Translational research using culture models to inform preclinical animal models is an ethical and efficient pathway to safe and effective clinical applications in human and veterinary patients. Dogs have been extensively explored as a preclinical model for human IVD degeneration with validated Thompson (gross evaluations) and Pfirrmann (MRI evaluations) grades.[29] Based on anatomical and clinical similarities in development of IVDD for dogs and humans, preclinical canine models can provide highly relevant translational evidence for application to human IVDD.[13,30,31] In conjunction with similarities in cell populations, ECM composition and structure, and mechanical loading properties for canine and human IVDs[12,13,32,33], it is highly desirable to establish a canine culture model that is valid for foundational translational research.

The overarching hypothesis for this chapter is that the healthy NCD and CD IVD tissues would show similar biomarker activities. In order to test this hypothesis, the following

specific aims were performed in this chapter: 1) compare biomarkers produced by cervical and lumbar IVDs from NCD and CD dogs during an initial culture period, 2) compare biomarkers produced by different tissue types (AF or NP) and culture systems (monoculture of AF or NP and co-culture of AF and NP) of IVD tissues from NCD and CD dogs, 3) compare biomarkers produced by the control and cytokine stimulated IVD tissues from NCD and CD dogs, and ultimately 4) compare the levels of biomarkers from NCD and CD dog IVD tissues (AF and NP).

Materials and Methods

Preparation of IVD explants

With Institutional Animal Care and Use Committee (IACUC) approval (MU ACUC #9163 & #9164), lumbar and cervical IVDs were aseptically recovered from skeletally mature (age ranged one to four years) and female chondrodystrophic (n=7) and non-chondrodystrophic (n=15) dogs without history of neck or back pain were euthanized for reasons unrelated to this study. All isolated IVDs were examined for evidence of IVD degeneration, and grossly normal (NP: clear to opaque, soft, discretely round gel; AF: intact layers of rings without fissures or neovascularization) IVDs were selected for study. AF and NP explants were created using 6 mm sterile dermal biopsy punches. The tissue explants were cultured as mono- (AF or NP separately; AF and NP) or co-culture (AF and NP explants together; CO) in supplemented DMEM at 37°C and 5% CO2 with or without 10 ng/ml of canine recombinant IL-1β for 21 days of culture. Media were

changed every 3 days and collected on days 3, 9, 15, and 21 for biomarker analyses as described below.

Media biomarker assay

Media were tested for MMP-1, MMP-2, MMP-3, KC, MCP-1, IL-4, IL-6, IL-8, IL-10, and IL-18 using commercially available assays according to the manufacturer's protocol.

Statistical analysis

The resulting data was tested for normality using Shapiro-Wilk test in R. Significant differences between the control and cytokine stimulated metabolism between the tissue types (AF, NP, and CO) were determined using Kruskal-Wallis test followed by Dunn's test with Benjamini-Hochberg adjustments and Wilcoxon test was used for pairwise comparisons with FSA and rstatix packages in R. Statistical significance was set at $p \leq 0.05$.

Results

Aim 1: Characterization of biomarker differences between cervical and lumbar regions during the initial culture period

NCD AF tissues produced detectable levels of IL-6, IL-8, KC, MCP-1, MMP-1, MMP-2 and MMP-3. NCD NP tissues produced detectable levels of IL-8, KC, MCP-1, and MMP-2. For both AF and NP, IL-10 levels were near the detection limit of the assay and was not included in the analysis. For AF, MMP-2 levels were low and near the detection limit of the assay.

NCD dogs: The biomarkers (Fig. 3-1) produced by AF tissues from cervical and lumbar IVDs did not show significant differences between cervical and lumbar regions except for production of IL-6 in the control group in the initial culture period (day 3). There were no significant differences between cervical and lumbar NP tissues (Fig. 3-2).

CD dogs: The biomarkers (Fig. 3-3) produced by AF tissues from cervical and lumbar IVDs did not show significant differences between the cervical and lumbar regions except for production of MMP-1 in the control group in the initial culture period (day 3). The biomarkers (Fig. 3-4) produced by NP tissues from cervical and lumbar IVDs did not show significant differences between the cervical and lumbar regions.

Aim 2: Comparisons of biomarkers produced by IVD tissue types and culture systems

NCD dogs cervical IVD explants (Figs. 3-5 and 3-6): IL-6 and MMP-3 concentrations for AF and CO were significantly higher than NP on day 3. KC and MCP-1 concentrations for CO were significantly higher than AF or NP on day 3. With IL-1β stimulation, IL-6 concentrations were significantly higher in AF and CO compared to NP on day 3 and significantly higher in AF compared to NP on day 21. IL-8 and MCP-1 concentrations were significantly higher in AF compared to NP on days 15 and 21. MMP-1 production by AF was significantly higher compared to NP on day 3. MMP-2 production by NP was significantly higher compared to AF on days 3, 15, and 21 and MMP-2 production by CO was significantly higher than AF on day 21.

NCD dog lumbar IVD explants (Figs. 3-7 and 3-8): IL-6 concentrations were significantly higher for AF and CO compared to NP on day 3 and significantly higher for CO compared

to NP on day 9. IL-8 production was significantly higher by CO compared to AF or NP on day 3 and IL-8 production by CO was significantly higher compared to AF on day 9. KC production was significantly higher by CO compared to AF or NP on days 3 and 9. MCP-1 production by CO was significantly higher compared to AF or NP on day 3. MMP-1 concentrations for AF and CO were significantly higher compared to NP on day 3 and MMP-1 production by CO was significantly higher than NP on day 9. MMP-3 concentrations for AF and CO were significantly higher compared to NP on all time points. With IL-1β stimulation, IL-6 and MCP-1 concentrations for AF were significantly higher compared to NP on day 3.

CD dog cervical explants (Figs. 3-9 and 3-10): IL-6 production by CO was significantly higher compared to NP on day 3. With IL-1β stimulation, IL-6 production by AF was significantly higher than NP on day 3.

CD dog lumbar explants (Figs. 3-11 and 3-12): IL-6 concentrations for AF and CO were significantly higher compared to NP on day 3 and production by CO was significantly higher compared to NP on day 9. IL-8 production by CO was significantly higher compared to AF or NP on day 3 and AF on day 9. KC production by CO was significantly higher compared to AF or NP on days 3 and 9. MCP-1 production by CO was significantly higher compared to AF or NP on day 3. MMP-3 concentrations for AF and CO were significantly higher compared to NP on all time points. With IL-1β stimulation, there were no significant differences in biomarker productions noted.

Aim 3: Comparisons of biomarkers released by control and cytokine stimulated groups

Monoculture of cervical AF tissues from NCD dogs (Fig. 3-13): IL-6, IL-8, KC and MMP-1 concentrations were significantly higher in the cytokine stimulated group on day 3.

Monoculture of cervical NP tissues from NCD dogs (Fig. 3-14): IL-6 and MCP-1 productions were significantly higher in the cytokine stimulated group on days 9, 15, and 21. IL-8 concentration was significantly higher in the cytokine stimulated group on day 3, 9, 15, and 21. KC production was significantly higher in the cytokine stimulated group on day 3, 9, and 15.

Co-culture of cervical AF and NP from NCD dogs (Fig. 3-15): IL-6 concentration was significantly higher in the cytokine stimulated group on days 3, 9, and 21. IL-8, KC, and MCP-1 concentrations were significantly higher in the cytokine stimulated group on days 3 and 9. MMP-3 concentration was significantly higher in the cytokine stimulated group on day 21.

Monoculture of lumbar AF tissues from NCD dogs (Fig. 3-16): IL-10 levels were below the detectable assay limits. IL-6 and MCP-1 concentrations were significantly higher in the cytokine stimulated groups on day 3. IL-8 and KC concentrations were significantly higher in the cytokine stimulated groups on days 3 and 9.

Monoculture of lumbar NP tissues from NCD dogs (Fig. 3-17): IL-10, MMP-1, and MMP-3 levels were below the detectable assay limits. No significant differences between control and cytokine groups were detected in the tested analysts.

Co-culture of lumbar AF and NP tissues from NCD dogs (Fig. 3-18): IL-10 levels were below the detection limit of the assay. IL-8 and MCP-1 concentrations were significantly higher in the cytokine stimulated groups on day 3.

Monoculture of cervical AF tissues from CD dogs (Fig. 3-19): IL-10 levels were below the detectable assay limits. IL-8 production was significantly higher in the cytokine stimulated group on day 3.

Monoculture of cervical NP tissues from CD dogs (Fig. 3-20): IL-10, MMP-1, and MMP-3 levels were below the detectable assay limits. IL-8 and KC concentrations were significantly higher in the cytokine stimulated group on day 3.

Co-culture of cervical AF and NP tissues from CD dogs (Fig. 3-21): IL-10 levels were below the detection limit of the assay. No significance difference between the control and cytokines stimulated groups were found for all detectable biomarkers throughout the 21-day culture period.

Monoculture of lumbar AF tissues from CD dogs (Fig. 3-22): IL-10 levels were below the detection limit of the assay. IL-8, KC, and MMP-1 concentrations were significantly higher in the cytokine stimulated groups on day 3.

Monoculture of lumbar NP tissues from CD dogs (Fig. 3-23): IL-10, MMP-1, and MMP-3 levels were below the detection limit of the assay. IL-6 levels were significantly higher in the cytokine stimulated group on days 3, 9, and 15. KC production levels were significantly higher in the cytokine stimulated group on days 9, 15, and 21. MCP-1 production was significantly higher in the cytokine stimulated group on day 15.

Co-culture of lumbar AF and NP tissues from CD dogs (Fig. 3-24): IL-10 level was below the detection limit of the assay. IL-6 and MMP-3 levels were significantly higher in the cytokine stimulated groups on day 21.

Aim 4: Comparisons of biomarkers produced by CD and NCD dogs

Comparisons of biomarker production by cervical AF from CD and NCD dogs (Fig. 3-25): MMP-2 levels were below the detection limit of the assay. IL-6 production was significantly higher in the AF tissues from CD dogs compared to NCD dogs on days 3 and 21. MCP-1 production was significantly higher in the AF tissues from CD dogs compared to those of NCD dogs on day 15. MMP-1 and MMP-3 concentrations were significantly higher in the AF tissues of CD dogs compared to those of NCD dogs on day 3. MMP-2 production was significantly higher in the AF tissues of CD dogs compared to NCD dogs on days 15 and 21.

Comparisons of biomarker production by cervical NP from CD and NCD dogs (Fig. 3-26): IL-6 levels were below the detection limit of the assay. IL-8, MCP-1, and MMP-3 concentrations were significantly higher in the NP tissues of CD dogs compared to those of NCD dogs on day 3.

Comparisons of biomarker production by cervical AF from CD and NCD dogs with IL-1β stimulation (Fig. 3-27): MMP-2 levels were below the detection limit of the assay. IL-6, KC, and MCP-1 concentrations were significantly higher in the AF tissues of CD dogs compared to those of NCD dogs with cytokine stimulation on days 15 and 21. IL-8 production was significantly higher in the AF tissues of CD dogs compared to those of

NCD dogs with cytokine stimulation on day 21. MMP-1 production was significantly higher in the AF tissues of CD dogs compared to NCD dogs with cytokine stimulation on days 3, 15, and 21. MMP-3 production was significantly higher in the AF tissues of CD dogs compared to NCD dogs with cytokine stimulation on day 3.

Comparisons of biomarker production by cervical NP from CD and NCD dogs with IL-1β stimulation (Fig. 3-28): IL-6 levels were below the detection limit of the assay. MCP-1 production was significantly higher in the NP of CD dogs compared to NCD dogs with cytokine stimulation on days 3 and 21. MMP-1 and MMP-3 concentrations were significantly higher in the NP of CD dogs compared to those of NCD dogs with cytokine stimulation on day 3.

Comparisons of biomarker production by lumbar AF from CD and NCD dogs (Fig. 3-29): MMP-1 levels were below the detection limit of the assay. MMP-3 production was significantly higher in the AF tissues of CD dogs compared to NCD dogs on day 3.

Comparisons of biomarker production by lumbar NP from CD and NCD dogs (Fig. 3-30): MMP-1 levels were below the detection limit of the assay. IL-8, MCP-1, and MMP-3 concentrations were significantly higher in the NP tissues of CD dogs compared to those of NCD dogs on day 3.

Comparisons of biomarker production by lumbar AF from CD and NCD dogs with IL-1β stimulation (Fig. 3-31): MMP-2 levels were below the detection limit of the assay. KC production was significantly higher in the AF tissues of NCD dogs compared to CD dogs with cytokine stimulation on day 3. MMP-1 concentrations were significantly higher in

the AF tissues of CD dogs compared to NCD dogs with cytokine stimulation on days 3, 15, and 21. MMP-3 production was significantly higher in the AF tissues of CD dogs compared to NCD dogs with cytokine stimulation on days 3 and 21.

Comparisons of biomarker production by lumbar NP from CD and NCD dogs with IL-1β stimulation (Fig. 3-32): IL-6 levels were below the detection limit of the assay. IL-8 production was significantly higher in the NP tissues of CD dogs compared to NCD dogs with cytokine stimulation on days 3 and 21. KC production was significantly in the NP tissues of CD dogs compared to NCD dogs with cytokine stimulation on all time points. MCP-1 production was significantly higher in the NP tissues of CD dogs compared to NCD dogs with cytokine stimulation on days 3, 15, and 21. MMP-1 and MMP-3 concentrations were significantly higher in the NP tissues of CD dogs compared to those of NCD dogs with cytokine stimulation on day 3.

Discussion

Aim 1: Characterization of biomarker differences between cervical and lumbar regions during the initial culture period

For non-chondrodystrophic dogs, basal biomarker production profiles for AF and NP tissues were not markedly different between cervical and lumbar IVDs except for IL-6 production in the control group. Increased IL-6 has been indicated in IVD degeneration and is thought to have various effects by modulating gene expression and cell survival,

proliferation, and differentiation.[34] This data may indicate lumbar IVDs may undergo early IVD degeneration before cervical IVDs do.

For chondrodystrophic dogs, basal MMP-1 production showed regional differences between cervical and lumbar IVDs with higher MMP-1 production for cervical AF. Interestingly, this significance difference was not maintained in the presence of cytokine stimulation. This may indicate different mechanisms for CD-driven degeneration in the neck versus the low back of dogs whereby cervical IVDD is more AF dependent and lumbar IVDD is primarily NP driven. It was also interesting to note that the inflammatory and degradative biomarker signatures for cervical and lumbar IVDs were quite similar. While these findings are interesting and informative, they must be interpreted with caution at this point as the IVDs that were used in this study were from young adult animals without gross evidence for IVD degeneration and might be significantly different with aging and symptomatic IVDD.

Aim 2: Comparisons of biomarkers produced by IVD tissue types and culture systems

In the AF and NP tissues from NCD dogs, many of the biomarkers decreased in concentrations over the 21-day culture period. While the initial high concentrations and subsequent diminishing levels may have been influenced by the perturbations involved in explant culture, the respective differences between AF and NP noted have important relevance. AF explants had more robust biomarker production while NP tissues had more muted responses. Co-culture of AF and NP appeared to capture the activities of both tissue types without obvious positive or negative feedback between them. This

likely represents the clinical scenario of the intact IVD in which AF and NP both produce cytokines, chemokines, and matrix metalloproteinases (MMP) that drive degeneration, suggesting that this model has translational relevance. Interestingly, MMP-3 production was predominantly from AF while MMP-2 production was predominantly from NP, suggesting tissue-specific ECM remodeling and degradation mechanisms. Biomarker production patterns changed significantly with pro-inflammatory cytokine stimulation as well. Biomarker production levels did not diminish after day 3 of culture for cytokine-stimulated cervical IVDs, but did decrease for cytokine-stimulated lumbar IVDs, which may indicate a lower pro-inflammatory sensitive for lumbar IVDs in NCD dogs. MMP-1 production for AF was enhanced by cytokine stimulation, suggesting that AF has an ability to respond to an inflammatory environment by increasing MMP-1 production.

In CD dogs, biomarker production patterns were similar to those for NCD dogs. AF produced higher levels of biomarkers overall, and co-culture of AF and NP did not produce obvious negative or positive feedback effects. One major difference noted for the chondrodystrophic tissues was the finding that a greater number of biomarkers maintained elevated concentrations throughout the culture period, unlike NCD dogs. Specifically, MCP-1 and MMP-2 stay elevated without cytokine stimulation while most biomarkers from NCD dogs progressively decreased after 3 days in explant culture. With cytokine stimulation, IL-8, KC, MCP-1, and MMP-2 continued to stay elevated in cervical IVDs, but not in cytokine-stimulated lumbar IVDs. It is interesting that biomarker profiles for lumbar IVDs were similar for CD and NCD dogs. This may indicate that the increased rate of lumbar IVD degeneration in CD dogs compared to NCD dogs is not driven by

differences in metabolic responsiveness to insult, injury, or overuse by the IVD tissue, but may be more governed by genetically related differences between CD and NCD dogs.

Aim 3: Comparisons of biomarkers released by control and cytokine stimulated groups

In cervical IVDs of NCD dogs, IL-8, KC, and MMP-1 productions were significantly increased by pro-inflammatory cytokine stimulation. The biomarker production patterns were similar for lumbar IVDs, except that lumbar NP tissues IVDs appeared to be less sensitive to cytokine stimulation.

In CD dogs, significant differences for biomarker profiles between the treatment groups were fewer. While this could be related to the smaller sample size for CD dogs, the data suggest that it is more likely the result of true differences in cytokine responsiveness related to chondrodystrophy.

Aim 4: Comparisons of biomarkers produced by CD and NCD dogs

Basal biomarker production comparisons between breed types revealed significantly higher levels of IL-6, IL-8, MCP-1, MMP-1, MMP-2, and MMP-3 for cervical IVDs from CD dogs. With cytokine stimulation, CD IVDs also produced higher levels of increased KC compared to NCD IVDs. Lumbar IVDs showed fewer significant differences in biomarker production between CD and NCD dogs apart from IL-8, MCP-1, and MMP-3. With cytokine stimulation, more significant differences between CD and NCD dogs in biomarker production profiles were noted. These differences in tissue metabolic responses may contribute to the disparate clinical presentations for IVDD phenotypes

observed for chondrodystrophic versus non-chondrodystrophic breeds of dogs. Lastly, CD dogs appear to maintain pro-inflammatory biomarker production levels with cytokine stimulation throughout the culture period while NCD dogs do not. This could indicate that the IVDs from the CD dogs may be more vulnerable to increased inflammation.

Conclusion

The data from this study elucidate important differences in intervertebral disc biomarker production profiles based on tissue type, anatomical location, pro-inflammatory cytokine stimulation, and breed type. Based on the data, the healthy cervical and lumbar IVDs are similar in terms of biomarker profiles. Between AF and NP, AF appears to have more robust biomarker production and responses to inflammation compared to NP. Co-culture of both AF and NP show activities from the two tissue types, but it makes it more difficult to discern unique biomarker production patterns in each tissue. Overall, biomarker production responses to cytokine stimulation were similar for CD and NCD IVDs, however, there were potentially clinically relevant differences between breed types with respect to basal chemokine and MMP production levels. Taken together, this novel characterization of differences in biomarker profiles provided foundational data for subsequent experiments targeting specific disease mechanisms for IVDD in chondrodystrophic and non-chondrodystrophic breeds of dogs for translational application to canine and human patients across the spectrum of degenerative disc disease phenotypes.

Figures

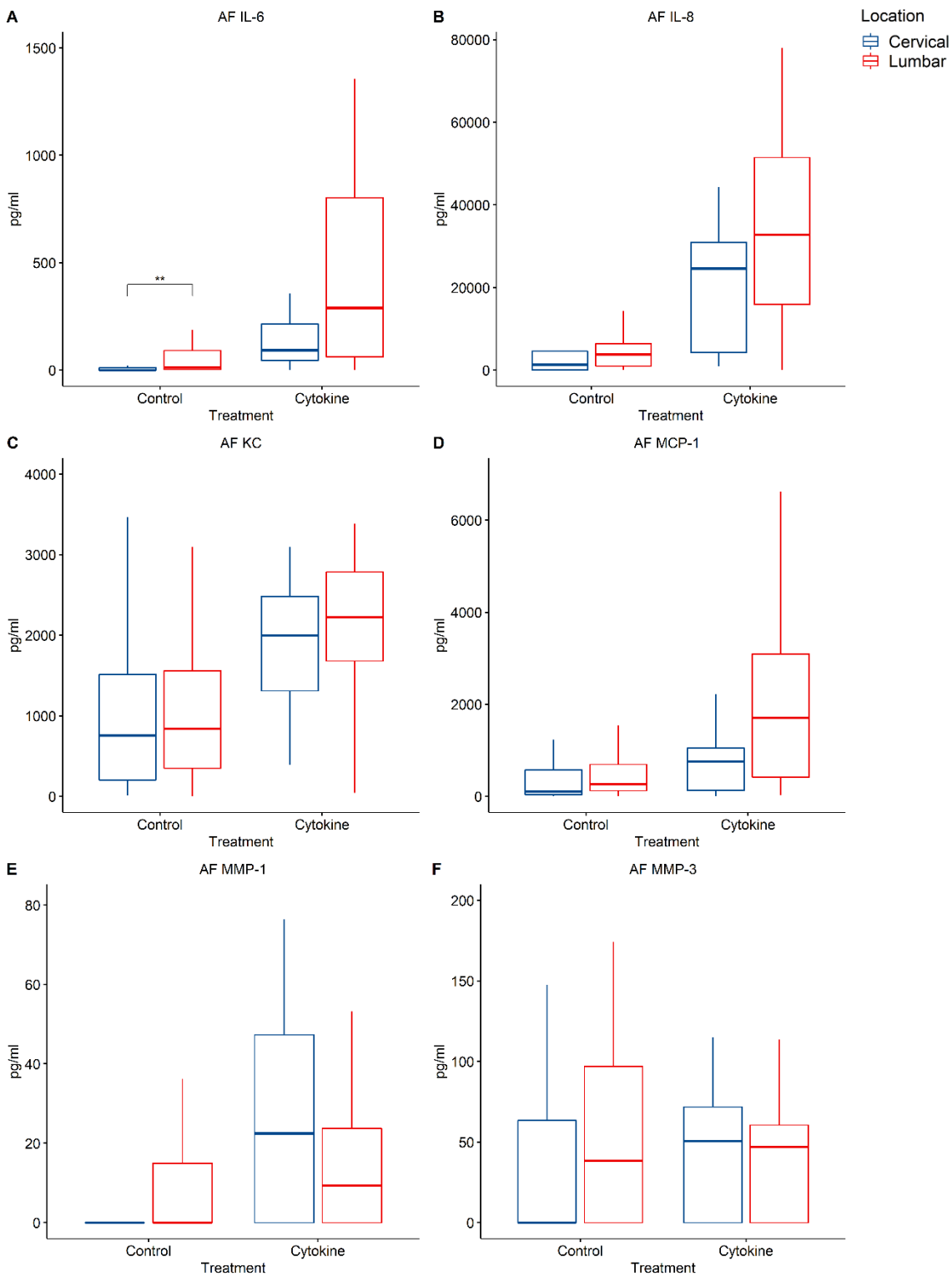

Figure 3-1 NCD AF cervical vs. lumbar regions on day 3

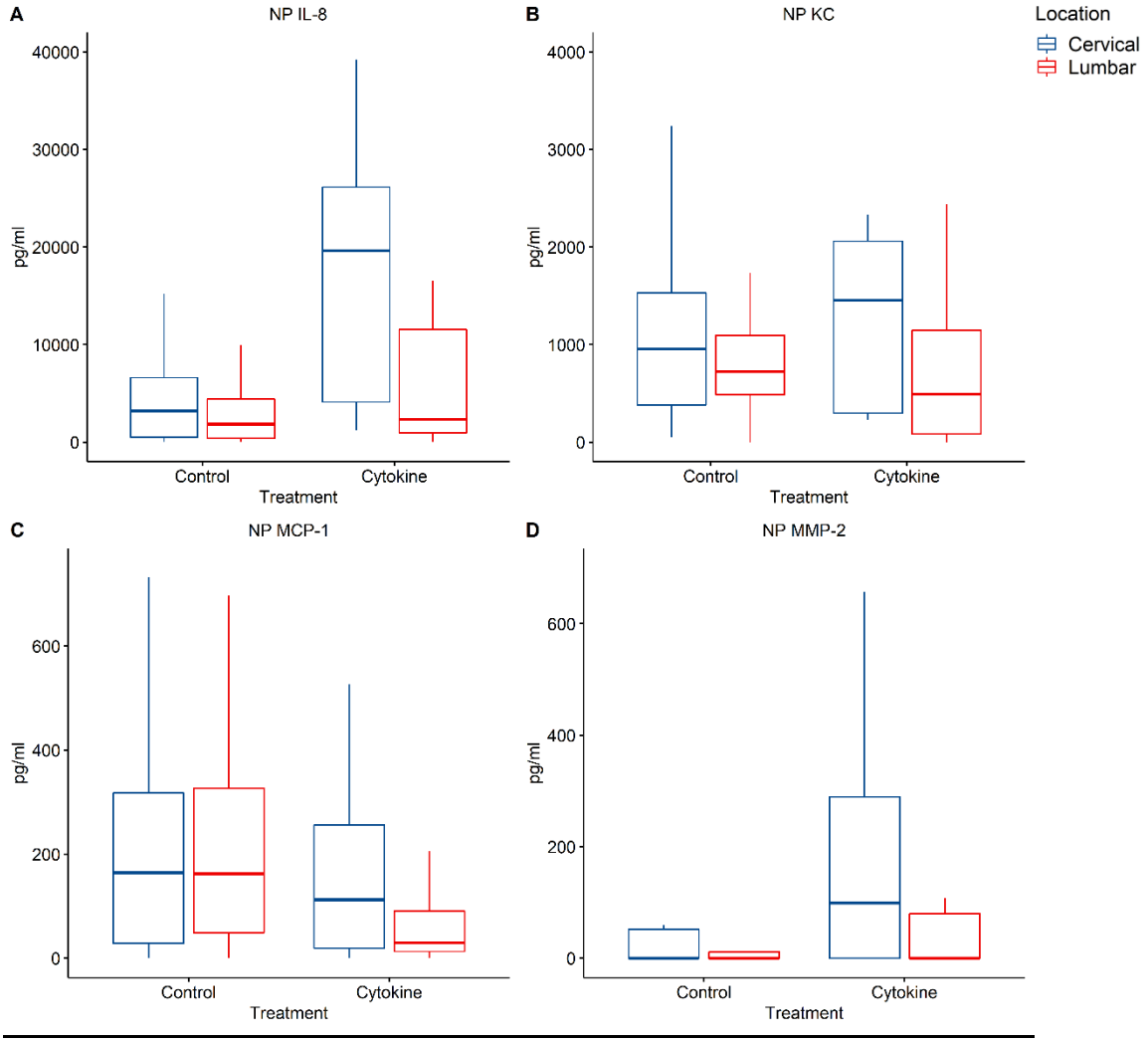

Figure 3-2 NCD NP cervical vs. lumbar regions on day 3

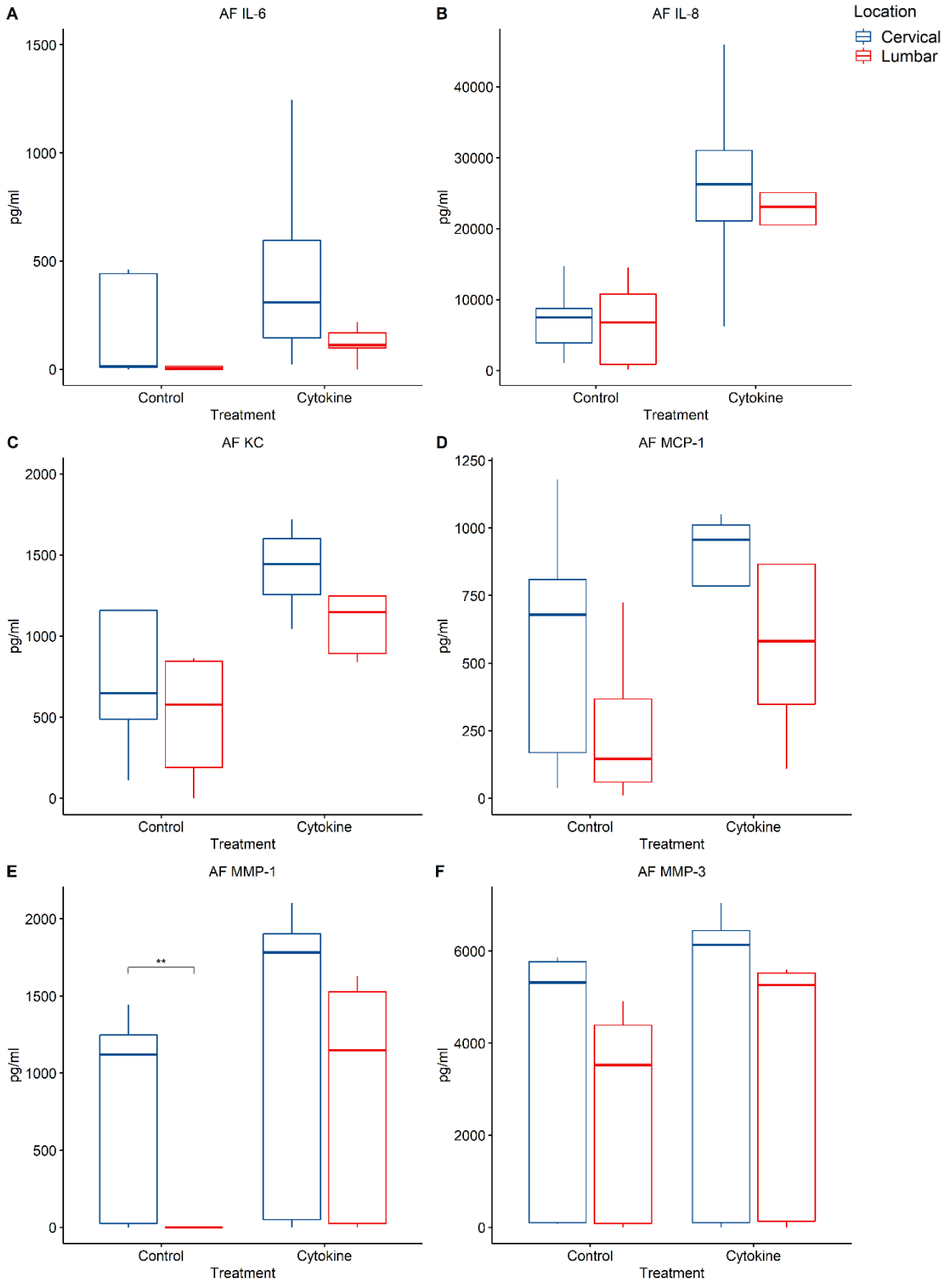

Figure 3-3 CD AF cervical vs. lumbar regions day 3

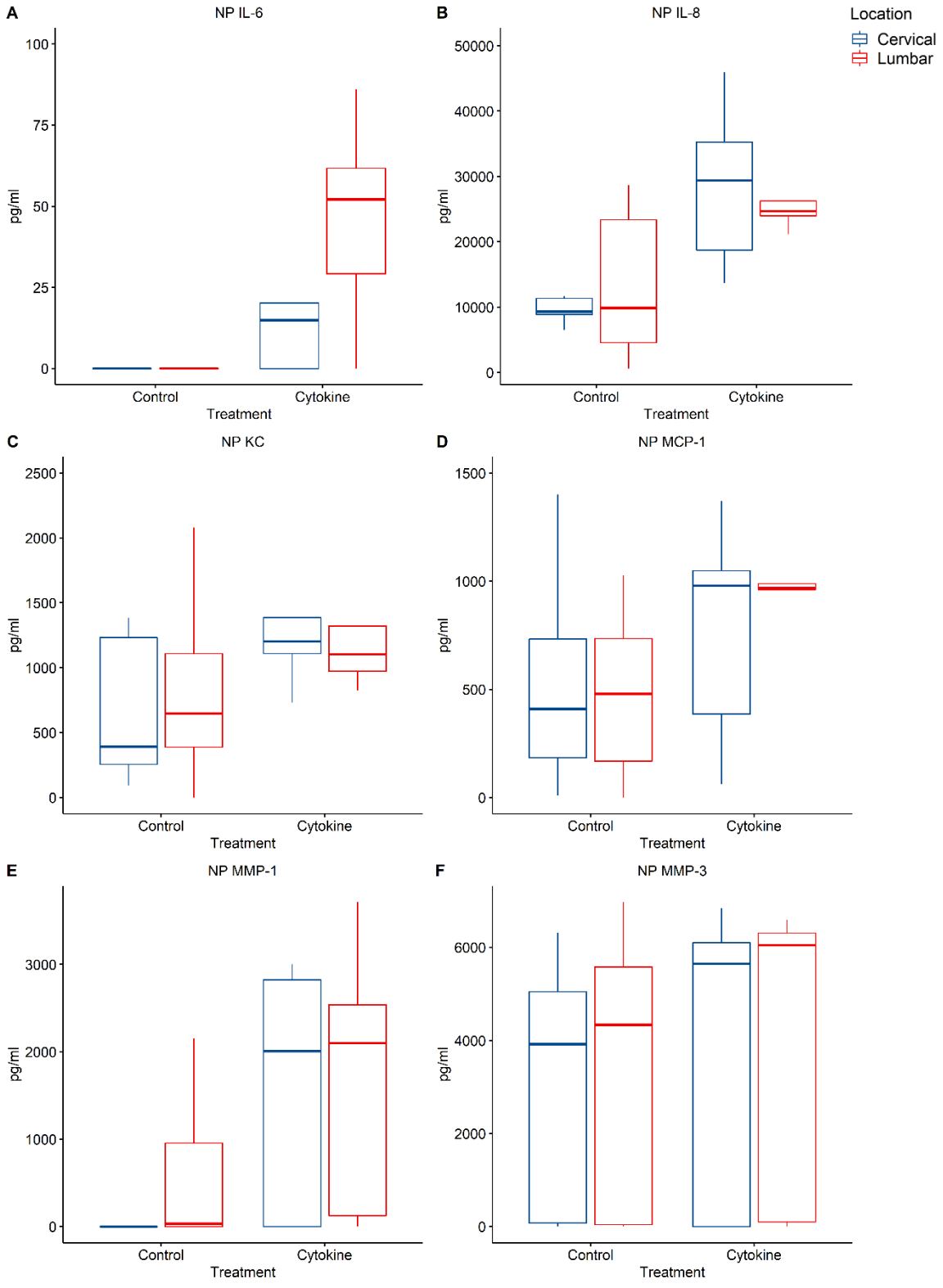

Figure 3-4 CD NP cervical vs. lumbar regions on day 3

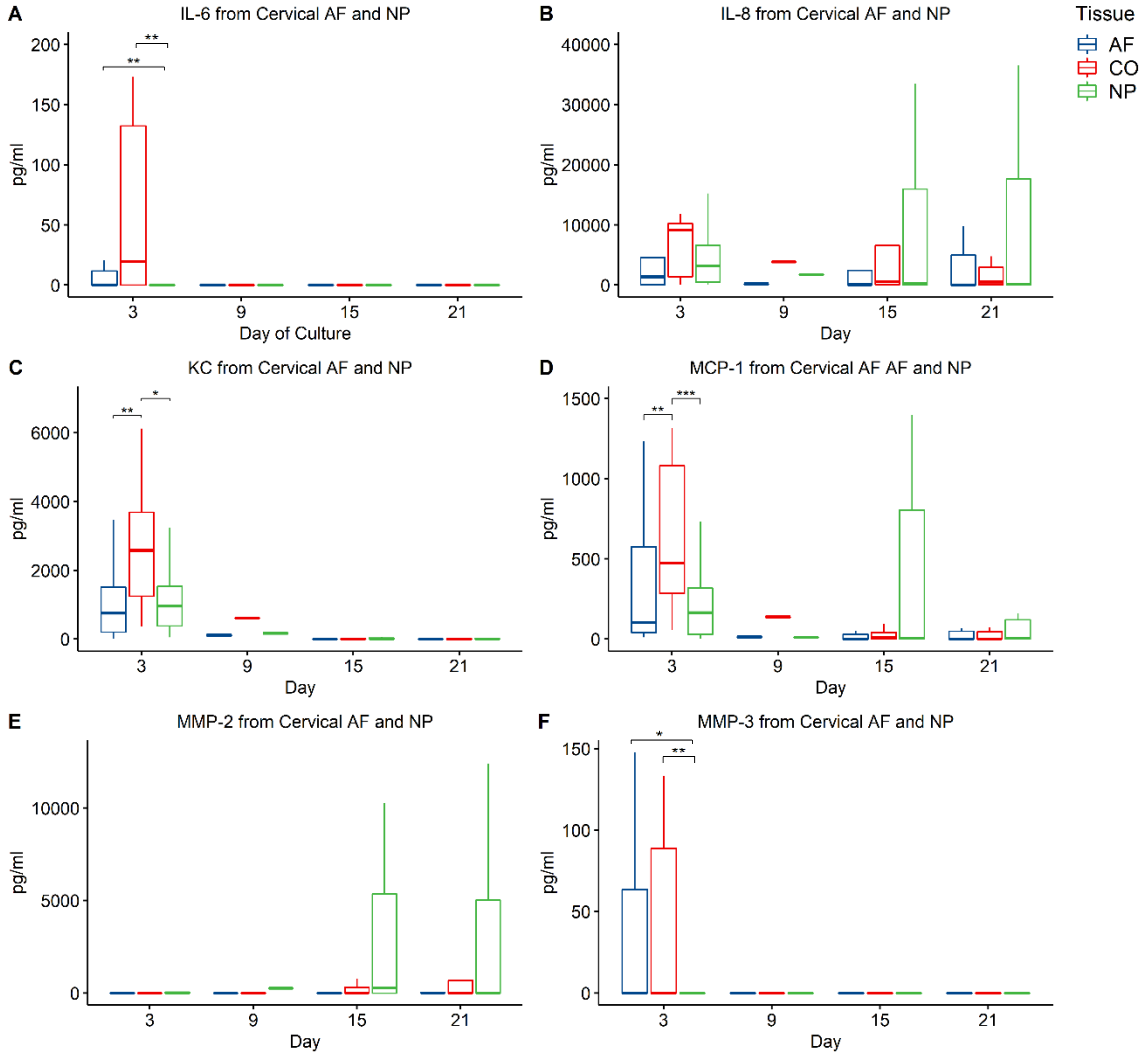

Figure 3-5 NCD cervical mono- and co-culture of AF and NP

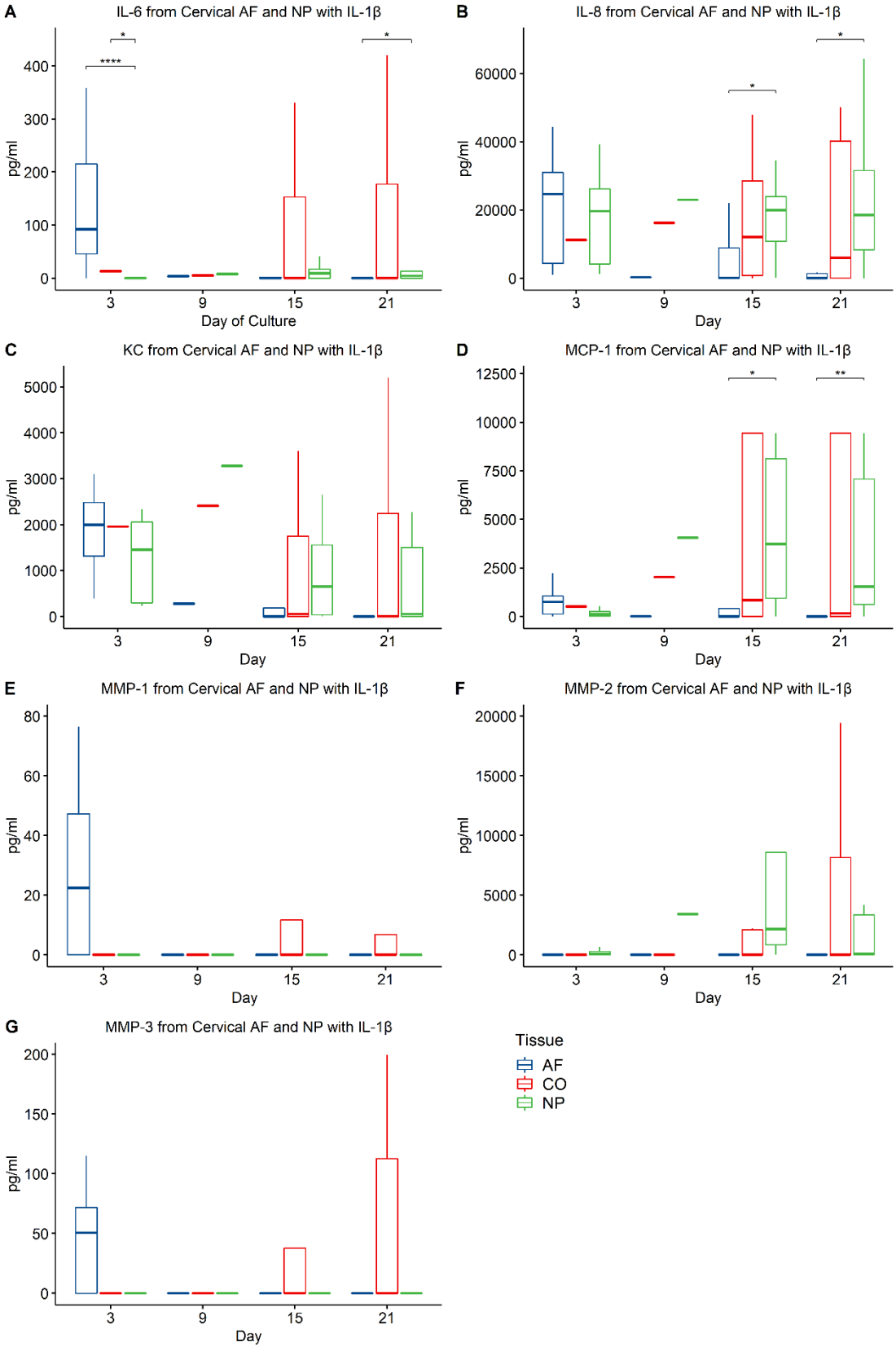

Figure 3-6 NCD cervical mono- and co-culture of AF and NP with IL-1β

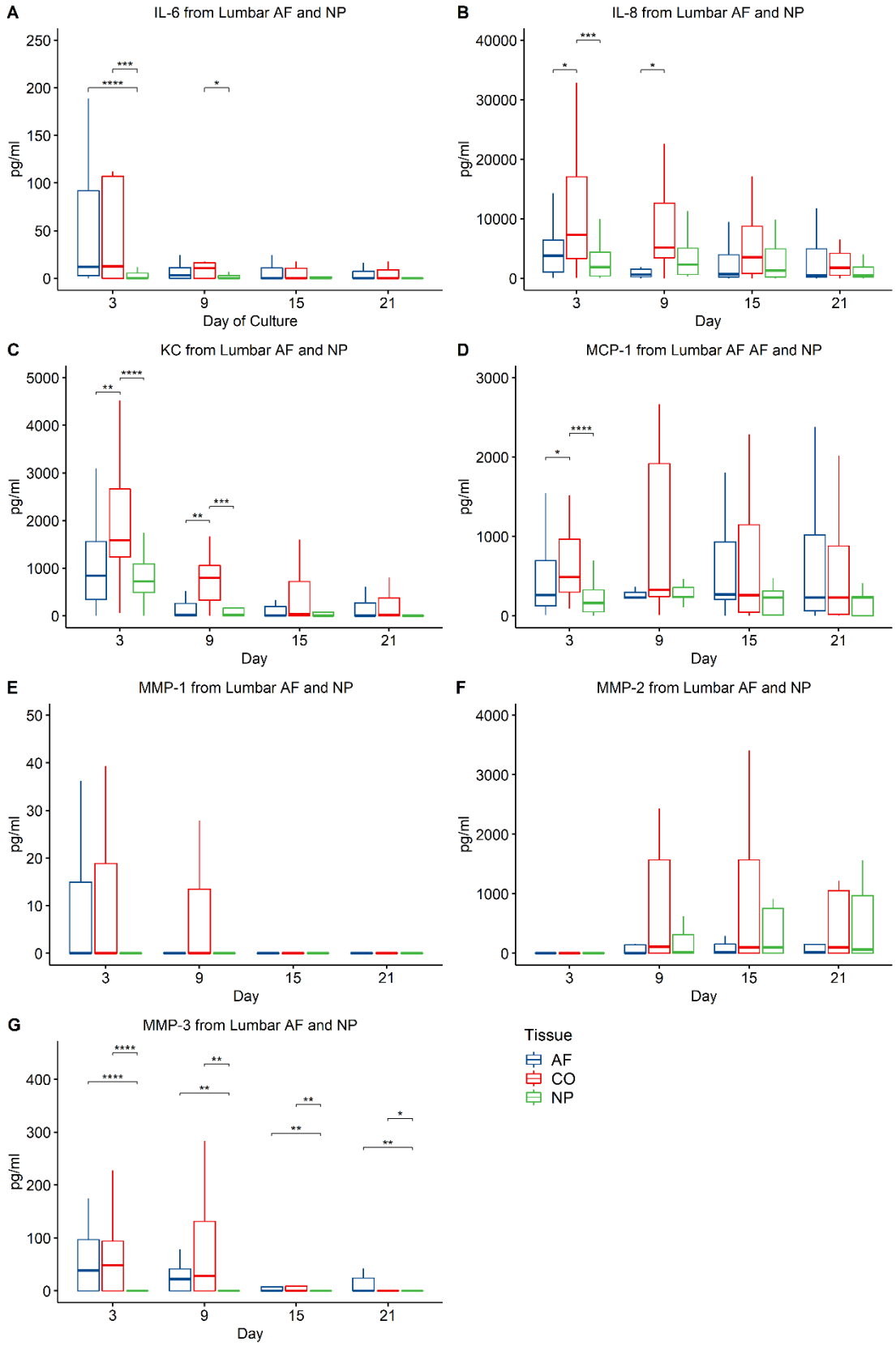

Figure 3-7 NCD lumbar mono- and co-culture of AF and NP

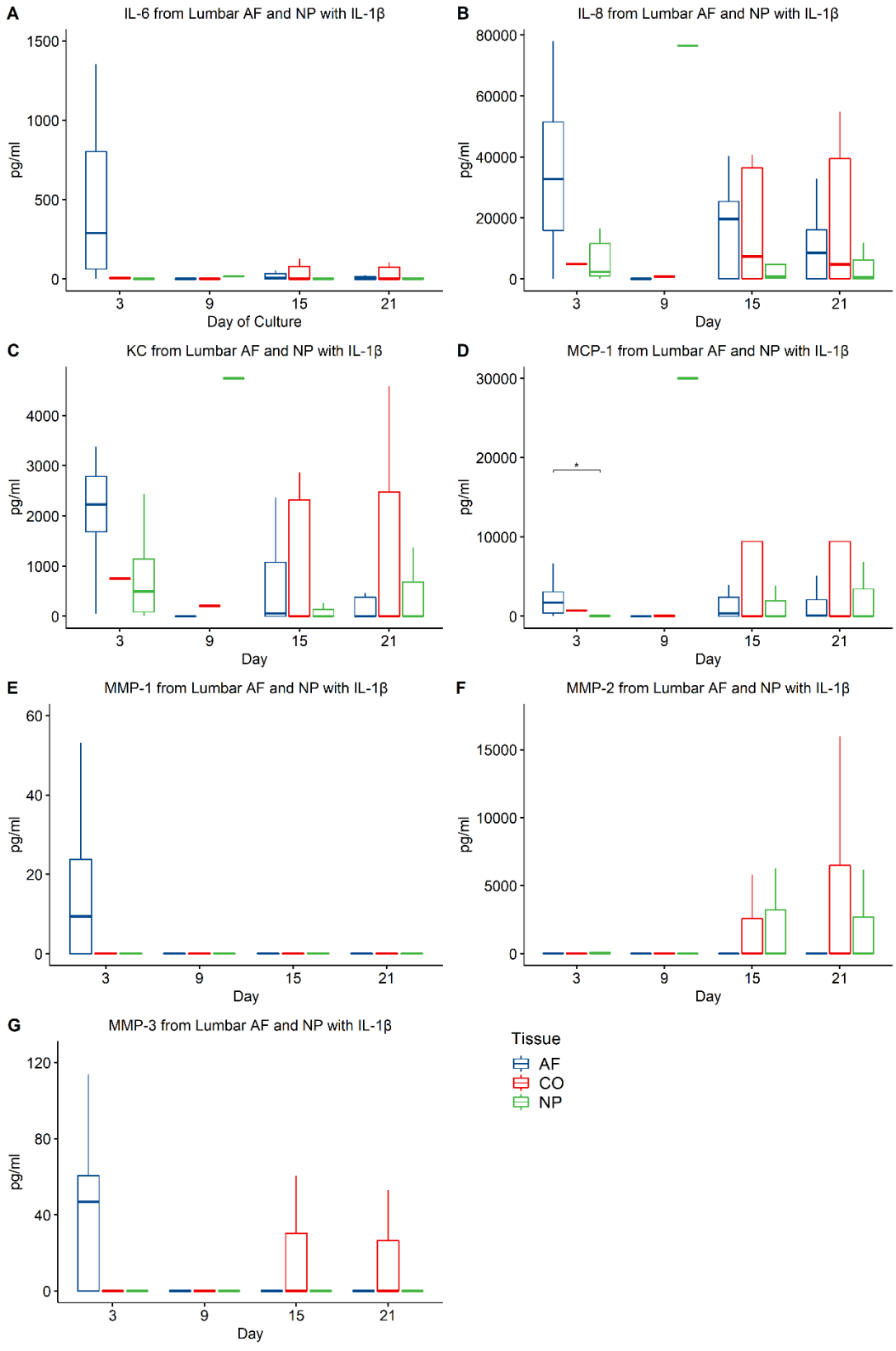

Figure 3-8 NCD lumbar mono- and co-culture of AF and NP with IL-1β

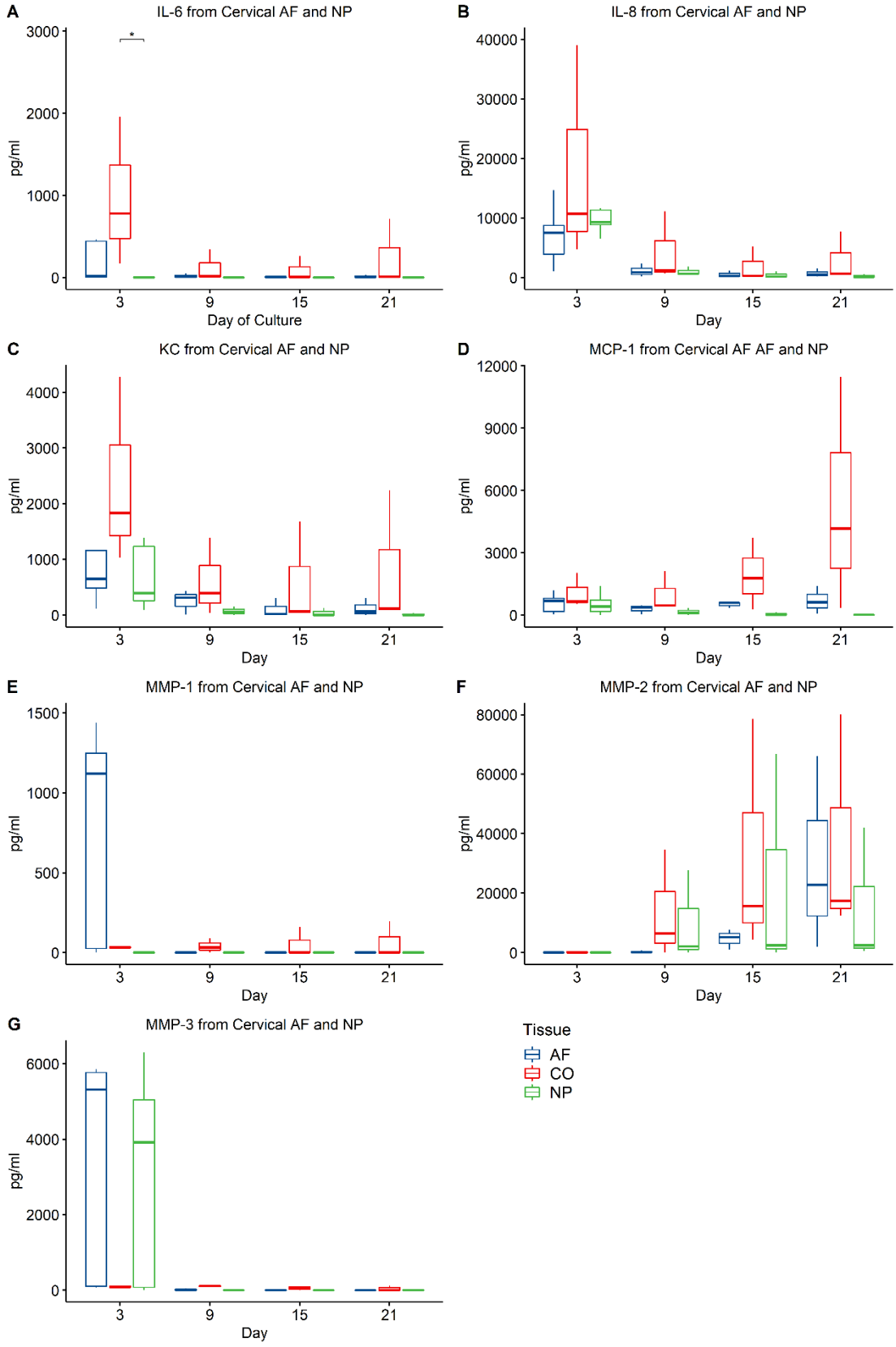

Figure 3-9 CD cervical mono- and co-culture of AF and NP

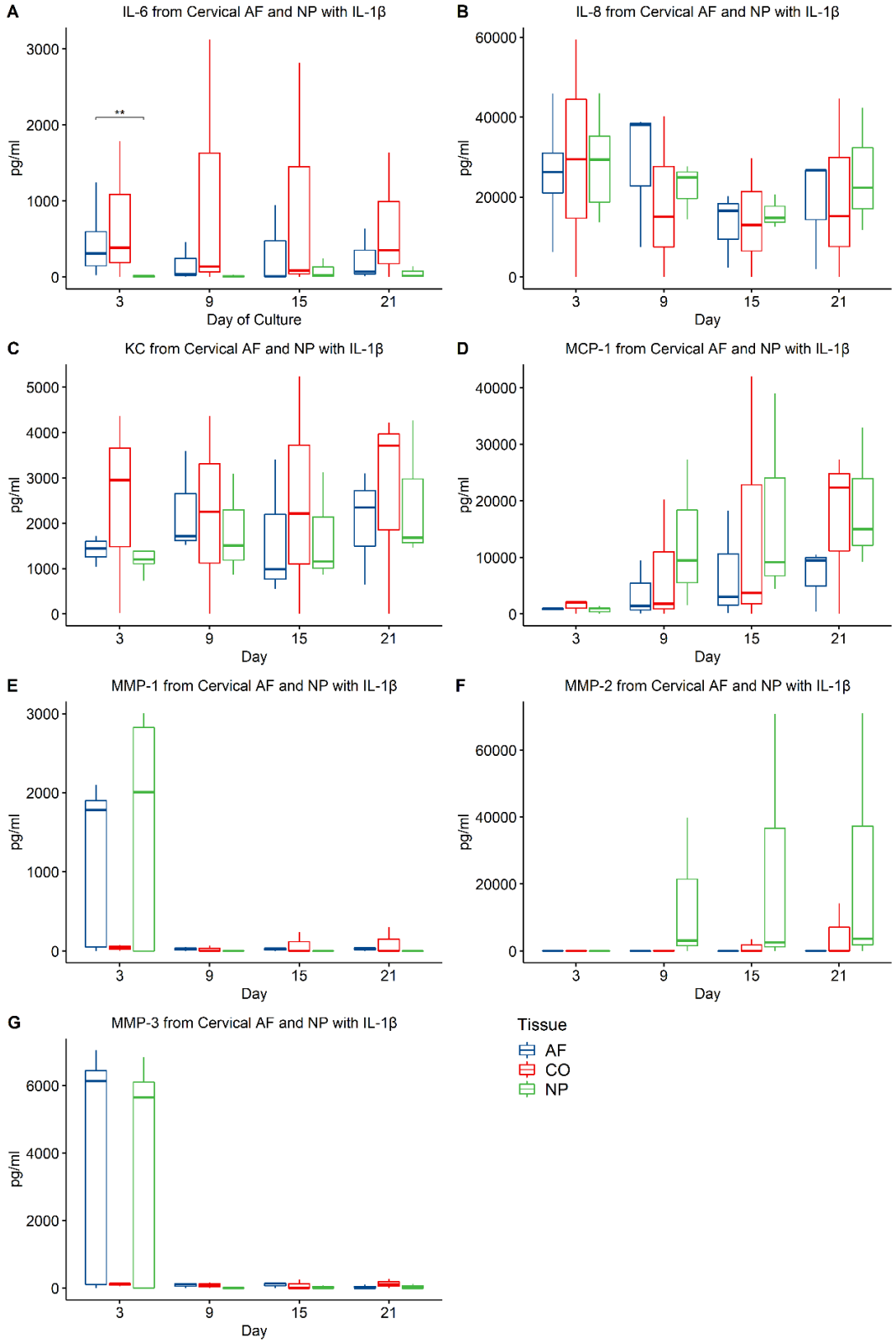

Figure 3-10 CD cervical mono- and co-culture of AF and NP with IL-1β

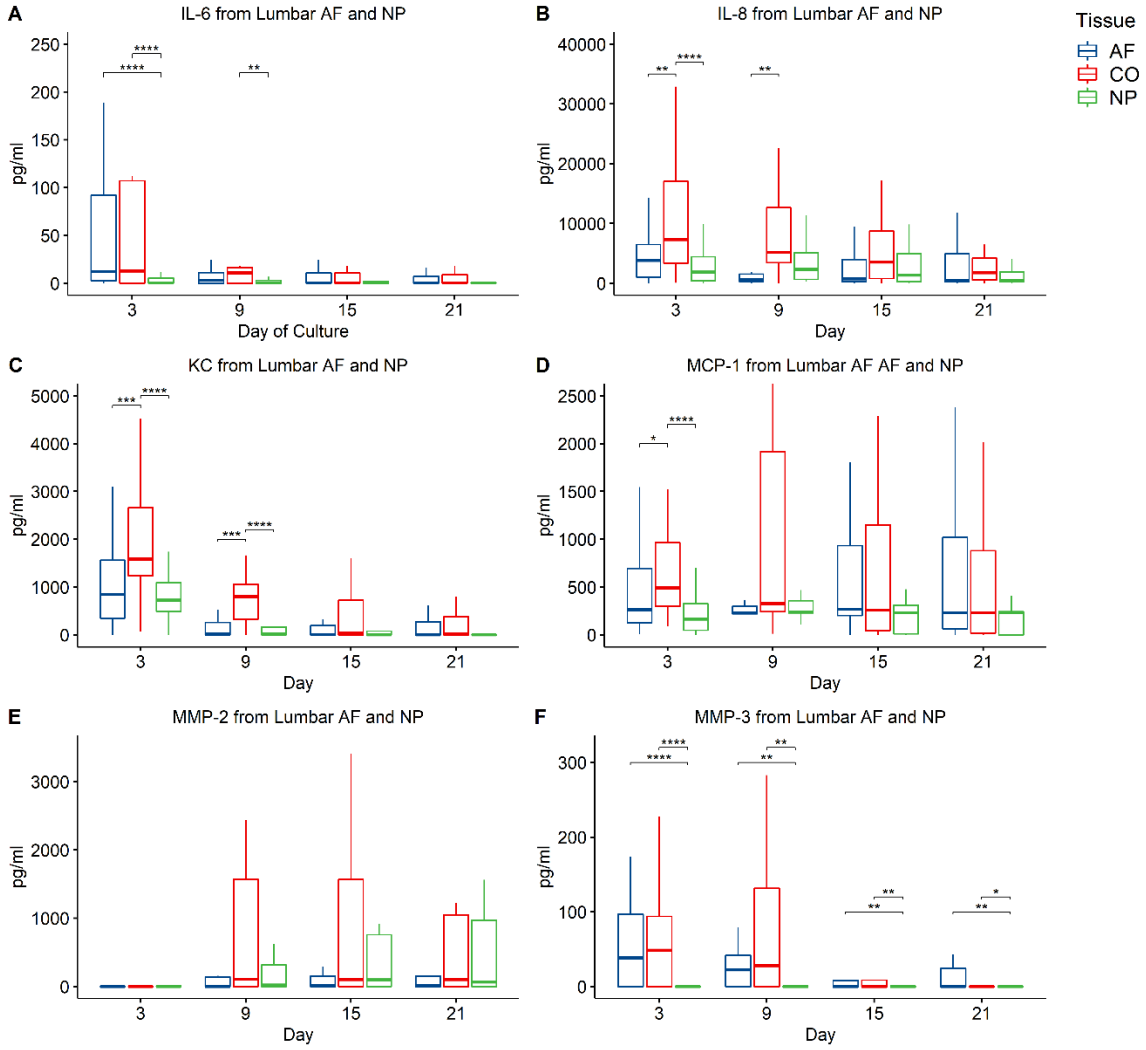

Figure 3-11 CD lumbar mono- and co-culture of AF and NP

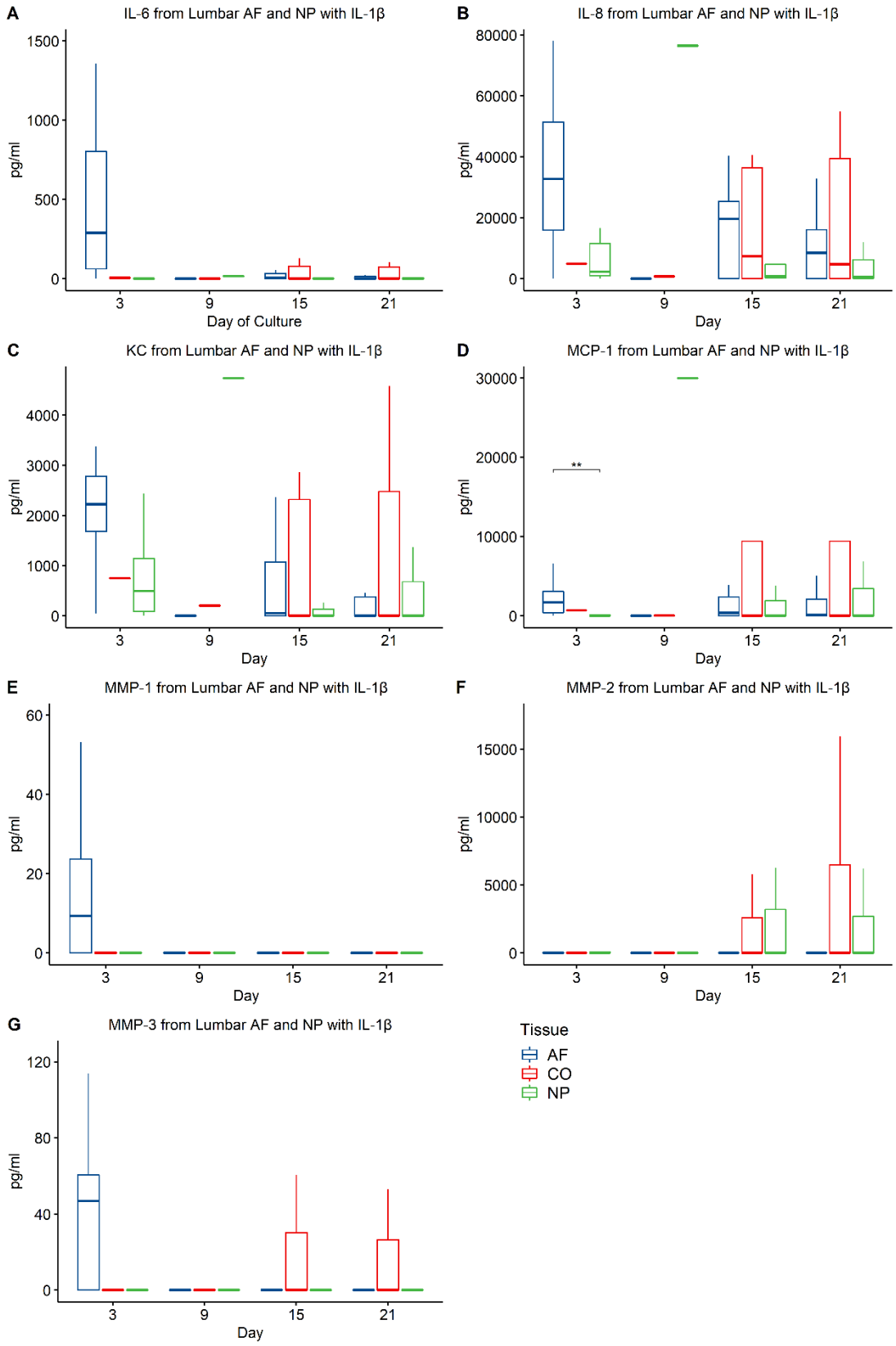

Figure 3-12 CD lumbar mono- and co-culture of AF and NP with IL-1β

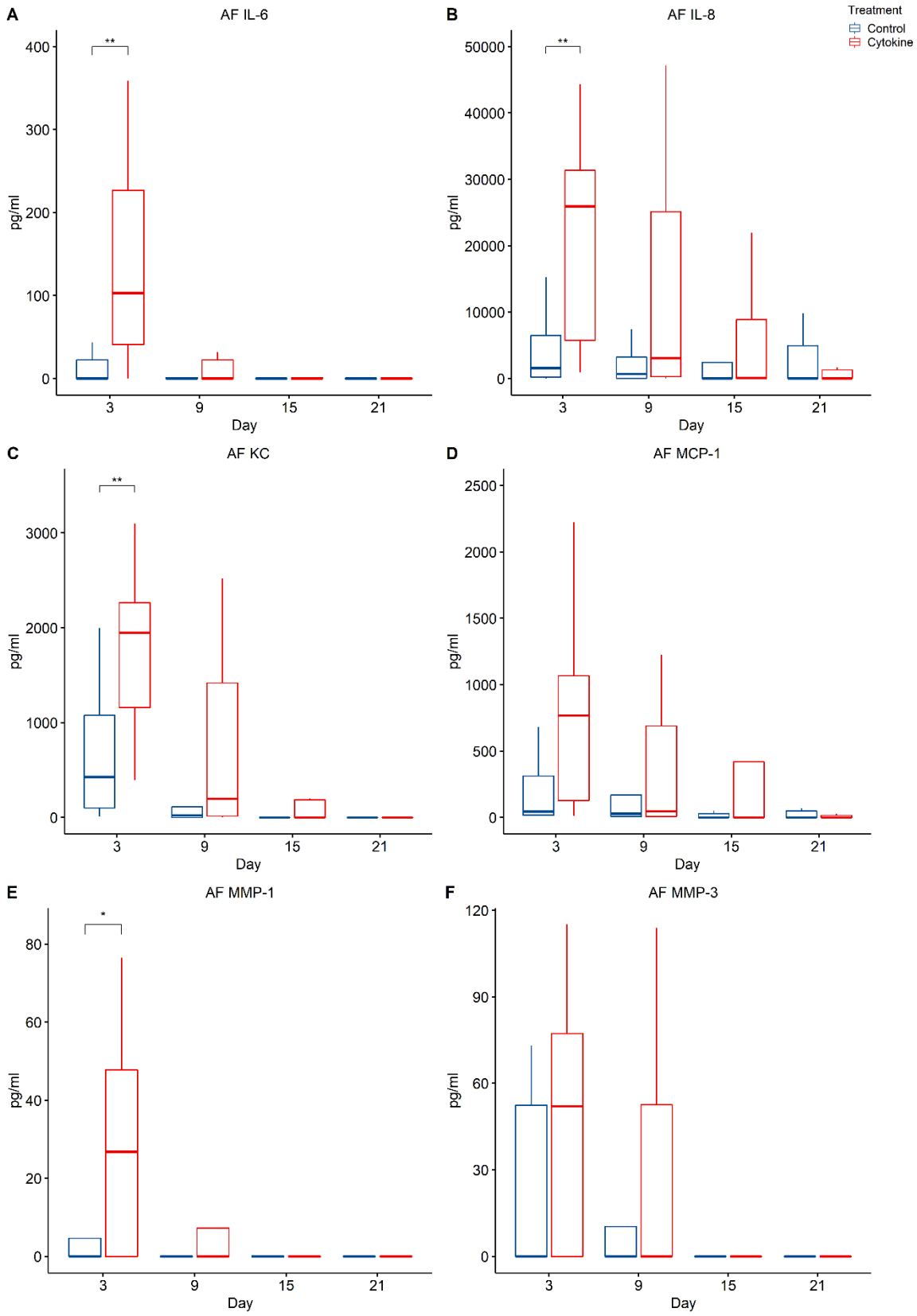

Figure 3-13 NCD AF cervical control vs cytokine over 21 days

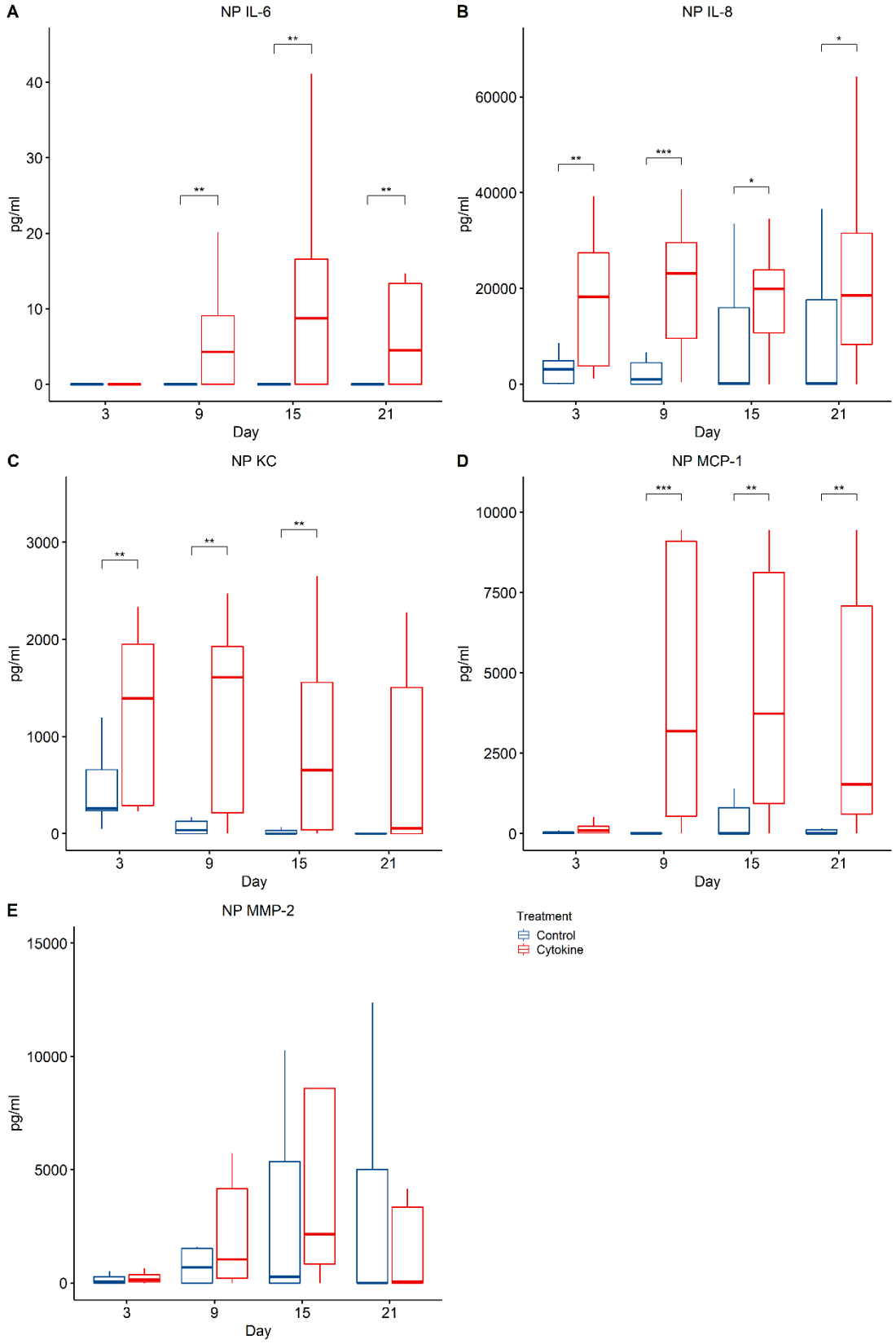

Figure 3-14 NCD NP Cervical control vs cytokine over 21 days

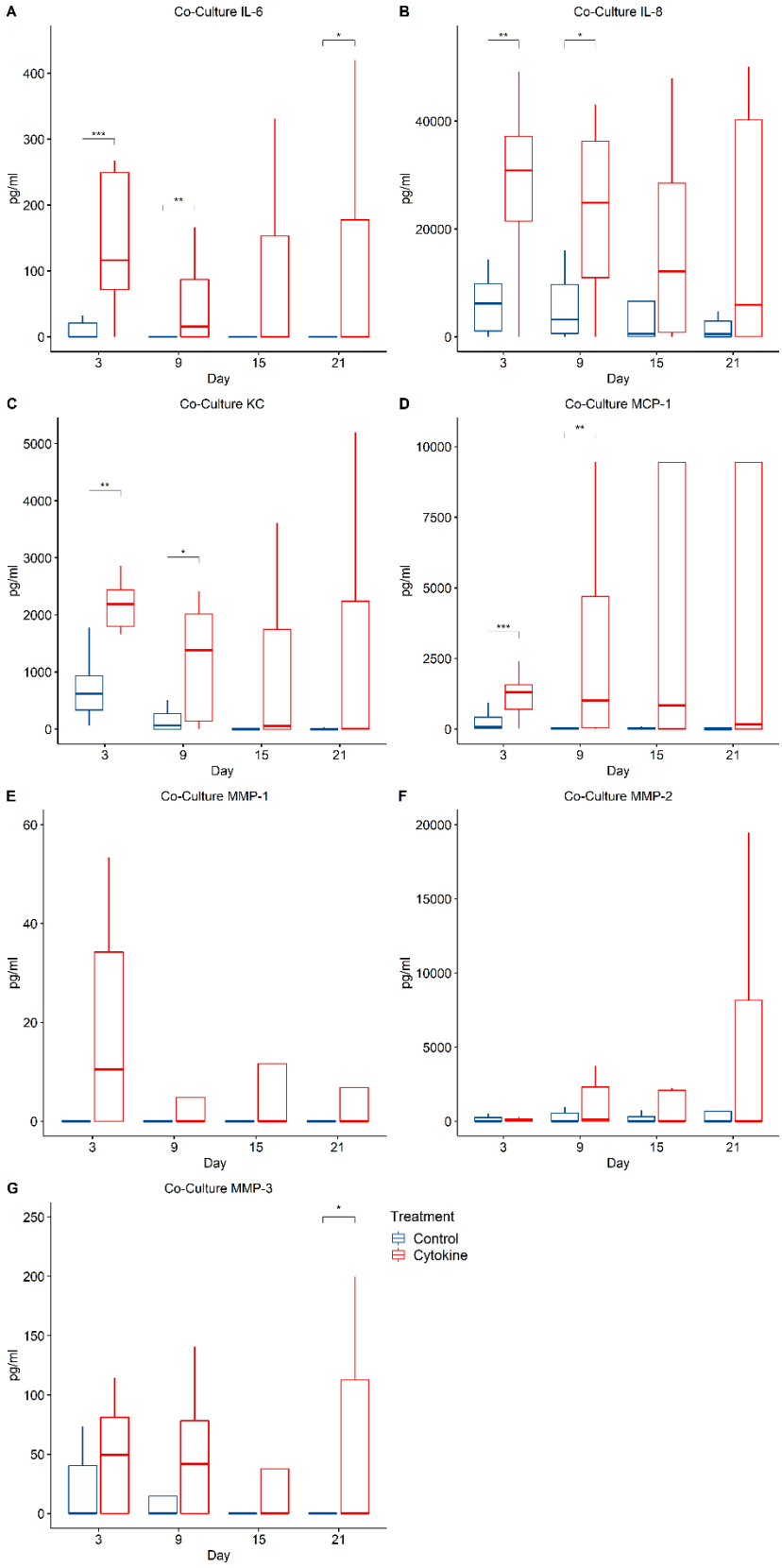

Figure 3-15 NCD CO cervical control vs cytokine over 21 days

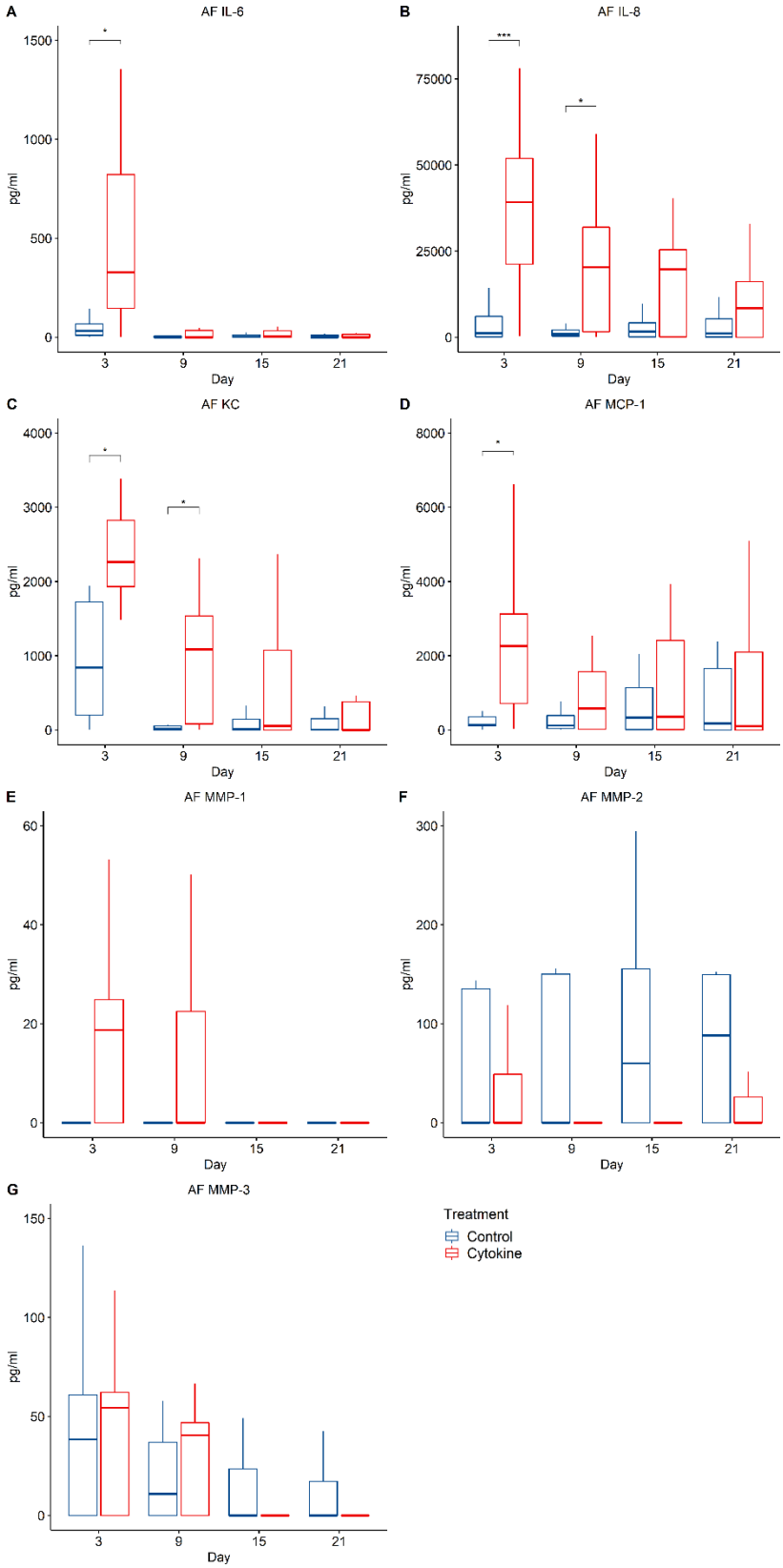

Figure 3-16 NCD AF lumbar control vs. cytokine over 21 days

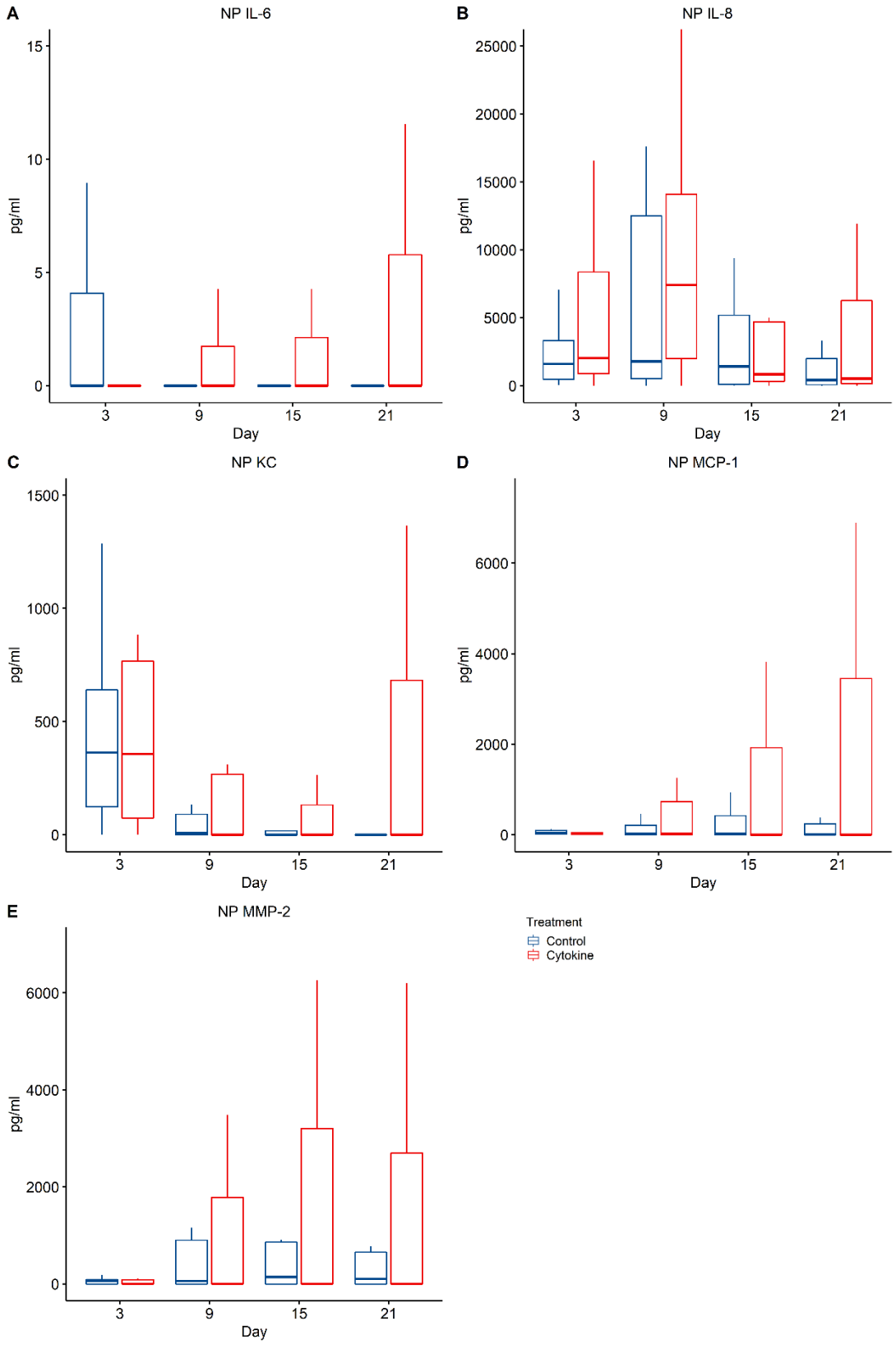

Figure 3-17 NCD NP lumbar NP control vs cytokine over 21 days

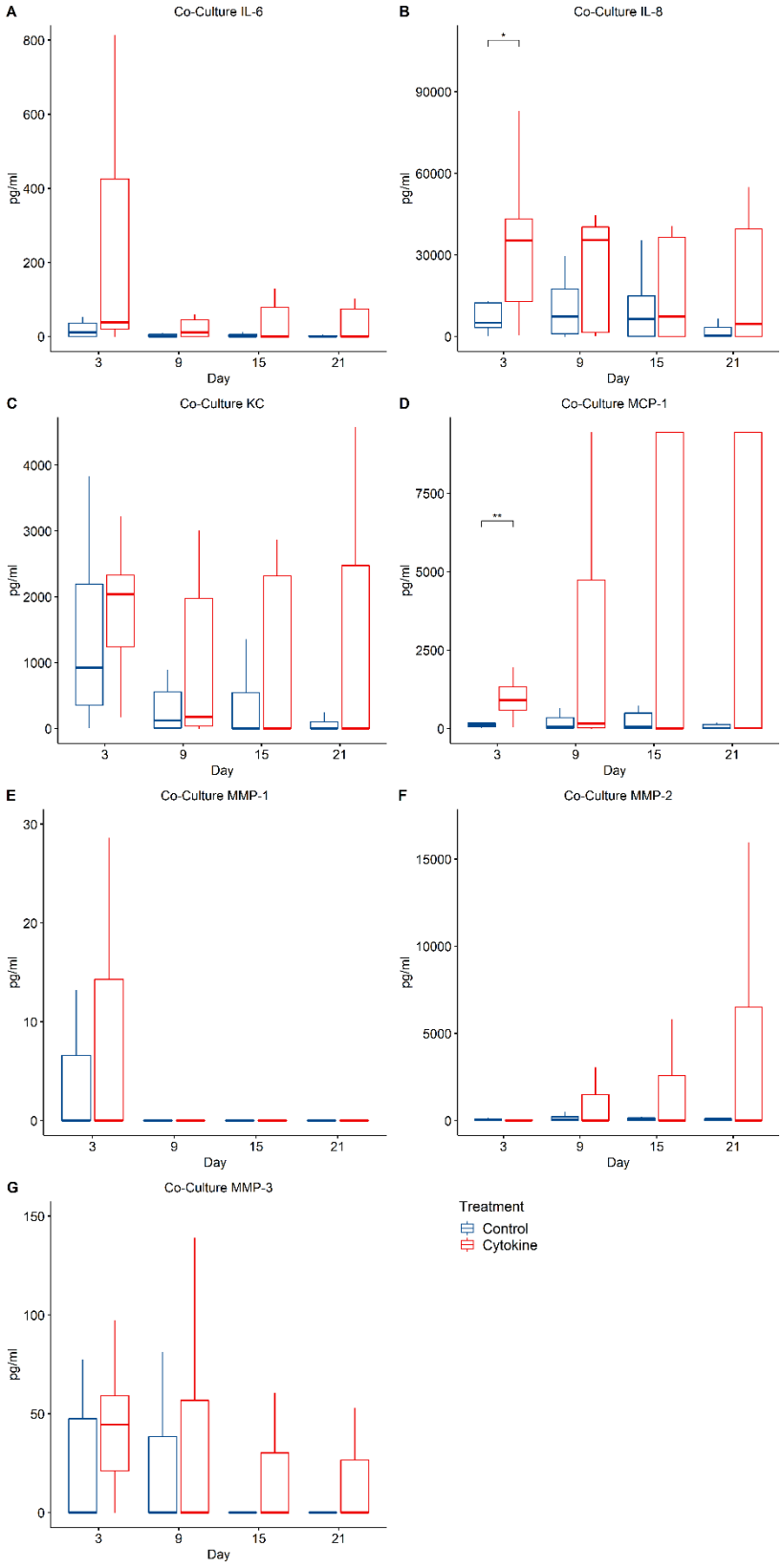

Figure 3-18 NCD CO lumbar control vs cytokine over 21 days

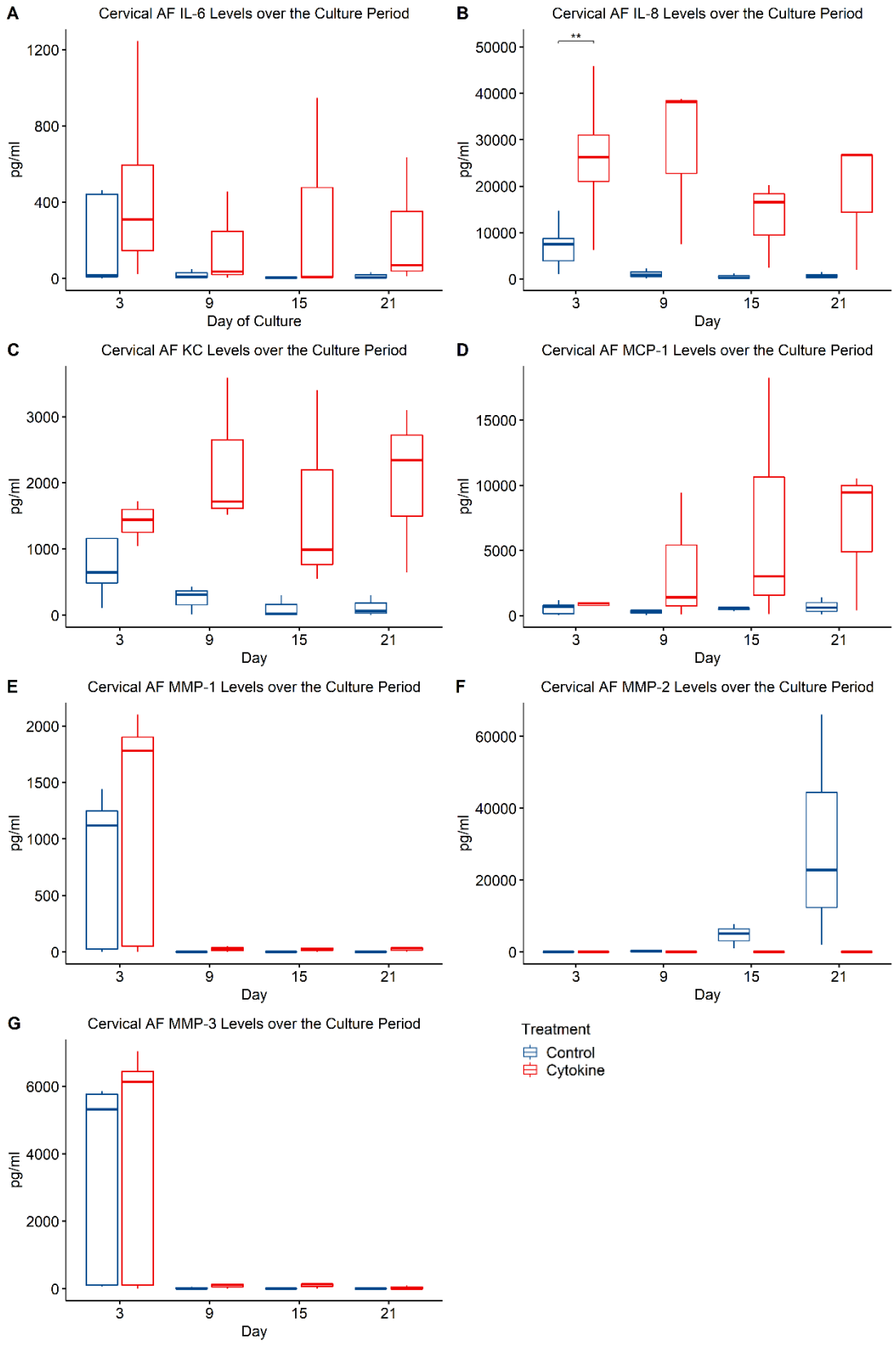

Figure 3-19 CD AF cervical control vs cytokine over 21 days

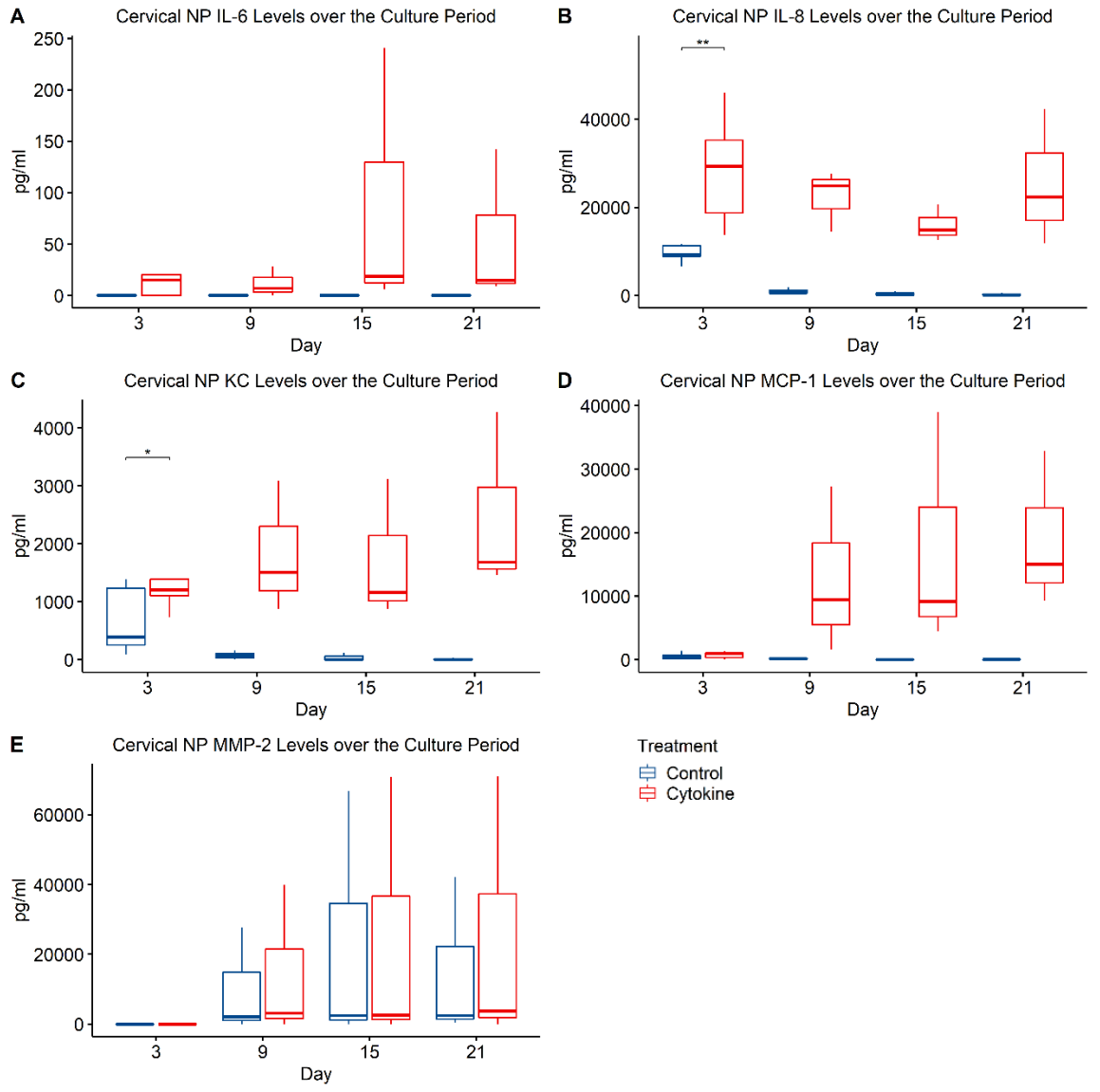

Figure 3-20 CD NP cervical control vs. Cytokine over 21 days

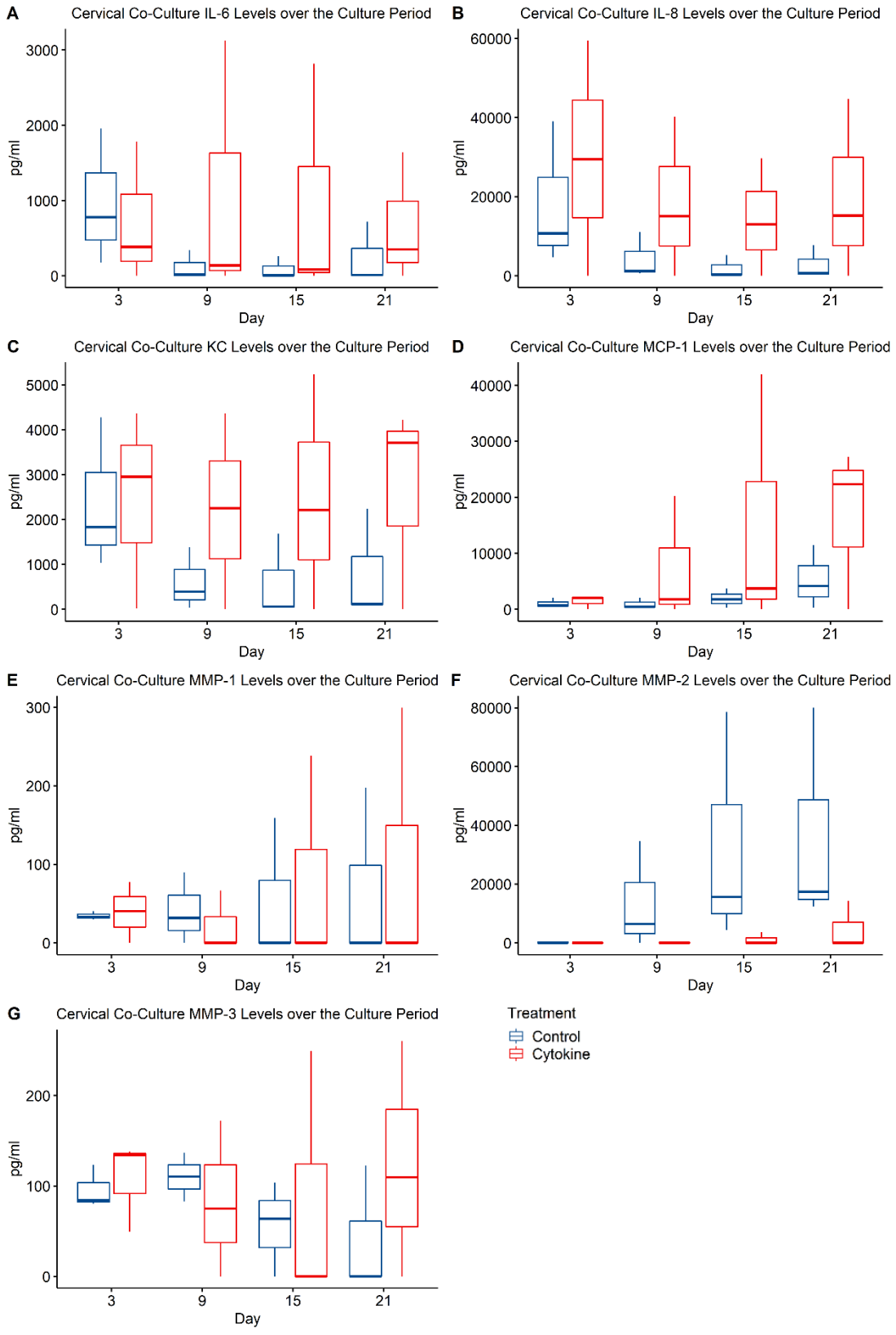

Figure 3-21 CD CO cervical control vs. cytokine over 21 days

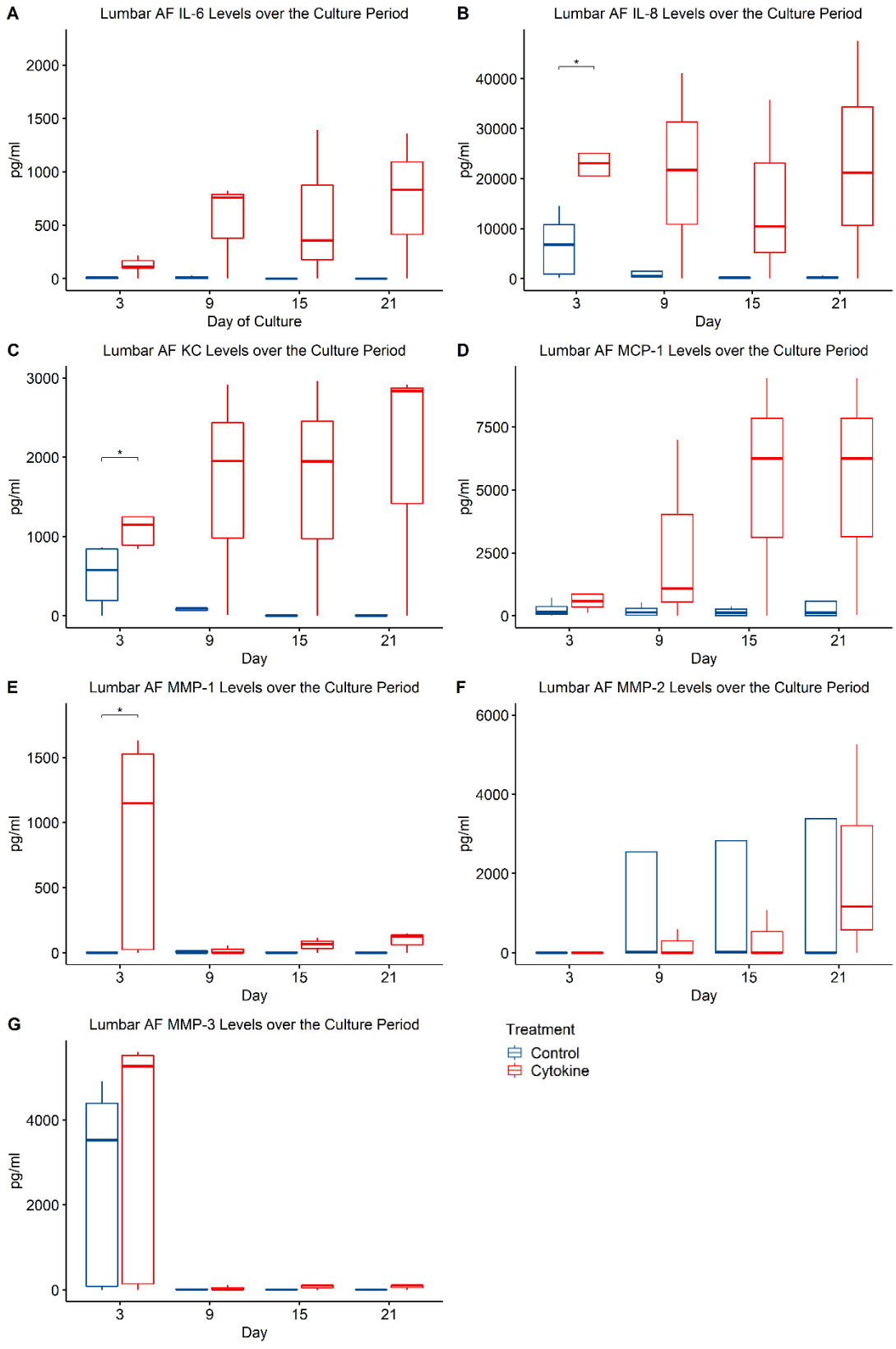

Figure 3-22 CD AF lumbar control vs. cytokine over 21 days

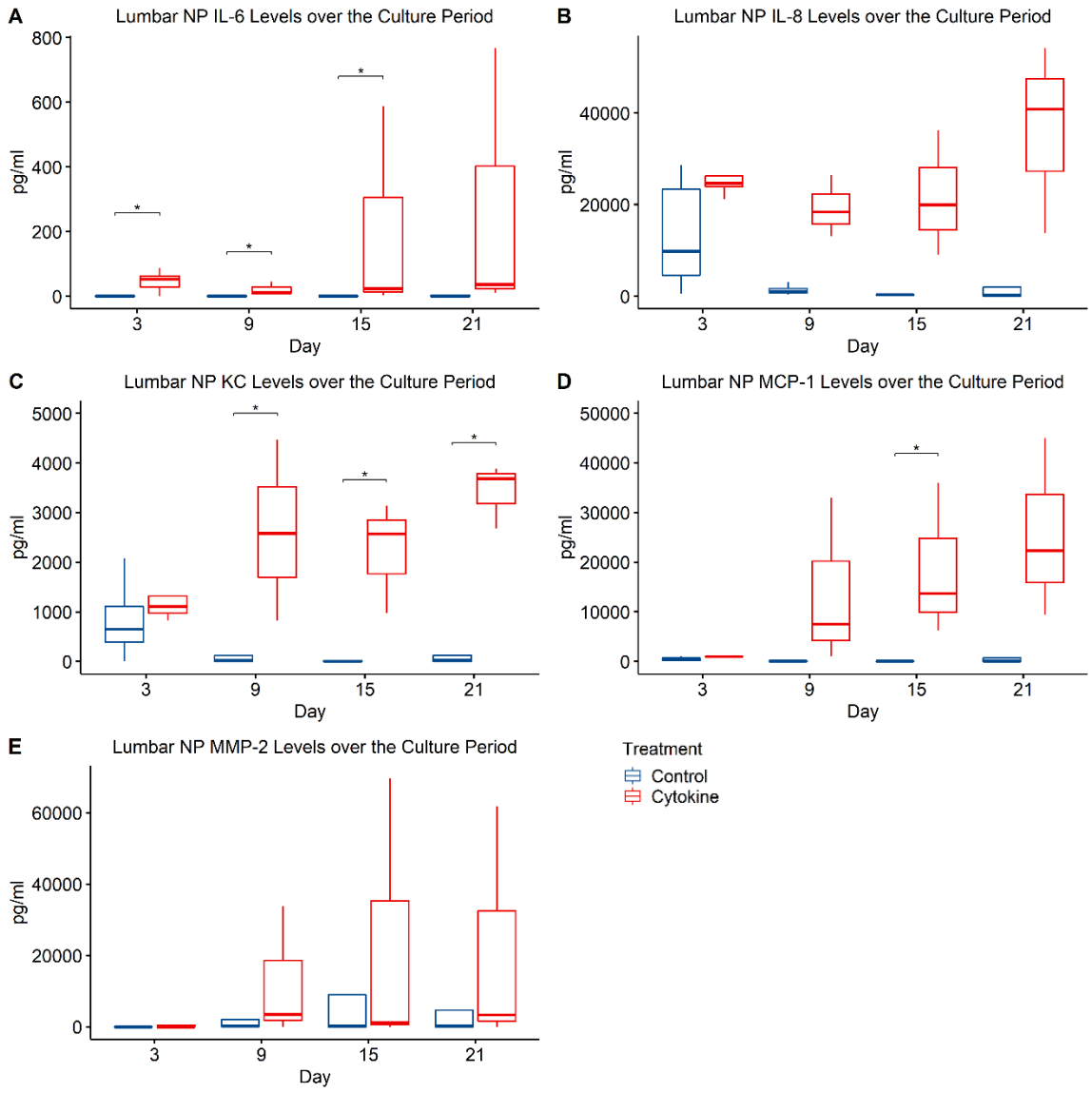

Figure 3-23 CD NP lumbar control vs. cytokine over 21 days

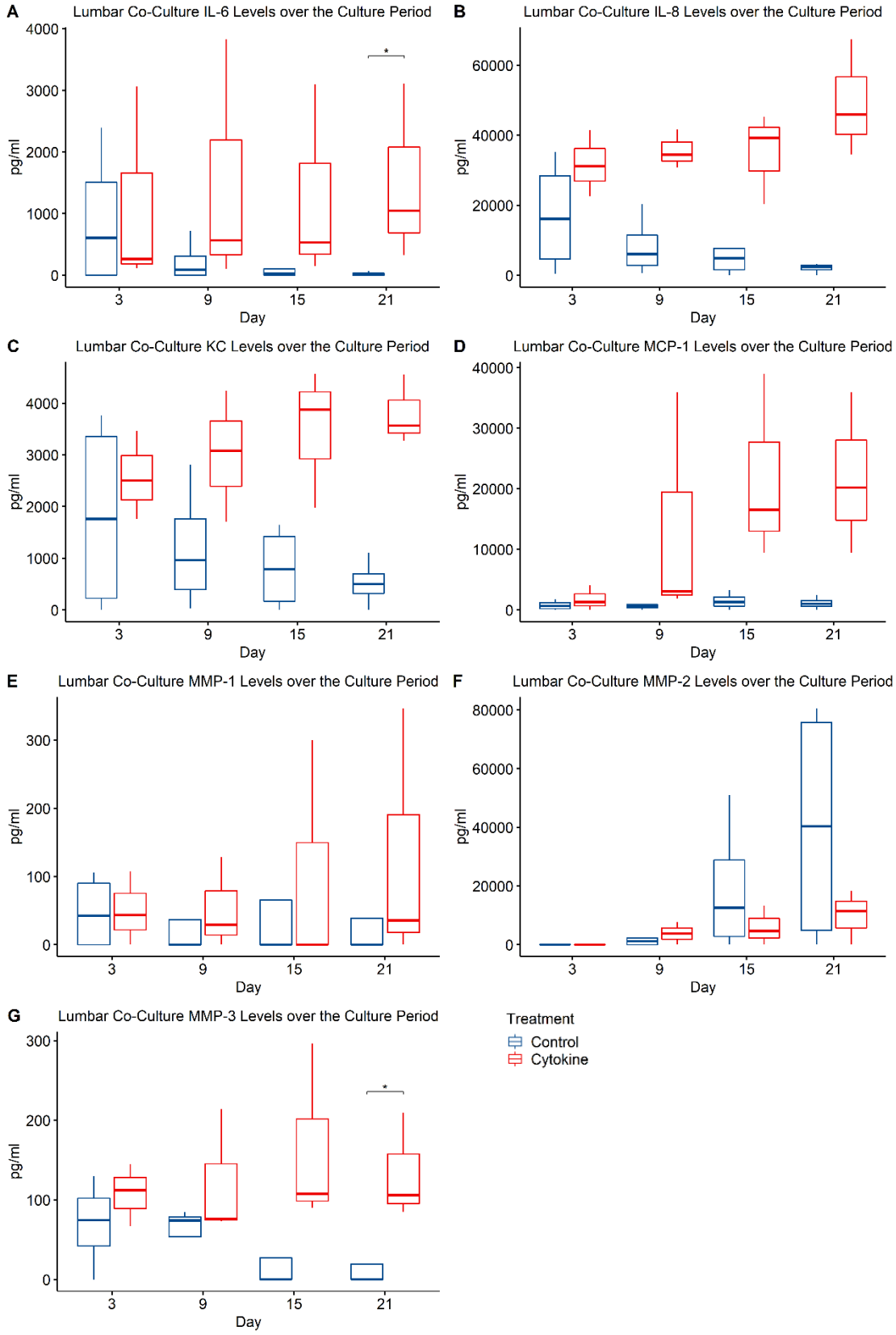

Figure 3-24 CD CO lumbar control vs. cytokine over 21 days

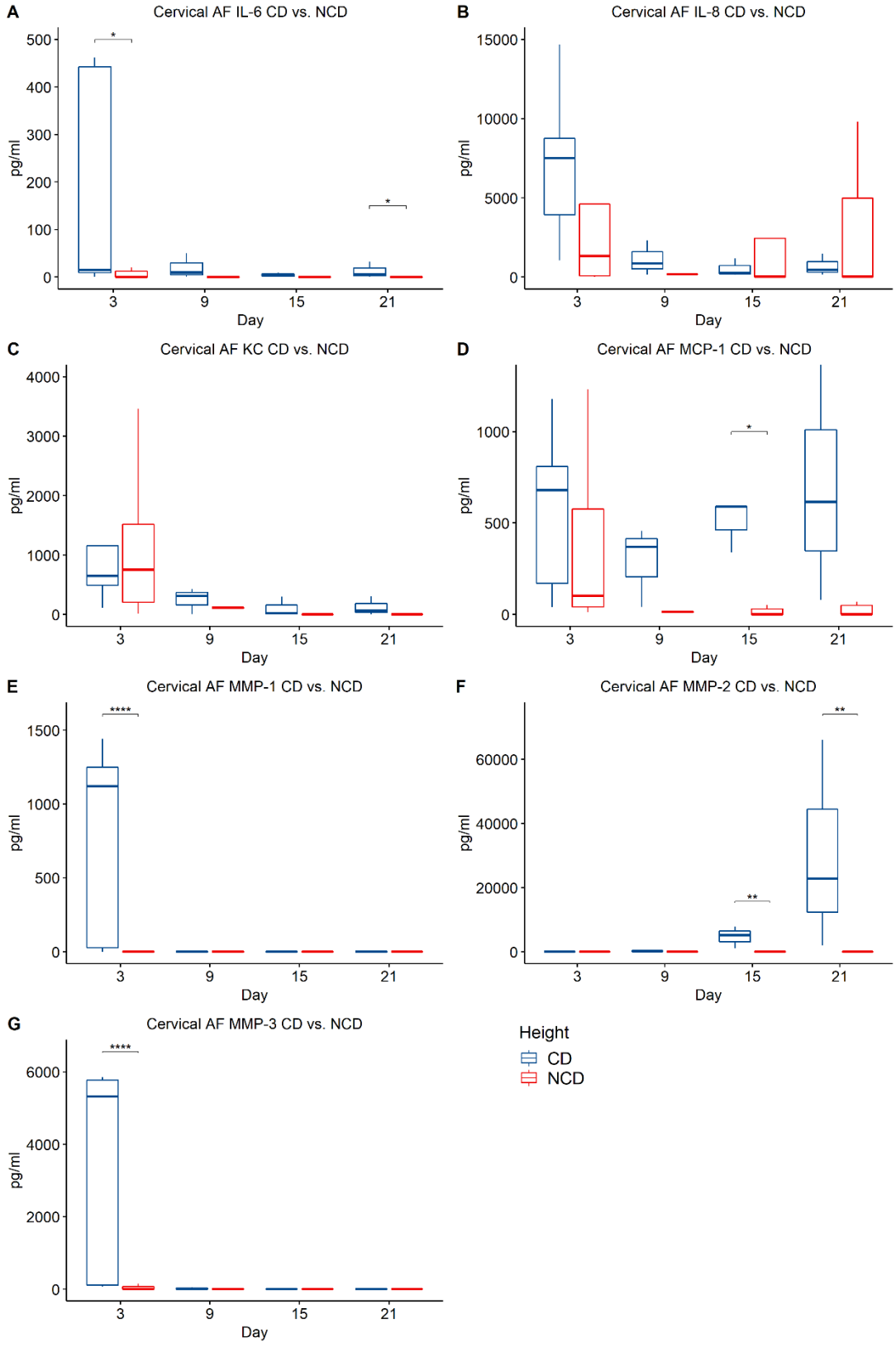

Figure 3-25 Cervical AF CD vs NCD

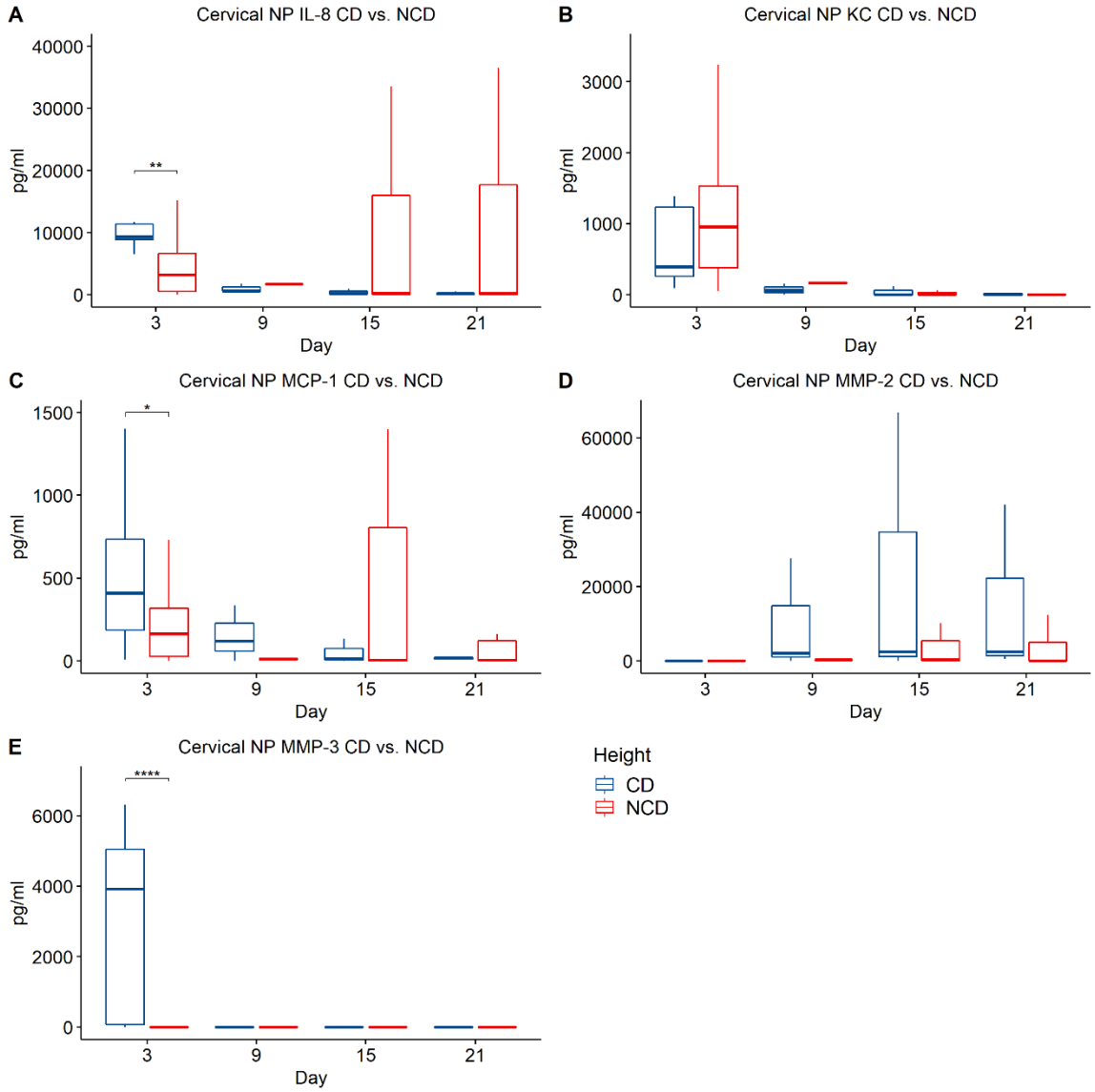

Figure 3-26 Cervical NP CD vs. NCD

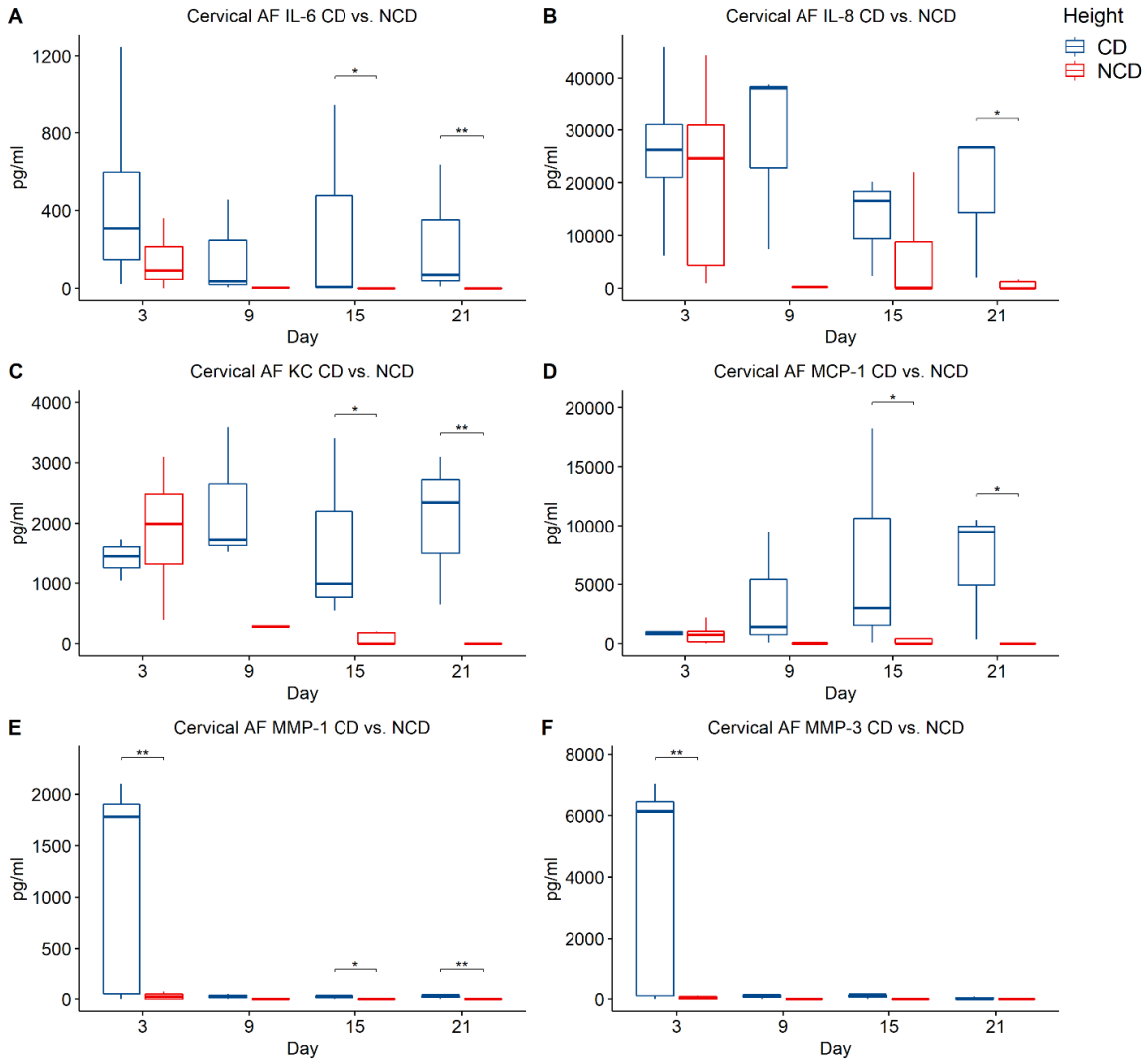

Figure 3-27. Cervical AF CD vs. NCD with IL-1β Stimulation

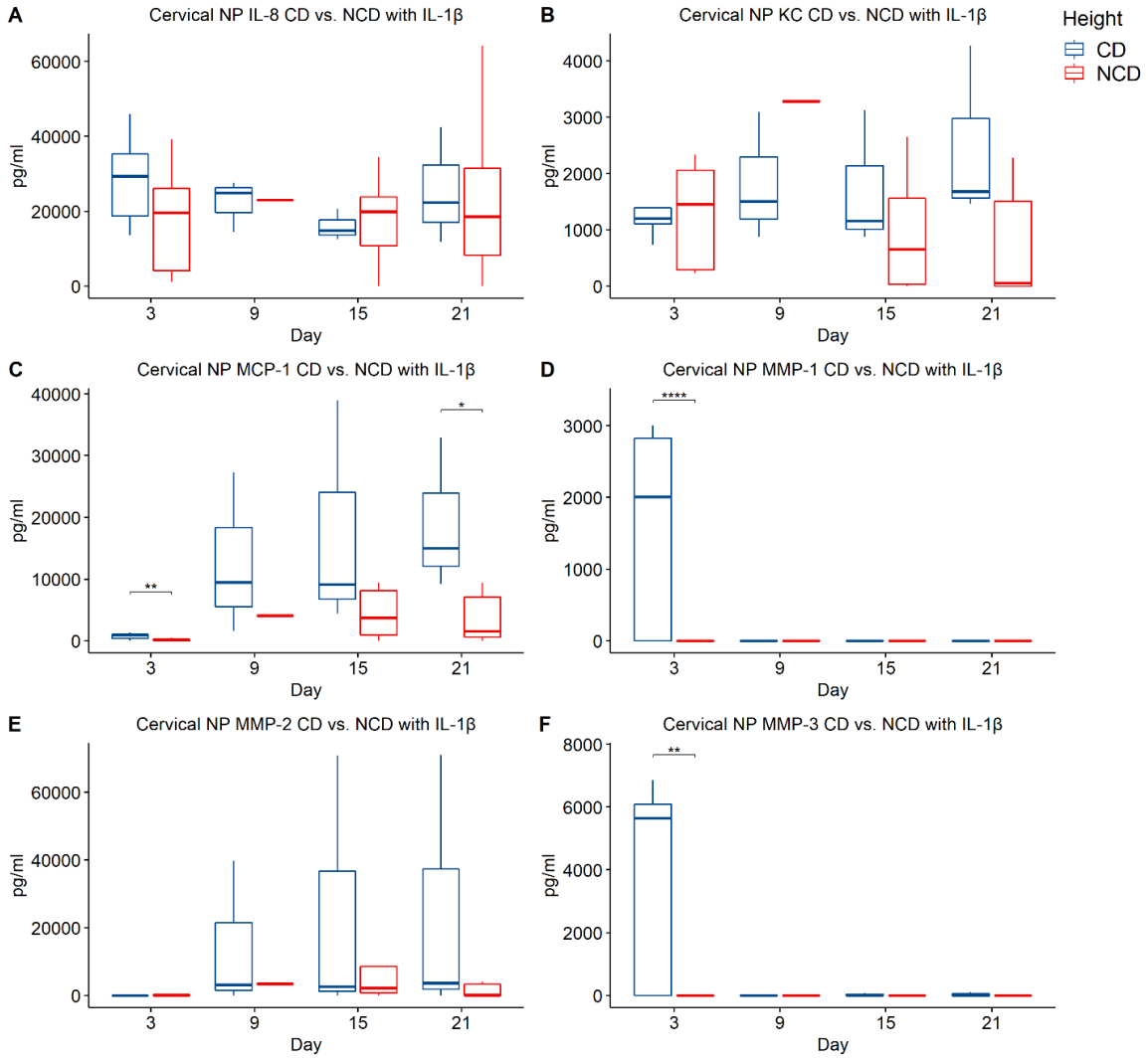

Figure 3-28. Cervical NP CD vs. NCD with IL-1β Stimulation

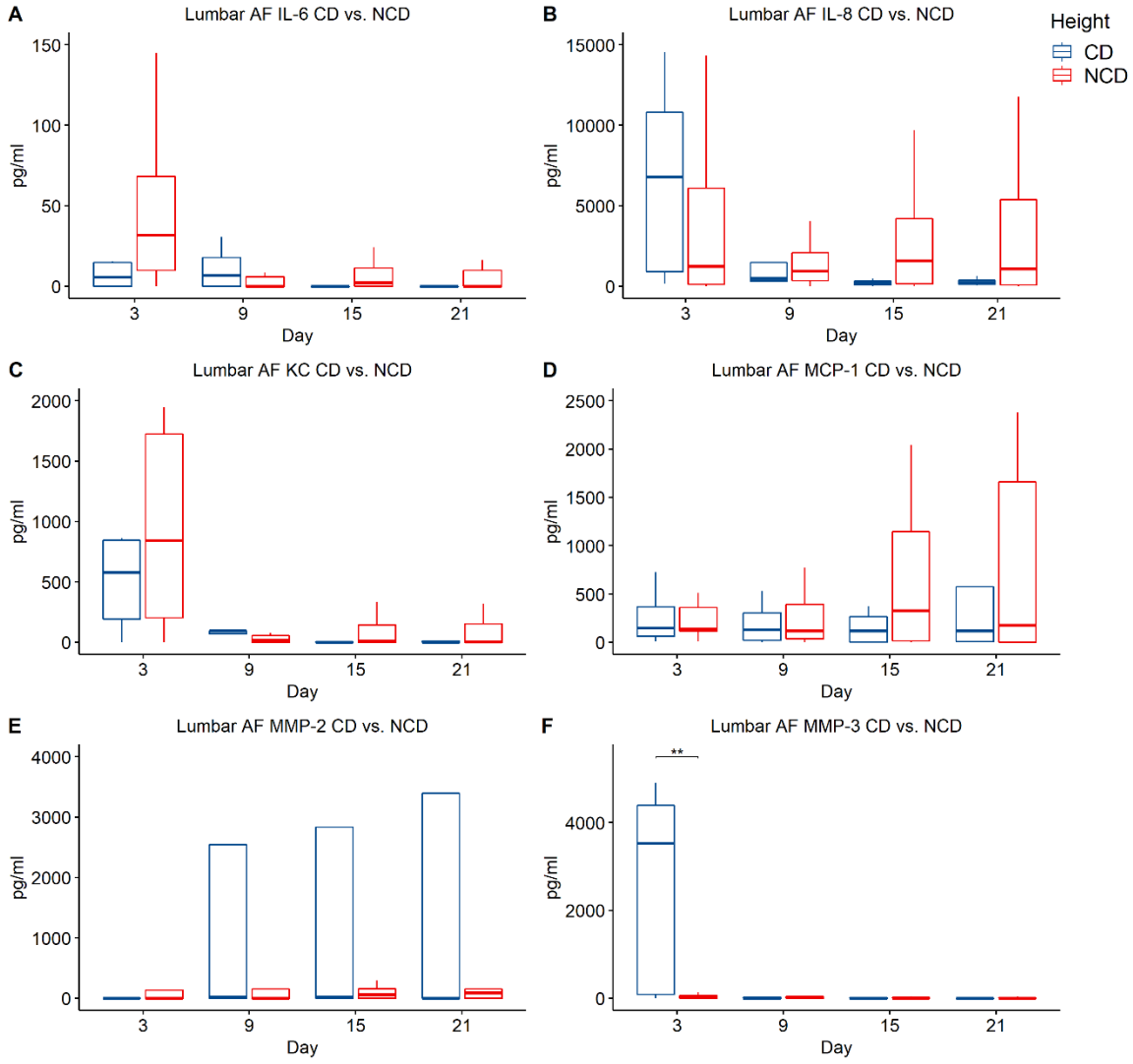

Figure3-29. Lumbar AF CD vs. NCD

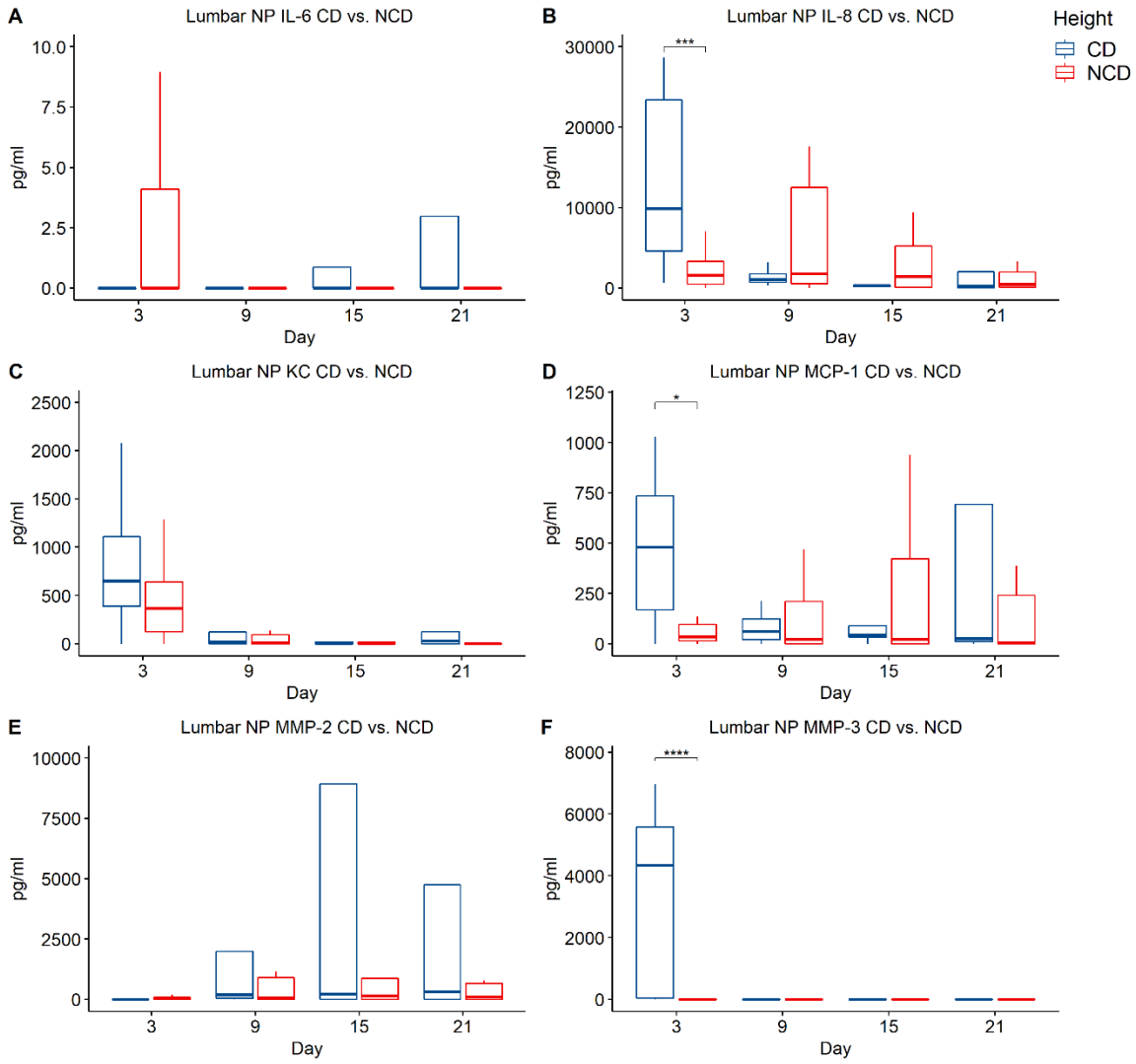

Figure 3-30. Lumbar NP CD vs. NCD

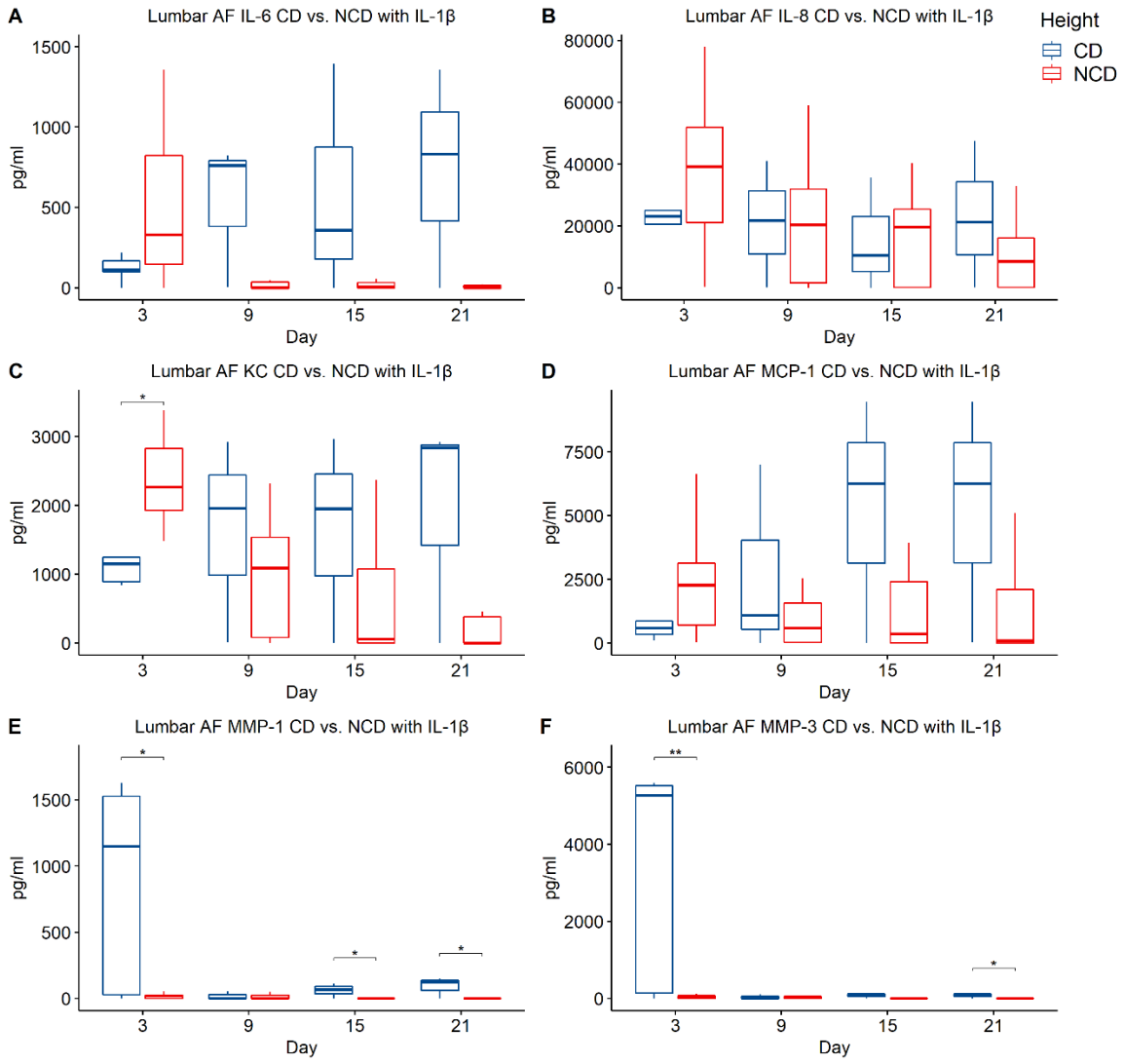

Figure 3-31: Lumbar AF CD vs. NCD with IL-1β stimulation

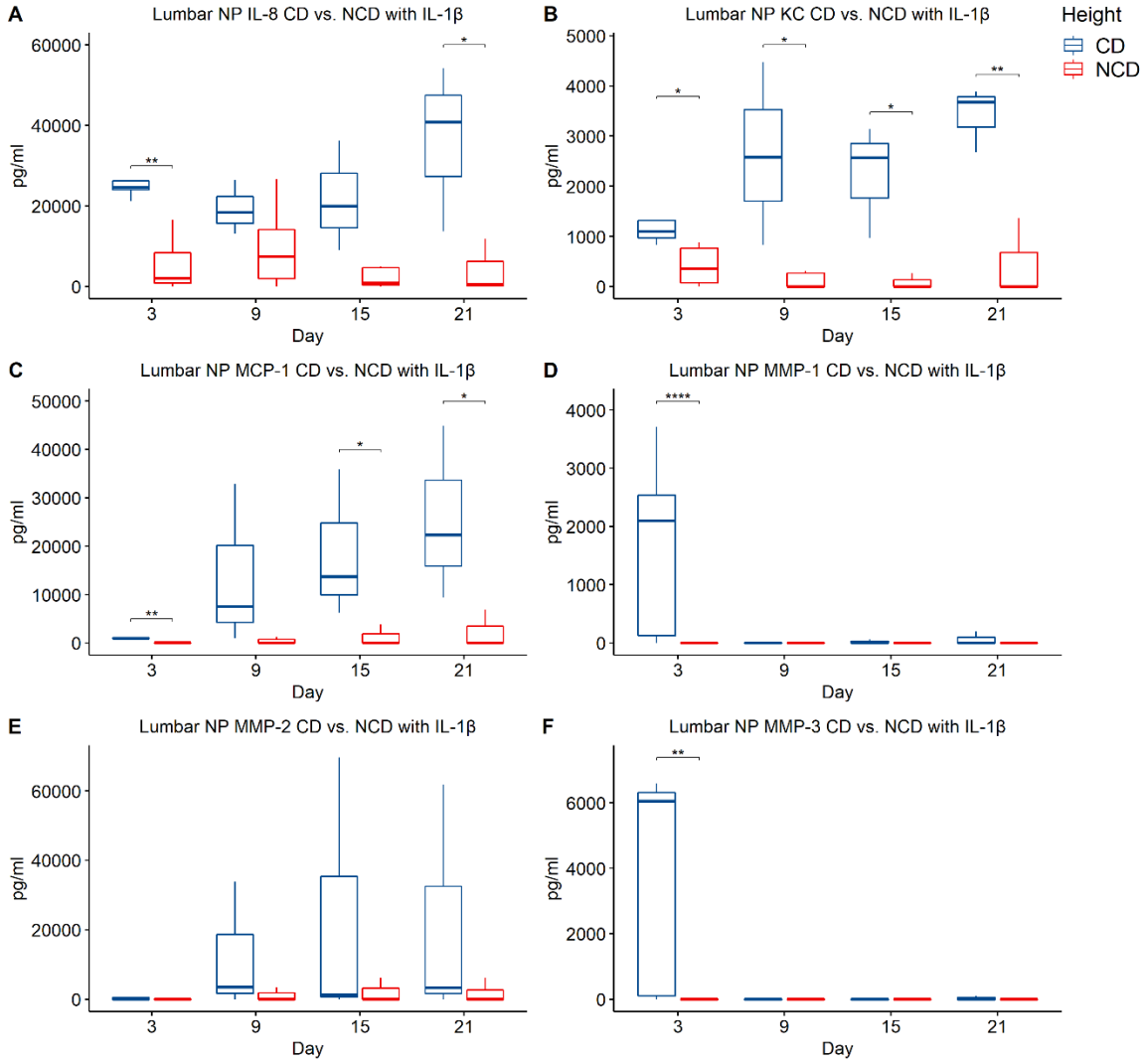

Figure 3-32: Lumbar NP CD vs. NCD with IL-1β stimulation

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Chapter 4: Comparisons of Biomarkers from Normal and Abnormal Canine IVDs

Introduction

Intervertebral disc degeneration (IVDD) is a common debilitating and costly medical problem in canine companion animals. IVDD has been reported to affect 0.3 – 2% of all dogs[1], and occurs more frequently in certain breeds[2,3]. There are several different clinical manifestations of IVDD in dogs with the most common being thoracolumbar (TL) disc herniation, degenerative lumbosacral stenosis (DLSS), and cervical spondylomyelopathy (CSM). Non-traumatic IVD abnormalities are classified as either Hansen type I or 2. Most often, chondrodystrophic (CD) dogs get Hansen type I IVDD which is caused by degeneration and calcification of NP and high-velocity herniation of the abnormal NP into the spinal canal. This leads to spinal cord trauma and Wallerian degeneration.[4] IVDs around the TL junction region are most commonly affected and type I IVDD is most often observed in young to middle aged CD dogs. Clinical presentations include acute paresis/paralysis of the hindlimbs, urinary and/or fecal incontinence, and loss of motor function and/or pain perception. In contrast, Hansen type 2 IVDD occurs more frequently in non-chondrodystrophic (NCD) breeds, specifically in working and performance dogs.[5] In most NCD dogs, clinical manifestations of IVDD emerge in the later stages of life. Common presentations are chronic and progressive paresis in the hindlimb with muscle atrophy, lumbosacral (LS) pain, and hunched posture with hindlimbs tucked underneath. While these are largely considered

neurologic conditions, the root cause of pain and functional deficits are attributable to abnormal disc tissue compromising the adjacent nerves and/or spinal cord. Regardless of Hansen type, symptomatic IVDD leads to decreased quality of life, associated financial costs, and may be cause for euthanasia.[6,7] Methods for diagnosis, treatment, and prognostication for canine IVDD are very similar to those employed for IVDD in human patients, and neck and low back pain due to IVD degeneration are common health and economic burdens for millions of individuals. [8] So, while human IVDD entails some important differences with respect to anatomy, mechanisms and phenotypes, the similarities in clinical manifestations, diagnostics, and treatments provide impetus for use of canine IVDD models for translational research towards further characterizing pathomechanisms and pathobiology of IVDD in both human and veterinary patients with a targeted emphasis on early stages of degeneration. Toward this goal, a critical first step is to characterize relevant changes related to canine IVDD and we hypothesized that abnormal IVD tissues would have higher inflammatory and degradative biomarker activities compared to the healthy IVD tissues. Therefore, the specific aims of this study were to 1) delineate differences in IVD biomarker production in normal versus abnormal canine IVDs, and 2) assess normal and abnormal canine IVDs' responses to pro-inflammatory stimuli as inflammation has been indicated as a key mechanism for all types of IVDD. [9]

Materials and Methods

Animals

Clinically normal dogs: With Institutional Animal Care and Use Committee (IACUC) approval (MU ACUC #9163), thoracolumbar IVDs were aseptically isolated from skeletally mature (age ranged one to three years) and female chondrodystrophic (n=6) dogs (purpose-bred laboratory Beagles donated by Sinclair Research) without history of neck or back pain that were euthanized for reasons unrelated to this study.

IVDD dogs: with IACUC approval (#9535), herniated, abnormal IVD materials were collected from chondrodystrophic dogs (n = 6) that presented at the University of Missouri Veterinary Health Center for thoracolumbar IVD herniation with owners' informed consent. This study did not influence the medical decisions made by the veterinary surgeons and the anormal IVD tissues would normally get discarded.

Preparation of healthy IVD explants and culture

All isolated IVDs were examined for evidence of IVD degeneration, and grossly normal (NP: clear to opaque, soft, discretely round gel; AF: intact layers of rings without fissures or neovascularization) IVDs were selected for study. AF and NP explants were created using 6 mm sterile dermal biopsy punches. The tissue explants were cultured as monoculture of AF or NP in supplemented DMEM at 37°C and 5% CO_2 with or without 10 ng/ml of recombinant canine (rc) IL-1β for 3 days. On day 3 media was collected and stored at -20˚C until used for biomarker analysis.

Surgical IVD tissue and culture

Herniated abnormal IVD tissues collected from the clinical IVDD canine patients were placed in room temperature sterile saline during the surgical interventions and were

transported to the laboratory within an hour of post-operative recovery. Surgical IVD tissues often resulted in smaller sizes and the tissues were divided into three pieces. The two tissues used for culture were randomly assigned to either the control (0 ng/ml rcIL-1β) or cytokine stimulated (10 ng/ml rcIL-1β) group and cultured for 3 days as described above. On day 3 media was collected and stored at -20˚C until used for biomarker analysis. *Media biomarker assay*

Day 3 culture media were tested for MMP-1, MMP-2, MMP-3, KC, MCP-1, IL-4, IL-6, IL-8, IL-10, and IL-18 using commercially available assays according to the manufacturer's protocol.

Statistical analysis

Raw media data were normalized based on the wet weight of the tissue to address the significant size differences between the healthy and surgical IVD tissues. The resulting data was tested for normality using Shapiro-Wilk test in R. Significant differences between the control and cytokine stimulated metabolism between the tissue types (AF, NP, and CO) were determined using Kruskal-Wallis test followed by Dunn's test with Benjamini-Hochberg adjustments and Wilcoxon test was used for pairwise comparisons with FSA and rstatix packages in R. Statistical significance was set at $p \leq 0.05$.

Results (fig. 4-1)

Aim 1: Comparisons of biomarkers produced by healthy and abnormal IVD tissues from CD dogs.

IL-8 and MMP-2 production by cytokine stimulated abnormal IVDs were significantly higher than by cytokine stimulated healthy AF (red bracket and asterisks). The cytokine stimulated abnormal IVD tissues produced significantly higher levels of KC compared to cytokine stimulated healthy AF or NP (red brackets and asterisks). The basal production of MCP-1 by the abnormal IVD tissues was significantly higher compared to that by healthy AF tissues. The basal production of MCP-1 by healthy NP tissues was significantly higher than that by healthy AF (blue brackets and asterisks). Cytokine stimulated abnormal IVD tissues produced significantly higher levels of MCP-1 compared to healthy AF or NP. The cytokine stimulated healthy NP produced significantly higher levels of MCP-1 compared to healthy AF (red brackets and asterisks). The basal production of MMP-1 by the abnormal IVD tissues was significantly higher than that by healthy AF (blue bracket and asterisk). The cytokine stimulated healthy NP tissues produced significantly higher levels of MMP-1 than the cytokine stimulated healthy AF (red bracket and asterisks). The basal production levels of MMP-3 by healthy AF and NP were significantly higher than for abnormal IVDs (blue bracket and asterisks). The cytokine stimulated healthy NP tissues produced significantly higher levels of MMP-3 compared to those for healthy AF or abnormal IVD tissues. The cytokine stimulated AF tissues produced significantly higher levels of MMP-3 compared to abnormal IVD tissues (red brackets and asterisks).

Aim 2: Comparisons of responses to increased pro-inflammatory stimuli by healthy and abnormal IVD tissues from CD dogs

IL-6 production levels were significantly higher in the cytokine stimulated healthy AF and NP compared to respective control groups. IL-8, KC, MCP-1, MMP-1, and MMP-3 productions were significantly higher in the cytokine stimulated healthy AF tissues compared to their control groups. Cytokine stimulation of abnormal IVD tissues was not associated with significant differences in biomarkers measured.

The abnormal IVDs did not produce detectable levels of MMP-3, while the healthy AF and NP tissues produced high levels of MMP-3. In contrast, the production of MMP-2 by healthy AF and NP tissues was minimal, with most samples being below the detection limit of the assay, while abnormal IVD tissues consistently produced measurable levels of MMP-2.

Discussion

This study has identified biomarkers that are differentially expressed by healthy and degenerative IVD tissues from CD dogs. Biomarkers that are associated with increase inflammation and degradative processes were generally higher in the degenerative IVD tissues obtained from the clinical CD dogs. Basal and cytokine stimulated levels of MCP-1 were higher for abnormal IVD tissues recovered from canine patients undergoing spine surgery. Importantly, MCP-1 has been implicated in back pain in human patients as well.[13,14]

MMP-1 production was significantly higher for herniated IVD tissues recovered at surgery when compared to healthy AF explants. MMP-1 is involved in extracellular

matrix (ECM) remodeling and catabolic activities associated with interstitial collagen types I, II, and III, and can be upregulated through biologic and biomechanical stimuli. Both collagen types I and II are found in healthy AF (type I is predominant) while predominantly collagen type II is found in healthy NP. However, collagen content shifts from type II to types I and III in degenerative NPs. These ECM characteristics likely explain the differences in predominant MMP production noted in the present study. MMP-2, which mainly degrades collagen type I, was higher in abnormal herniated IVDs tissues. MMP-2 has also been implicated in neovascularization[15] and ECM remodeling, both of which occur during IVD degeneration.[16–18] In contrast, abnormal IVD tissues had minimal production of MMP-3 which was much higher in healthy AF and NP tissues in CD dogs. MMP-3 activity is directed toward collagen type II rather than collagen type I.[18]

KC production was increased in abnormal IVD tissues with cytokine stimulation. This important chemokine has been implicated in angiogenesis and arteriogenesis through monocytic recuritment.[19] In degenerative disc disease, KC likely contributes to degeneration by increasing IVD vascularity with resultant inflammatory responses and structural alterations. IL-8 production by the degenerative IVDs from painful IVDD dogs was significantly higher compared to the healthy IVD tissues. This is also observed in human IVD tissues as well and IL-8 upregulation in IVDs was observed with experimental treatments of Substance P.[20] Furthermore, IL-8 upregulation has also been indicated in attenuated disc inflammation in the rodent model.[21]

In terms of responses to cytokine stimulation, it is interesting many biomarkers did not increase production in the healthy NP and surgical IVD tissues. This could potentially due to smaller samples with widespread data points or it could indicate that the AF tissues are more active at producing biomarkers associated with remodeling. While inflammation has been associated with increased degeneration, inflammation also has important roles in tissue healing and remodeling.[22] NP tissues from CD dogs may have reduced ability to respond to increased inflammation, which may be indicative of mechanisms behind NP's propensity to degeneration. Unlike healthy AF or NP tissues, the general lack of responses to increased inflammation in the degenerative IVD tissues may be potentially due to the fact that the tissues were in the end-stage IVDD and have decreased cellular viability and ability to respond to inflammation.

Potential limitations

While the findings in this chapter provide novel insights to biomarker regulation in health and disease, there are potential ways to improve the current study design. Diseased IVD tissues from the clinical patients were not clear of tissue type unlike the health IVD tissues that were selected based on distinctive gross tissue morphology. Furthermore, including more samples would be able to overcome the statistical insignificance with the non-normally distributed data as the general trend is that the healthy NP and surgical IVD tissues appear to increase the levels of the tested biomarkers in response to IL-1β stimulation.

Conclusion

To summarize the findings from this set of analyses, there are several differences between the normal and abnormal IVD tissues from CD dogs in respect to chemotactic and proteolytic activities. The data from this study confirm previous findings that were found in degenerative IVDs from other preclinical models and human subjects, however, this study has identified biomarkers such as IL-6 and IL-8 that have not been identified in the degenerative canine tissues.[12] The unique responses to cytokine stimulation in normal and abnormal IVD tissues from CD dogs have not yet been explored prior to this study. The results provide direct clinical relevance for the potential novel diagnostic, prognostic, disease staging, and therapeutic strategies that utilize biomarker panels which can be developed towards optimizing management of patients with IVDD in dogs and humans.

Figures

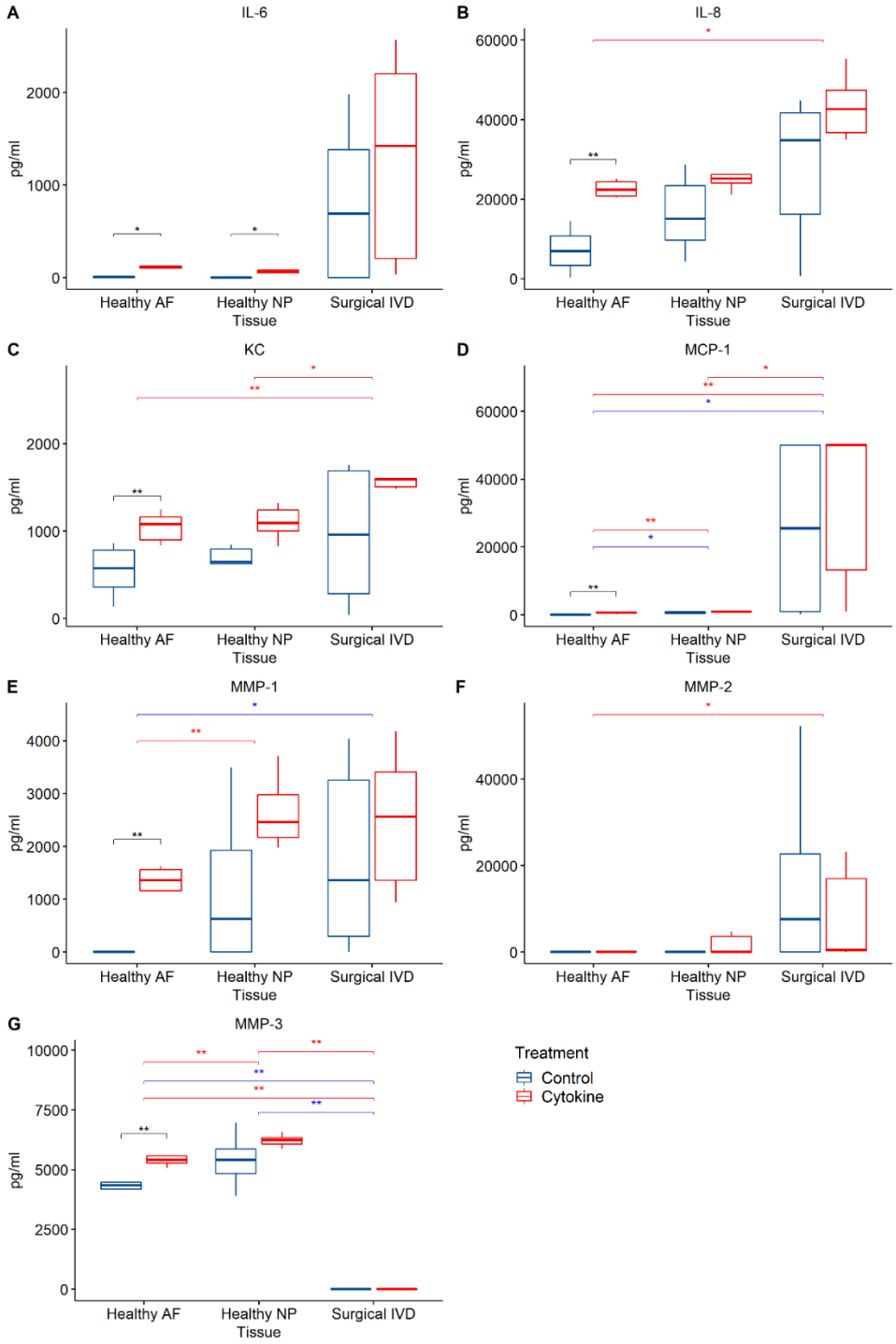

Figure 4-1: Comparisons of biomarker productions by healthy CD AF and NP and surgical IVD tissues with or without cytokine stimulation. Black brackets depict the significant differences between control and cytokine stimulated groups. Red brackets denote the significant differences between tissue types in the control groups. Blue brackets denote the significant differences between tissue types in the cytokine stimulated groups.
*: p≤0.05. **:p ≤0.01.

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Chapter 5: Comparison of canine and human IVDs

Introduction

Intervertebral disc diseases (IVDD) are significant health concerns in both canine and human populations. The Lancet's Global Burden of Disease report in 2016 has reported back and neck pain to be among the top five diseases affecting people, which continue to increase based on relative aging of the population.[1] Degenerative IVDD may affect up to 90% of people and may remain asymptomatic for decades.[2–5] In dogs, IVDD may also be asymptomatic, but affected dogs can be severely debilitated such that euthanasia is needed if treatment is not successful.[6] For canine and human IVDD patients, different etiologies including chronic inflammation, aging, genetics, poor biomechanics (posture, repetitive loading, etc.), trauma, and obesity have been associated with symptomatic disease. Unique structural changes are observed in IVD degeneration characterized by notochordal cells (NC) in healthy nucleus pulposus (NP) shifting phenotype to chondrocyte-like-cells and associated tissue remodeling to resemble the more fibrotic annulus fibrosus (AF).[7,8] The progressive disc height changes that occur with degeneration lead to cumulative consequences in terms of altered dynamics of the entire spine.[9]

Dogs and humans share many important similarities regarding IVD degeneration and clinical IVDD, including the established phenotypes reported. For example, chondrodystrophic (CD) dogs develop Hansen type I IVDD, which is developed from degenerative, calcified NP that causes high velocity herniation and compression of

spinal cord or other surrounding tissues.[10] Thoracolumbar (TL) junction IVD abnormalities are observed in the human population as well, however, it is not as common as in the CD dog population.[11] In addition, calcification of human NP and/or inner AF has not been fully explored though there is some evidence of calcification and degeneration in scoliotic spines and older adult spines.[12,13] In non-chondrodystrophic (NCD) dogs, chronic and progressive IVDD develops most commonly in the low lumbar or lumbosacral (LS) joint involving Hansen type II, which is characterized by the progressive structural weakening and protrusion of IVD into the spinal column. Many active, working dogs used in military, performance, rescue, and farming suffer from IVDD which could result in their early retirement.[14] The lower lumbar or LS joint is the most common site reported for human low back pain as well.[15] Diagnostic methods and treatments to address IVDD are quite similar between the two species as well.

Various management guidelines have been established for both canine and human IVDD, however, these guidelines have not proven consistently effective in preventing IVDD or altering its typical course in patients.[16] Further, there are no available regenerative techniques to restore the structure and function of degenerative IVDs. Current treatments focus on palliative management through physical therapy and pharmacological interventions or surgical removal of offending disc material with or without vertebral body fusion to treat instability when present. These treatments can provide pain relief and resolution of neurologic deficits, however, none effectively restores IVD integrity or material properties, and some even potentiate the likelihood for adjacent segment disease (ASD).[17] In order to optimize prevention and treatment so

as to improve outcomes, it is crucial to more fully characterize the underlying causes of IVD degeneration towards development of early diagnostics and regenerative therapies. As such, a validated preclinical model for IVDD that can effectively assess tools for early degeneration detection, monitoring IVDD progression, and regenerative strategies for both dogs and humans is necessary. Given that dogs develop spontaneous IVDD that closely resembles human IVDD, the canine model has indisputable advantages. Therefore, this study was designed to further develop preclinical canine models for effective and ethical translational research for human IVDD by identifying molecular changes associated with IVD health and disease. The specific aims for this study were 1) to compare biomarker profiles of non-symptomatic canine (NCD and CD) and human IVD tissues, 2) to understand how non-symptomatic NCD IVDs respond to increased inflammation compared to the human IVDs, 3) to evaluate how non-symptomatic CD IVDs respond to increased inflammation compared to the human IVDs, and 4) to compare biomarker profiles of symptomatic canine (CD) and human IVD tissues.

Materials and methods

Preparation of canine IVD explants

With Institutional Animal Care and Use Committee (IACUC) approval (MU ACUC #9163 and #9164), lumbar IVDs were aseptically recovered from skeletally mature (1-4 years of age) female chondrodystrophic (n=7) and non-chondrodystrophic (n=18) dogs without history of neck or back pain euthanized for reasons unrelated to this study. All isolated

IVDs were examined for evidence of IVD degeneration, and grossly normal (NP: clear to slightly opaque, soft, discretely round gel; AF: intact layers of rings without fissures or neovascularization) IVDs were selected for study. AF and NP explants were created using 6 mm sterile dermal biopsy punches.

With IACUC approval (#9535), herniated, abnormal IVD materials were collected from chondrodystrophic dogs (n = 6) that presented at the University of Missouri Veterinary Health Center for thoracolumbar IVD herniation with owners' informed consent. This study did not influence the medical decisions made by the veterinary surgeons and the anormal IVD tissues would normally get discarded. Herniated abnormal IVD tissues collected from the clinical IVDD canine patients were placed in room temperature sterile saline during the surgical interventions and were transported to the laboratory within an hour of post-operative recovery. Surgical IVD tissues often resulted in smaller sizes and the tissues were divided into three pieces. Two of the pieces were used in culture study and one was stored in a -80°C freezer for future studies.

Preparation of human IVD explants

Midwest Transplant Network (MTN) screened and selected donors (n=7, mean age 47.1 yrs, 4 female) without known history of back pain were recovered and donated to the Thompson Laboratory for Regenerative Orthopaedics (TLRO) for research purposes with fully informed family consent. Radiographs were obtained from the donated spine segments to identify and confirm IVD levels. Then the spine segments were processed in aseptic manner to isolate individual IVDs. The IVDs were bisected to determine

Thompson grades. Depending on availability, the L3-4 or L4-5 or L5-S1 IVD was used for this study. From these IVDs, AF and NP explants were created using 6mm dermal biopsy punches.

With Institutional Review Board approval (#201692), surgical IVD tissues from patients (n=56, mean age 54.8 yrs, 36 female) were collected during surgical interventions by the spine surgeons at Missouri Orthopaedic Institute (MOI). This project did not influence the medical decision making of the surgeons. The surgical tissues would otherwise be discarded. Explants of the submitted surgical IVD tissues were created as described above.

Culture and media analysis

The NCD and CD canine IVD tissue explants were cultured as monoculture of AF or NP in supplemented DMEM at 37°C and 5% CO_2 with or without 10 ng/ml of recombinant canine IL-1β for 3 days.

The donor (cadaveric; CAD) AF and NP explants and surgical IVD explants were placed in supplemented DMEM at 37˚C and 5% CO_2 for 3 days with or without 10 ng/ml of recombinant human IL-1β.

Media from day 3 of culture were tested for MMP-1, MMP-2, MMP-3, IL-6, IL-8, KC (CXCL1; equivalent to human GRO-α), and MCP-1 (CCL2), using commercially available assays according to the manufacturer's protocol. Biomarker levels were normalized to tissue wet weights for analysis.

Statistical analysis for non-symptomatic canine and human IVDs

Because biomarker variables had large variability and followed a non-normal distribution, the natural log and square root transformation were considered. Because of the large differences among the ranges of all biomarkers, the natural log transformation was selected. A small value (0.01) was added to the zero values to avoid calculating the logarithm of zero. Wilcoxon rank sum test and the two-sample t-test were used to compare transformed biomarker values in different height groups. The Wilcoxon rank sum tests the null hypothesis that two groups have the same median, and the two-sample t test tests that two groups have the same mean. The null hypothesis would be rejected when the p-value is less than 0.05, which indicates that two height groups have significantly different median or mean. In order to control false discovery rate, which was the proportion of significant results that were actually false positives, the Benjamini-Hochberg (BH) procedure was used to adjust for the multiple testing p-values. The false discovery rate was set to 0.05 in this study. The null hypothesis would be rejected when the p-value was less than its Benjamini-Hochberg (BH) critical value which was calculated by Software R 3.6.2.

Statistical analysis for comparisons of clinical canine and human IVDs

Fixed effect regression models were used to investigate the relationship between Species (dog or human) and the logarithmic value of biomarkers, after controlling for subject effects. The significance for the Species coefficient in the linear model will tell if there is a significant mean difference in logarithmic biomarker values between dogs and

humans, after controlling for subject effects. The Benjamini-Hochberg procedure was used to adjusted all p-values for linear regression models. The assumptions for linear regression were checked for all models. The regression diagnosis QQ plot and residual plot are in the appendix, the log transformation helped the normality assumption as well as the assumption for equal variance. Some deviations were still observed, although linear regression is robust to small deviations from assumptions. Other transformations (log of square root and log of cubic root) were considered but were not used for analysis as small deviations from the assumptions still existed and these are less frequently used in the literature.

Results

Aim1: Comparisons of non-symptomatic NCD and CD canine and human IVD tissues

Figures 1 and 2 show boxplots for transformed biomarker values for Height (NCD, CD and CAD) in the control group. Based on the results in Table 1, for AF tissue, there were significant mean/median differences between NCD and CAD heights for transformed biomarkers IL-6, IL-8, KC, MCP-1, MMP-3, and significant mean/median differences between CD and CAD heights for IL-8, KC, MCP-1. Differences between other transformed biomarker values were not statistically significant. Based on the results in Table 2, for NP tissue, there were significant mean/median differences between NCD and CAD heights for transformed biomarkers IL-6, IL-8, KC, MCP-1, MMP-2, MMP-3, and significant mean/median differences between CD and CAD heights for IL-6, IL-8, KC,

MCP-1. Differences between other transformed biomarker values were not statistically significant.

Aim 2: Comparisons of responses to cytokine stimulation in non-symptomatic NCD canine and human IVD tissues

Figure 3 and 4 show boxplots for log biomarker values for NCD and CAD heights in the cytokine group. Based on the results in Table 3, there were significant mean/median differences between NCD and CAD heights for transformed biomarkers IL-6, IL-8, KC, MCP-1, MMP-1, MMP-3 in AF tissue, and IL-6, IL-8, KC, MMP-2, MMP-3 in NP tissue. Differences between other transformed biomarker values were not statistically significant.

Aim 3: Comparisons of response to cytokine stimulation in non-symptomatic CD canine and IVD tissues

Figures 5 and 6 show boxplots for log biomarker values for CD and CAD heights in the cytokine group. Based on the results in Table 4, there were significant mean/median differences between CD and CAD heights for transformed biomarkers IL-6, IL-8, KC, MCP-1, MMP-1, MMP-2 in AF tissue, and IL-6, IL-8, KC, MCP-1, MMP-2 in NP tissue. Differences between other transformed biomarker values were not statistically significant.

Aim 4: Comparisons of symptomatic IVDD canine and human IVD tissues

Figure 7 shows a boxplot for the transformed biomarkers by species in the control group. Table 5 shows the results to assess differences in logarithmic value of biomarkers for species (dogs vs humans) using linear regression after adjusting for a fixed subject effect in control group. Since none of the p-value is less than its Benjamini-Hochberg critical value, there was no statistically significant difference for logarithmic value of any biomarker between dogs and humans in the control group after adjusting for subject effect.

Figure 8 shows a boxplot for transformed biomarkers by species in the cytokine group. Table 6 shows the results to assess differences in logarithmic value of biomarkers for species (dogs vs humans) using linear regression after adjusting for a fixed subject effect. Since none of the p-values was less than its Benjamini-Hochberg critical value, there was no significant difference for logarithmic value of any biomarker between dogs and humans in the cytokine group, after adjusting for subject effect.

Discussion

The canine (NCD and CD) IVD tissues had different biomarker profiles compared to the non-symptomatic human IVDs. The non-symptomatic NCD and CD dogs showed significant differences in biomarker profiles when compared to the non-symptomatic human IVDs. Overall, the tissues from both canine groups produced higher levels of pro-inflammatory biomarkers with or without cytokine stimulation. Interestingly, the differences in MMPs were shown only in the NCD dogs when compared to the cadaveric

human IVDs, while CD dogs did not produce significantly different levels of MMPs in the present study. It is interesting that biomarkers associated with degradation and ECM remodeling were more similar for CD dogs compared to the human IVDs because LS degeneration in NCD and humans is thought to spawn from degenerative end plates leading to downstream events causing degradation of the IVD. These results are likely related to the use of grossly normal canine IVDs in the present study such that CD IVDs have molecular-level degenerative changes prior to grossly evident disease that more closely resemble IVDD in humans.

Responses to cytokine stimulation were also much different for healthy NCD and CD canine IVDs when compared to the human IVDs. NCD explants produced higher levels of MMP-1 and MMP-3 from AF, but lower levels of MMP-2 from NP when compared to the respective cadaveric human tissues. These differences were further supported by gross differences as well. The NP tissues from NCD dogs maintained a healthy appearance, which was not consistently noted for the adult human IVDs which undergo marked changes in adulthood even without symptomatic IVDD. Cytokine-stimulated biomarker profiles were also different for CD IVDs when compared to cadaveric human IVDs, however, biomarker production levels from CD AF and NP were more similar compared to the respective human tissues. Concentrations of MMP-2 for cadaveric human AF and NP were significantly lower than for the respective CD tissues, which may indicate mechanistic differences regarding ECM remodeling in IVDD between CD dogs and humans. Differences cytokine-stimulated responses between canine and human IVDs were also likely related to the nature and timing of degenerative processes between

species. Canine and human subjects were not age-matched based on any validated algorithm. With recent advances in epigenetics, a better way to translate dog to human years has been reported[18] and this tool could be utilized to further refine the model by appropriate age-matching.

Importantly, there were virtually no differences in biomarker profiles for IVD tissues from clinical IVDD patients between species. The biomarker profiles did not show altered patterns even with cytokine stimulation suggesting that the degenerative canine and human IVDs with symptomatic IVDD do not differ in their responses to increased inflammation. These findings are pivotal and foundational for the goal of developing a valid preclinical model for human IVDD because they highlight the high translational potential for using available canine tissues to model symptomatic human and canine degenerative disc disorders.

Conclusions

This series of analyses allowed comparisons of biomarkers associated with IVD degeneration in dogs and humans. The direct comparisons of the biomarkers at the basal level provide novel data for characterization of two canine models, chondrodystrophic and non-chondrodystrophic, that will allow for further refinement towards valid modeling mechanistic studies for IVDD. The IVD biomarker profiles for symptomatic IVDD in dogs and humans were very similar, even with cytokine stimulation, suggesting that late-stage degenerative canine and human IVDs share

important similarities that can be leveraged for translational research. Importantly, this dispels the historical notion that IVDD in CD dogs is vastly different from humans based on calcification of the NP. As such, the results of this study provide strong impetus for further development and validation of canine models for IVDD to provide relevant clinical insights and platforms that can lead to novel diagnostic tools and regenerative therapies in both species.

Figures

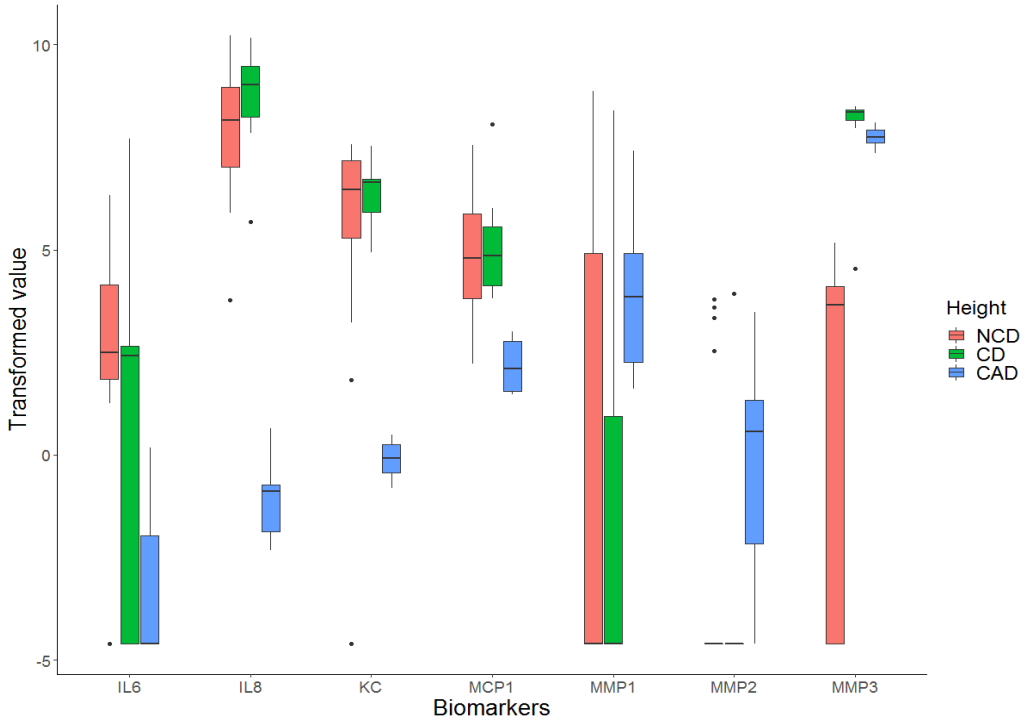

Figure 1: Transformed biomarker Boxplot in control group for AF comparing NCD, CD, and CAD

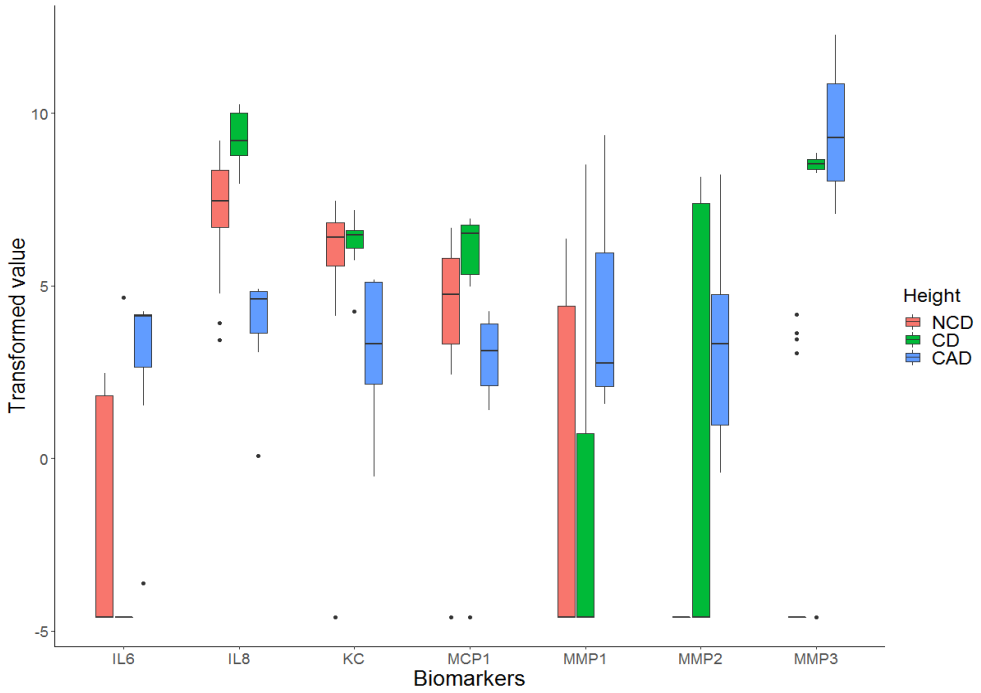

Figure 2: Transformed biomarker Boxplot in control group for NP comparing NCD, CD, and CAD

Treatment = Control, Tissue = AF					
Variable	**Height NCD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	2.51	-4.61	(3.86, 7.39)	**0.0014***	**Yes**
log (IL8)	7.89	-1.11	(7.59, 10.40)	**<0.0001**	**Yes**
log (KC)	6.47	-0.09	(5.03, 7.26)	**0.0002***	**Yes**
log (MCP1)	4.69	2.17	(1.53, 3.51)	**<0.0001***	**Yes**
log (MMP1)	-4.61	3.84	(-8.84, 0.62)	0.0503*	No
log (MMP2)	-4.61	0.58	(-5.50, 0.01)	0.1130*	No
log (MMP3)	3.65	7.74	(-12.26, -3.55)	**0.0001***	**Yes**
Treatment = Control, Tissue = AF					
Variable	**Height CD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	2.42	-4.61	(-2.45, 7.34)	0.2194*	No
log (IL8)	8.61	-1.11	(8.24, 11.20)	**<0.0001**	**Yes**
log (KC)	6.34	-0.11	(5.66, 7.23)	**<0.0001**	**Yes**
log (MCP1)	5.15	2.17	(1.64, 4.33)	**0.0004**	**Yes**
log (MMP1)	-4.61	3.84	(-10.20, 2.64)	0.1506*	No
log (MMP2)	-4.61	0.58	(-6.36, 0.01)	0.1196*	No
log (MMP3)	8.35	7.74	(-0.10, 0.84)	0.0530*	No

*p-value used Wilcoxon rank sum test comparing medians; others used two sample t test comparing means.

Table 1: Mean/median comparison test results in control group between NCD, CD and CAD for AF

Treatment = Control, Tissue = NP					
Variable	**Height NCD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	-4.61	4.12	(-8.74, -1.66)	**0.0006***	**Yes**
log (IL8)	7.47	4.63	(2.35, 4.30)	**0.0001***	**Yes**
log (KC)	6.40	3.33	(1.29, 4.24)	**0.0005***	**Yes**
log (MCP1)	4.76	3.12	(0.23, 2.74)	**0.0221***	**Yes**
log (MMP1)	-4.61	2.77	(-7.37, 0.04)	0.0537*	No
log (MMP2)	-4.61	3.32	(-8.64, -6.23)	**0.0001***	**Yes**
log (MMP3)	-4.61	9.29	(-14.51, -12.12)	**0.0001***	**Yes**
Treatment = Control, Tissue = NP					
Variable	**Height CD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	-4.61	4.12	(-8.82, -0.99)	**0.0238***	**Yes**
log (IL8)	9.21	4.63	(3.75, 7.02)	**0.0006***	**Yes**
log (KC)	6.19	3.22	(1.03, 4.91)	**0.0059**	**Yes**
log (MCP1)	6.51	3.12	(0.80, 4.59)	**0.0262***	**Yes**
log (MMP1)	-4.61	2.77	(-11.93, 1.45)	0.0894*	No
log (MMP2)	-4.61	3.32	(-8.64, 5.51)	0.4382*	No
log (MMP3)	8.53	9.29	(-3.81, 1.15)	0.2593*	No

*p-value used Wilcoxon rank sum test comparing medians; others used two sample t test comparing means.

Table 2: Mean/median comparison test results in control group between NCD and CD for NP

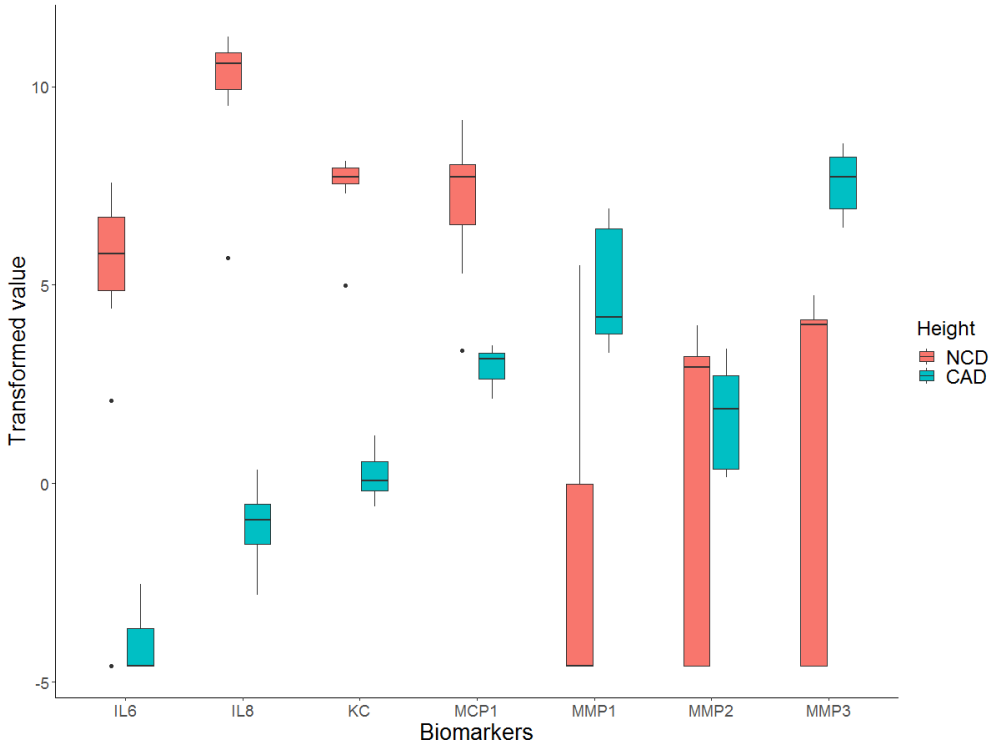

Figure 3: Transformed biomarker Boxplot in cytokine group for AF comparing NCD and CAD

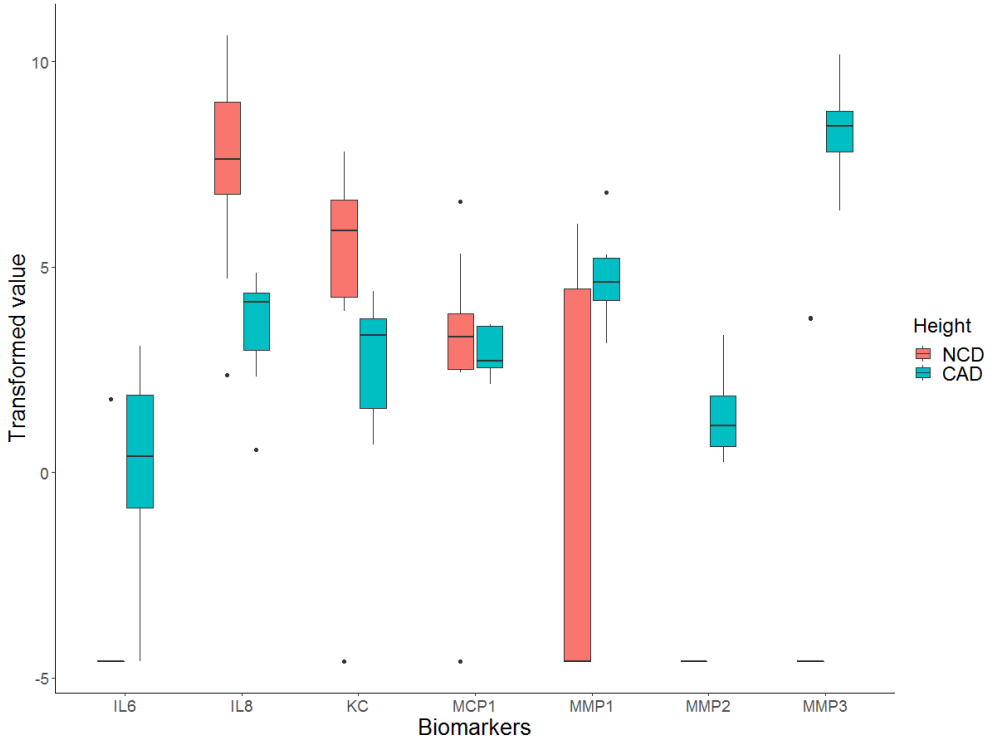

Figure 4: Transformed biomarker Boxplot in cytokine group for NP comparing NCD and CAD

Treatment = Cytokine, Tissue = AF					
Variable	**Height NCD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	5.79	-4.61	(7.88, 11.27)	**0.0020***	**Yes**
log (IL8)	10.58	-0.93	(10.35, 12.31)	**0.0001***	**Yes**
log (KC)	7.72	0.06	(6.78, 8.06)	**0.0001***	**Yes**
log (MCP1)	7.13	2.95	(3.01, 5.36)	**<0.0001***	**Yes**
log (MMP1)	-4.61	4.18	(-10.87, -1.79)	**0.0137***	**Yes**
log (MMP2)	2.93	1.87	(-6.48, 2.52)	0.8548*	No
log (MMP3)	4.00	7.72	(-11.56, -2.88)	**0.0005***	**Yes**
Treatment = Cytokine, Tissue = NP					
Variable	**Height NCD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	-4.61	0.39	(-7.00, -2.62)	**0.0039***	**Yes**
log (IL8)	7.46	3.46	(1.88, 6.12)	**0.0010**	**Yes**
log (KC)	5.88	3.33	(0.60, 4.52)	**0.0083***	**Yes**
log (MCP1)	3.29	2.71	(-1.13, 1.55)	0.6505*	No
log (MMP1)	-4.61	4.64	(-9.74, 0.12)	0.0763*	No
log (MMP2)	-4.61	1.13	(-6.77, -4.90)	**<0.0001***	**Yes**
log (MMP3)	-4.61	8.42	(-13.44, -10.97)	**0.0002***	**Yes**

*p-value used Wilcoxon rank sum test comparing medians; others used two sample t test comparing means.

Table 3: Mean/median comparison test results in cytokine group between NCD and CAD

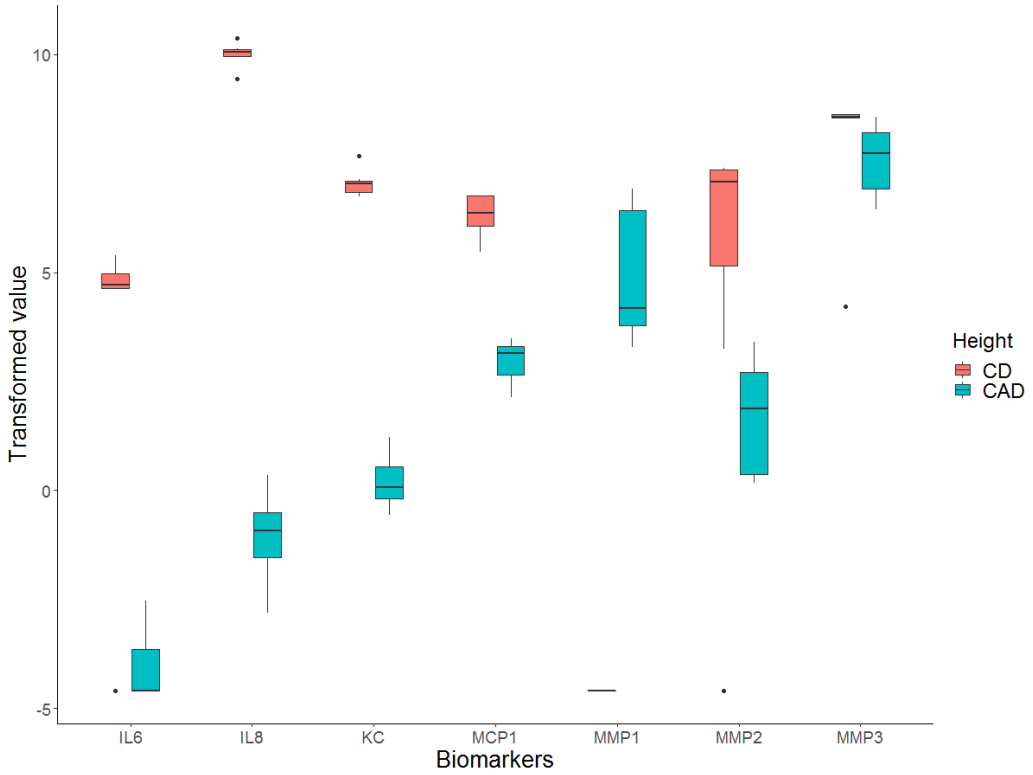

Figure 5: Transformed biomarker Boxplot in cytokine group for AF comparing CD and CAD

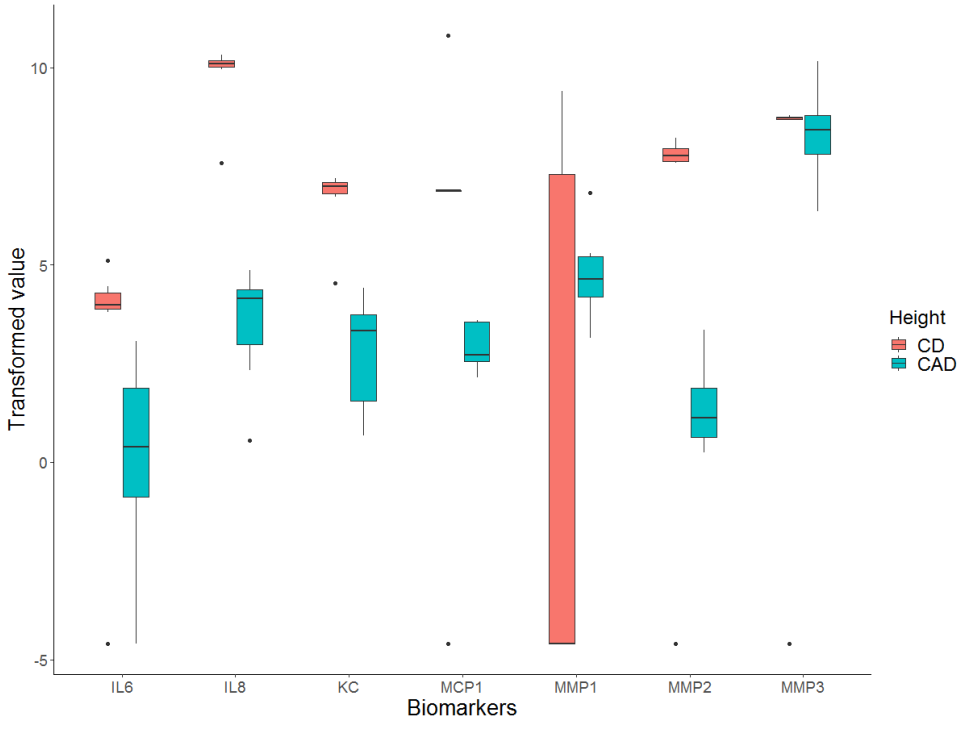

Figure 6: Transformed biomarker Boxplot in cytokine group for NP comparing CD and CAD

Treatment = Cytokine, Tissue = AF					
Variable	**Height CD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	4.71	-4.61	(7.25, 9.73)	**0.0095***	**Yes**
log (IL8)	10	-1.07	(10.10, 12.04)	**<0.0001***	**Yes**
log (KC)	7.05	0.2	(6.28, 7.41)	**<0.0001**	**Yes**
log (MCP1)	6.31	2.95	(2.79, 3.95)	**<0.0001**	**Yes**
log (MMP1)	-4.61	4.18	(-11.18, -7.98)	**0.0011***	**Yes**
log (MMP2)	7.08	1.87	(0.16, 6.92)	**0.0379***	**Yes**
log (MMP3)	8.57	7.72	(-0.03, 1.74)	0.0728*	No
Treatment = Cytokine, Tissue = NP					
Variable	**Height CD mean/median**	**Height CAD mean/median**	**95% CI mean/median difference**	**p-value**	**BH adjusted p-value significance**
log (IL6)	3.99	0.39	(0.88, 6.11)	**0.0252***	**Yes**
log (IL8)	10.09	4.14	(5.25, 7.85)	**0.0006***	**Yes**
log (KC)	6.99	3.33	(2.75, 5.65)	**0.0006***	**Yes**
log (MCP1)	6.88	2.71	(3.27, 4.75)	**0.0262***	**Yes**
log (MMP1)	-4.61	4.64	(-9.74, 4.10)	0.6053*	No
log (MMP2)	7.77	1.13	(4.31, 7.40)	**0.0262***	**Yes**
log (MMP3)	8.73	8.42	(-1.46, 1.23)	0.8048*	No

*p-value used Wilcoxon rank sum test comparing medians; others used two sample t test comparing means.

Table 4: Mean/median comparison test results in cytokine group between CD and CAD

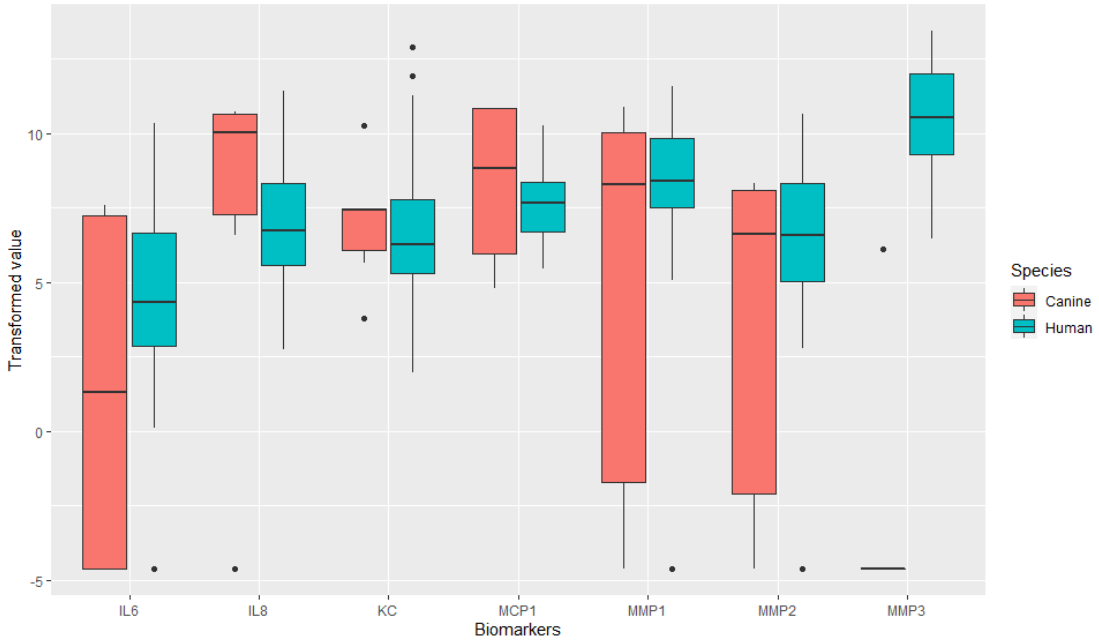

Figure 7: Transformed biomarker value by species in the control group

Logarithmic Biomarker	Independent Variable	Coefficient Estimate	Std Error	P-value	Benjamini-Hochberg critical value	Significance
log (IL6)	Species	11.56	4.18	0.0092	<0.0001	No
log (IL8)	Species	-1.99	2.20	0.3723	0.0003	No
log (KC)	Species	1.43	1.95	0.4699	0.0004	No
log (MCP1)	Species	1.59	1.10	0.1583	0.0001	No
log (MMP1)	Species	2.95	3.47	0.4022	0.0003	No
log (MMP2)	Species	14.30	2.60	<0.0001	<0.0001	No
log (MMP3)	Species	16.65	1.42	<0.0001	<0.0001	No

Table 5: Regression model results for comparing biomarkers between dogs and humans

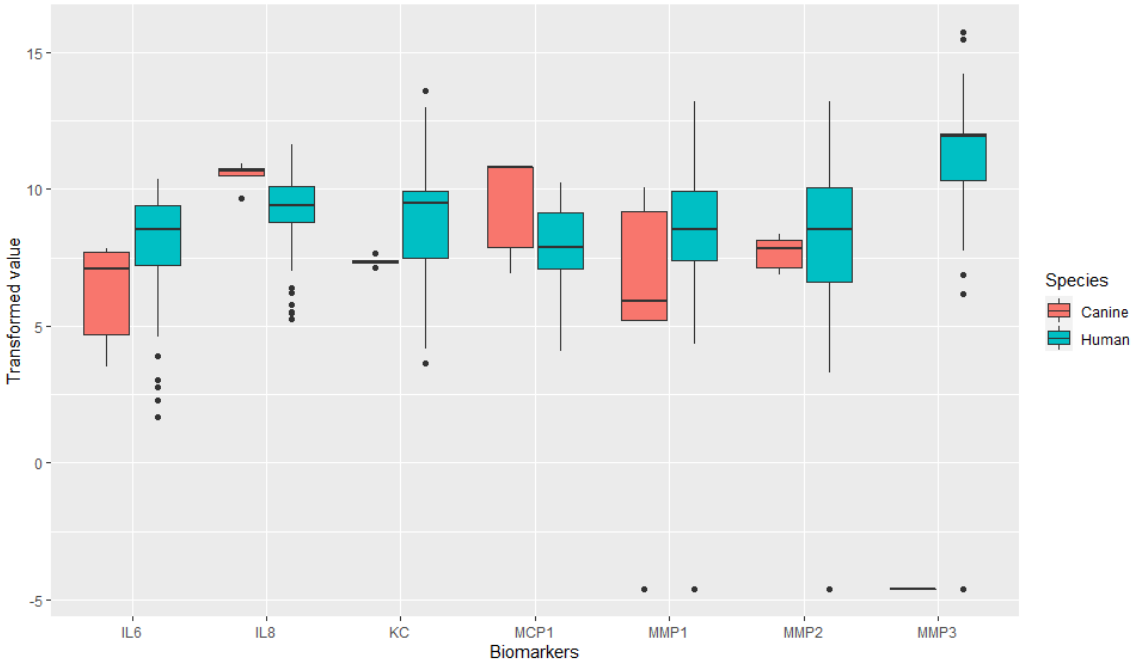

Figure 8: Transformed biomarker value by species in cytokine group

Logarithmic Biomarker	Independent Variable	Coefficient Estimate	Std Error	P-value	Benjamini-Hochberg critical value	Significance
log (IL6)	Species	-2.02	1.57	0.2086	0.0002	No
log (IL8)	Species	-3.44	1.05	0.0024	<0.0001	No
log (KC)	Species	-0.79	2.31	0.7345	0.0006	No
log (MCP1)	Species	-3.62	1.19	0.0046	<0.0001	No
log (MMP1)	Species	-0.32	3.32	0.9243	0.0008	No
log (MMP2)	Species	3.69	4.21	0.3865	0.0003	No
log (MMP3)	Species	16.65	1.68	<0.0001	<0.0001	No

Table 6: Regression model results for comparing biomarkers between dogs and humans

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Chapter 6: Comparison of human IVDs from donors without history of back pain and IVDs from patients undergoing surgery for IVDD-related back pain

Introduction

Intervertebral disc (IVD) disorders or degeneration associated with low back pain comprise a significant global healthcare burden.[1] While there are several factors involved in low back pain generation and disability, IVD degeneration has been strongly associated with symptomatic disease inpatients. Cell and extracellular matrix changers in cartilaginous end plate (CEP), annulus fibrosus (AF), and nucleus pulposus (NP) drive the degenerative process. Notochordal cells in NP gradually decrease[3] into adolescence such that very few, if any, notochordal cells survive into the adulthood.[4] The NP gets repopulated with chondrocyte-like cells (CLCs) and undergoes associated morphologic and structural changes to become a white to tan, opaque, and fibrotic tissue. AF cells likely contribute to this pathologic process.[5],[8,9] Cellular and structural changes in IVDs are age-dependent[6] and even normal adult human IVDs contain mostly large amount of extracellular matrix (ECM) with relatively low cell density.[7] Cell and matrix alterations can result in disc protrusion or extrusion causing inflammation, pain, and neurologic deficits. Continued progression and increasing involvement of additional discs and structures are common in IVDD.[10]

Current diagnostic tools are limited to tissue-level morphologic changes present at the time of symptomatic disease. Prognostic imaging studies are able to detect pre-symptomatic evidence of degeneration, but have not translated well to prediction of disease progression and associated clinical signs.[11] There have been reports of serum biomarkers that correlate to back pain[12,13], however, these biomarkers have not been validated for clinical application in screening, staging, or monitoring symptomatic IVDD. As such, there is a major unmet need in healthcare with respect to tools that can effectively screen, diagnose, stage, and prognosticate for degenerative disc disorders that cause low back pain in millions of individuals every year.

This study was designed to investigate and delineate biomarkers that are associated with symptomatic lumbar IVDD requiring surgery. We hypothesized that 1) IVDs from donors without known history of back pain would produce similar levels of biomarkers associated with structural degradation and remodeling, but will produce lower levels of biomarkers associated with inflammation compared to the abnormal IVD tissues recovered during surgeries, and 2) IVDs from donors without known history of back pain would have more pronounced responses to increased pro-inflammatory stimuli compared to the surgical tissues.

Materials and Methods

Donor IVD Sample Preparations

Midwest Transplant Network (MTN) screened and selected organ and tissue donors (n=7, mean age 47.1 yrs, 4 female) without known history of back pain. With donor

family consent, spines were recovered and donated to Thompson Laboratory for Regenerative Orthopaedics (TLRO) for research purposes. Radiographs were obtained from the donated spine segments to identify and confirm IVD levels. Then the spine segments were aseptically processed to isolate individual IVDs. During dissection and IVD isolation, Thompson grades were assigned and documented.[14–16] Depending on availability, L3-4 or L4-5 or L5-S1 IVD was enrolled in this study. From these IVDs, AF and NP explants were created using 6mm dermal biopsy punches.

Surgical IVD sample preparations

With Institutional Review Board approval (#201692), surgical IVD tissues were recovered during surgical interventions by the spine surgeons at Missouri Orthopaedic Institute (MOI). This project did not influence the medical decision making of the surgeons. The surgical tissues would otherwise be discarded. Explants of the submitted surgical IVD tissues were created as described above. Tissues from age, BMI, Pfirrmann grade, and IVD level matched subjects (n=14, mean age 49.8 yrs, 6 female) were used for study.

Culture and media analyses

The donor and surgical IVD explants were placed in supplemented DMEM at 37˚C and 5% CO2 for 3 days with or without 10 ng/ml of recombinant human IL-1β. After days, media were collected for biomarker analysis and tissues were weighed to determine tissue wet weight. Media from day 3 of culture were tested for PGE_2, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, TIMP-1, TIMP-2, TIMP-3, TIMP-4, GRO-α, MCP-3, PDGF-AA, PDGF-AB/BB, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, PGE_2, and

VEGF using commercially available assays according to the manufacturer's protocol. Biomarker levels were normalized to tissue wet weight for analysis.

Tissue protein extraction

The donor AF explants were pulverized using liquid nitrogen and BioPulverizer (BioSpec). The NP tissues were not pulverized as NP tissues were less fibrous. Instead of pulverization, the NP tissues were cut into smaller pieces (~ 2mm diameter) using sterile scalpel blades. AF and NP tissues were processed in metal tubes and 4-5 chromium beads and with cOmplete Lysis-M (Roche) protein extraction buffer (750 µL and 500 µL respectively) with Mini Protease Inhibitor Cocktail Tablet in a bead beater for 5 minutes. The lysates were centrifuged at ~14,000 x *g* for 10 minutes. The resulting supernatant was transferred to new clean tubes.

Data and statistical analysis

For statistical analysis, surgical tissues were separated into groups based on pre-operative MRI Pfirrmann grades, with a grade of 3 classified as 'Mid' Pfirrmann (n=7, mean age 47.9, 4 female) and grades of 4 and 5 classified as 'High' Pfirrmann (n=7, mean age 51.7, 2 female). Donor cadaveric IVDs with a Thompson grade ≥3 was considered degenerated and used for analysis (n=7, mean age 47.1, 4 female). For cytokine stimulation study, the % differences after cytokine treatment were calculated using 'quantmod' package in Software R 3.6.2. Significant differences between groups were determined by Kruskal-Wallis. In order to control false discovery rate, the Benjamini-Hochberg (BH) procedure was used to adjust for the multiple testing p-

values. The false discovery rate was set to 0.05 in this study. The null hypothesis would be rejected when the p-value was less than its Benjamini-Hochberg (BH) critical value which was calculated by using 'FSA' and 'rstatix' packages in Software R 3.6.2.

Results

Aim 1: Comparisons of released biomarkers from healthy donor and abnormal IVD tissues

Surgical and donor IVD tissues produced detectable levels of PGE2, GRO-α, IL-6, IL-8, MCP-1, MCP-3, MIP-1α, MIP-1β, and RANTES (Figure 1). The production levels of RANTES, IL-8 and PGE2 by the mid and high Pfirrmann grade groups were significantly higher than by donor AF and NP tissues. The production of MIP-1α by the mid Pfirrmann group was significantly higher than by donor AF and NP tissues. Surgical and donor IVD tissues produced detectable levels of VEGF, PDGF-AA, and PDGF-AB/BB (Figure 2). The production of VEGF in the mid and high Pfirrmann groups was significantly lower than in donor AF and NP tissues. The production of PDGF-AB/BB was significantly higher in the mid and high Pfirrmann groups compared to donor AF tissues. Surgical and donor IVD tissues produced detectable levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13 during culture (Figure 3). The production of MMP-9 was significantly higher in the mid and high Pfirrmann grade groups compared to donor NP and AF tissues. Surgical and donor IVD tissues produced detectable levels of TIMP-1, TIMP-2, TIMP-3, and TIMP-4 during culture (Figure 4). The mid and high Pfirrmann grade groups produced significantly lower TIMP-2 and TIMP-4 compared to donor AF and NP tissues.

Aim 2: Comparisons of responses to cytokine stimulation by healthy donor and abnormal IVD tissues

IL-6 production changes for donor AF were significantly higher than for mid Pfirrmann tissues after cytokine treatment. IL-8 production changes for high Pfirrmann tissues were significantly higher compared to changes for mid Pfirrmann tissues, but significantly lower compared to changes for donor NP tissues. The donor AF responses were significantly higher compared to mid Pfirrmann tissue responses to cytokine stimulation. MCP-1, MCP-3, GRO-α, MIP-1α, MIP-1β, RANTES, and PGE2 biomarker responses were similar across tissue types (Figure 5). The percent changes for growth factors, matrix metalloproteinases, inhibitors of tissue matrix proteinases after cytokine treatments were similar across tissue types (Figures 6, 7, and 8).

Aim 3: Comparisons of tissue biomarkers for healthy donor versus abnormal IVDs

Surgical and donor IVD tissues produced detectable levels of GRO-α, IL-8, MCP-1, MCP-3, RANTES, PGE2, VEGF, PDGF-AA, and PDGF-AB/BB (Figure 9). The tissue concentrations of RANTES and PDGF-AB/BB by the mid and high Pfirrmann grade groups were significantly higher compared to donor IVDs. The tissue concentration of VEGF in the high Pfirrmann grade group was significantly lower compared to donor AFs. The tissue concentration of MCP-1 in the mid Pfirrmann grade group was significantly lower Surgical and donor IVD tissues produced detectable levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13 (Figure 10). The tissue concentrations of MMP-9 in the mid and high Pfirrmann grade groups were significantly higher compared to donor

AFs. Surgical and donor IVD tissues produced detectable levels of TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Figure 11). The tissue concentrations of TIMP-4 in the mid and high Pfirrmann grade groups were significantly lower compared to donor NPs, and the tissue concentration of TIMP-4 in the high Pfirrmann grade group was significantly lower compared to donor AFs.

Discussion

The results show biomarker differences between IVD tissues from non-symptomatic and symptomatic IVDD and rejects the null hypotheses. The basal level biomarker production data indicate potentially important differences in the inflammatory and degradative metabolic responses of degenerative IVD tissues obtained from painful patients compared to those obtained from organ and tissue donors with no history of back pain. These data indicate that IVD tissues obtained from symptomatic patients produced significantly higher levels of pro-inflammatory biomarkers, IL-8, RANTES, MIP-1α, and PGE_2, when compared to asymptomatic IVD tissues. These differential pro-inflammatory responses may be indicative of mechanisms that promote the pain that is associated with symptomatic IVDD in patients indicated for surgery. The data from the present study also elucidate a potential role for neovascularization in IVDD. The increased VEGF production by asymptomatic IVDD tissues suggest a potentially effective repair response or new vessel formation which can potentially lead to further degeneration and pain, while the increased PDGF-AB/BB production in symptomatic IVDD patients may signal ineffective repair and remodeling and more advanced stages

of neovascularization and severe inflammation and degeneration. The major difference between VEGF and PDGF-AB/BB is that VEGF modulates new vessel formation while PDGF-AB/BB stimulates vessel growth on existing vasculature. Vascularization of inner structures of IVD is one of characteristics of degenerating IVDs. It is interesting to find lower PDGF-AB/BB in the IVD tissues from non-symptomatic IVDD which is likely due to the fact that non-symptomatic IVD tissues have less vascularization. The significant decrease in TIMP production noted may be a factor in the shift towards a more degradative phenotype that results in pain, dysfunction, and the need for surgery.

In order to better understand responses to cytokine stimulation by each group, the % differences were taken instead of comparing the raw values of biomarker levels because the % differences would directly indicate the changes with increased inflammation. Based on this method of analysis, the majority of biomarkers show similar % changes except for IL-6 and IL-8. The percent change of IL-6 and IL-8 biomarker production by the IVD tissues from non-symptomatic IVDD were significantly higher than the degenerative IVD tissues from the surgical patients. IL-6 and IL-8 have both been implicated in inflammatory responses involved in disc degeneration as well as in pain pathways. These findings suggest that healthy IVDs may be better able to mitigate pro-inflammatory insults that drive degeneration and pain compared to herniated abnormal IVDs. Interestingly, healthy donor AF and NP responded to cytokine stimulation in very similar ways based on biomarker profiles, suggesting that the NP becomes metabolically similar to AF during aging even without evidence for symptomatic IVDD.

The results of this study provide novel data indicating potentially important differences in tissue levels of inflammatory and degradative biomarkers in degenerative IVDs recovered from clinical patients seeking treatment for symptomatic disc disease compared to those recovered from organ and tissue donors with no history of back pain. The increased level of RANTES in IVD tissues from symptomatic IVDD patients compared to asymptomatic donors, may indicate a role for the infiltration of specific immune cells during development and progression of symptomatic IVDD. Further, the lower levels of VEGF and higher levels of PDGF-AB\BB in the high Pfirrmann grade samples compared to the asymptomatic donors may indicate a role for neovascularization during IVDD, where stimulation of vascular infiltration occurs during asymptomatic degeneration (as indicated by VEGF). Whereas in symptomatic IVDD patients, neovascularization may have already progressed to a point to allow for hypervascularity (as indicated by PDGF-AB\BB). Finally, the data from this study point to a pathomechanism involving loss of degradative enzyme regulation associated with progression of IVDD and the development of clinical signs. The significantly lower levels of TIMP-4 in tissues from symptomatic IVDD patients may point to degradative enzyme activity imbalance fostering progressive degradation of the disc leading to pain, dysfunction, and need for surgery.

Conclusion

In this series of experiments and analyses, unique IVD biomarker profiles and expressions were identified for non-symptomatic versus symptomatic human IVDs. The data from this study provide initial characterization of inflammatory and degradative responses from IVD tissues, as well as tissue-level inflammatory and degradative biomarkers that differentiate symptomatic from asymptomatic intervertebral disc degeneration. While the majority of responses to cytokine stimulation were similar between IVD tissues from non-symptomatic and symptomatic specimens, inflammatory responses were heightened in non-symptomatic patients. The unique capabilities for comparing IVD tissues from symptomatic patients with those from organ and tissue donors with no history of IVDD-related pain provide direct clinical relevance for the data such that novel diagnostic, prognostic, disease staging, and treatment monitoring biomarker panels can be developed towards optimizing management of patients with IVDD and low back pain.

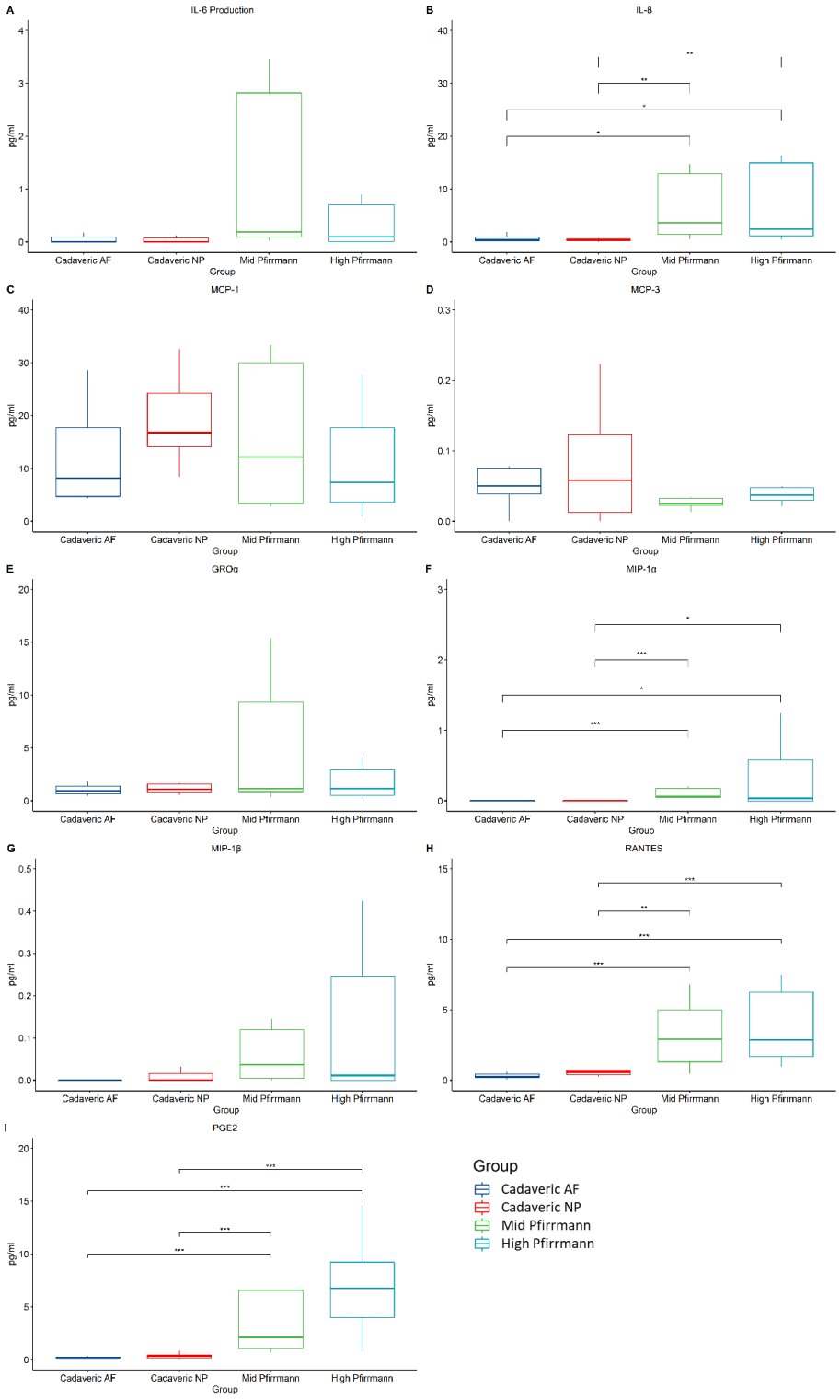

Figure 1: Boxplots for pro-inflammatory biomarker production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01. ***: p ≤0.001.

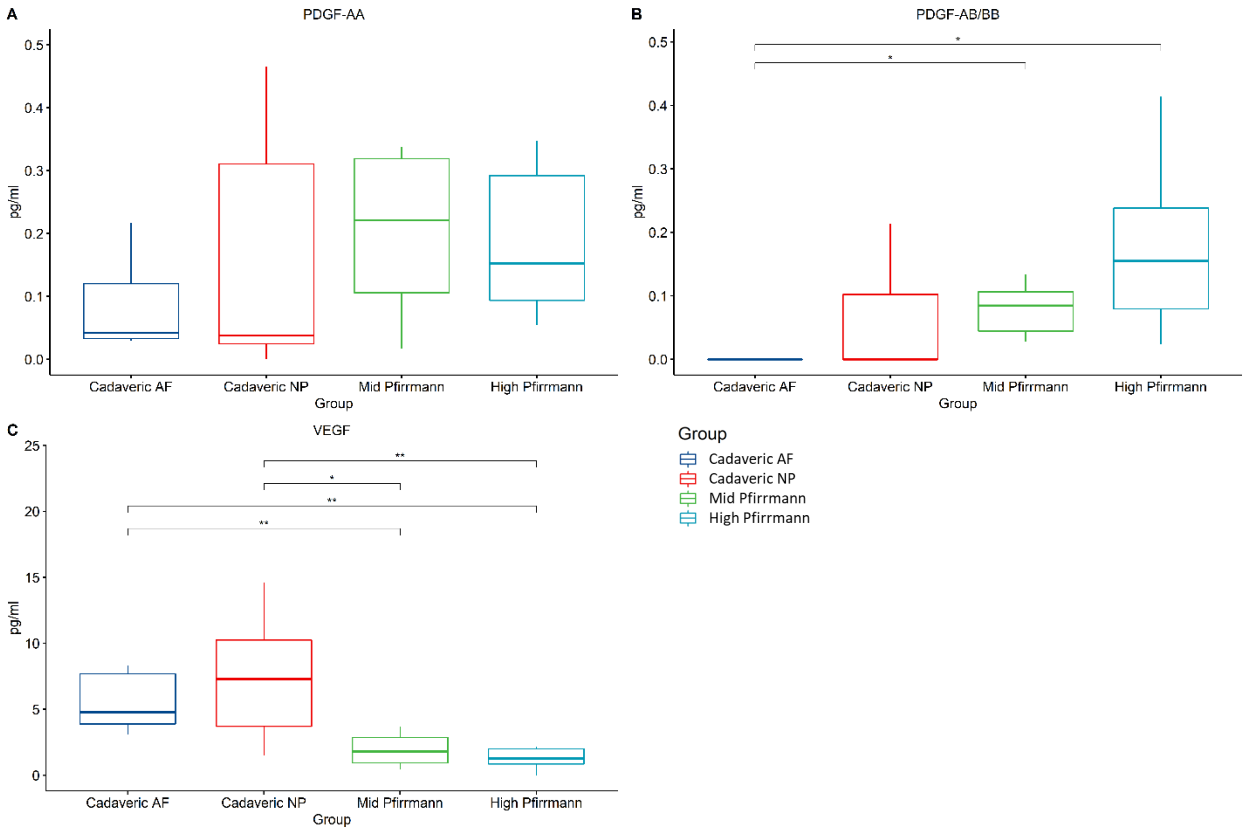

Figure 2: Boxplots for growth factor biomarker production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) during ex vivo culture for 3 days.
*: p≤0.05. **:p ≤0.01. ***: p ≤0.001.

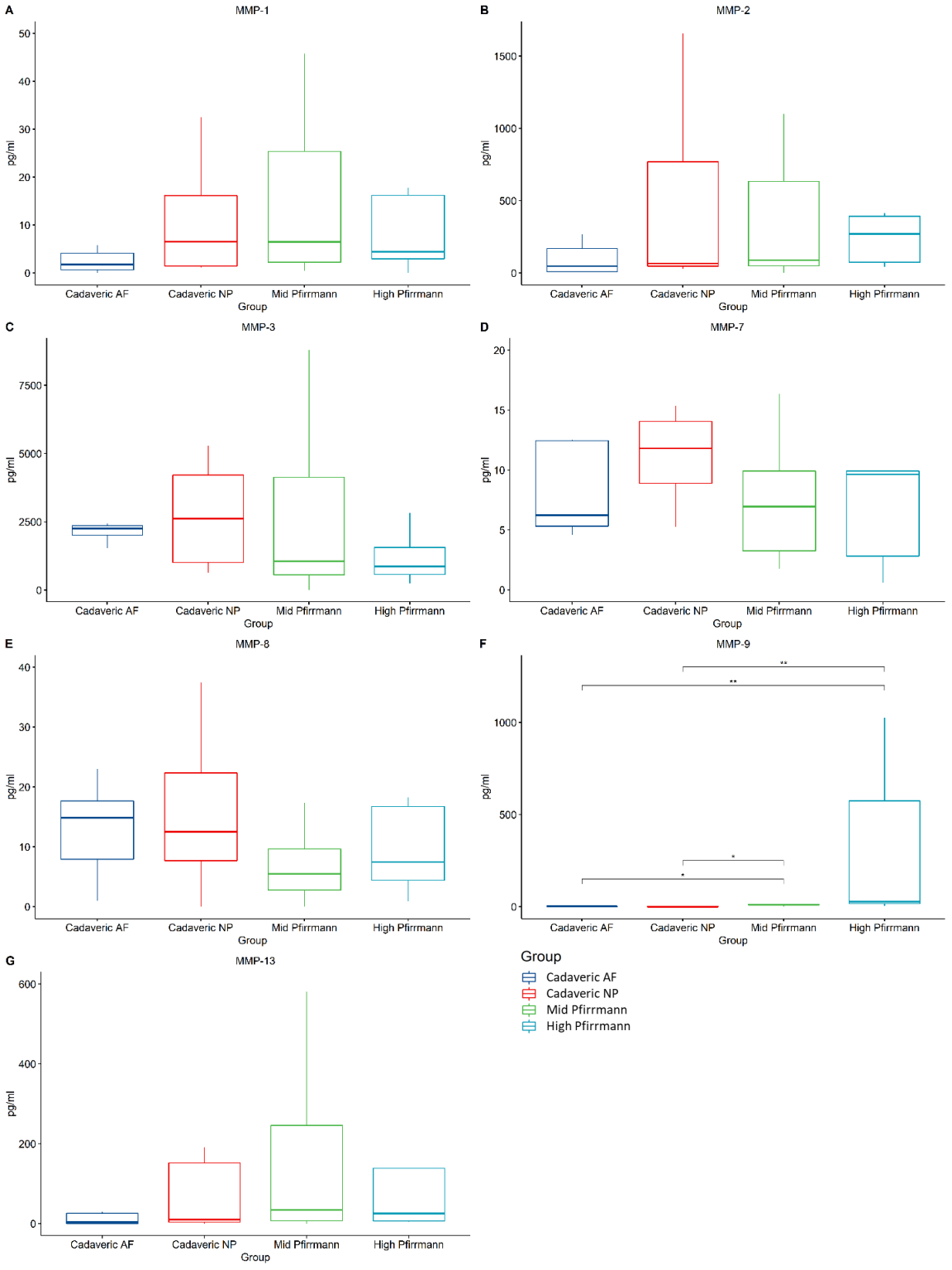

Figure 3: Boxplots for matrix metalloproteinases production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01.

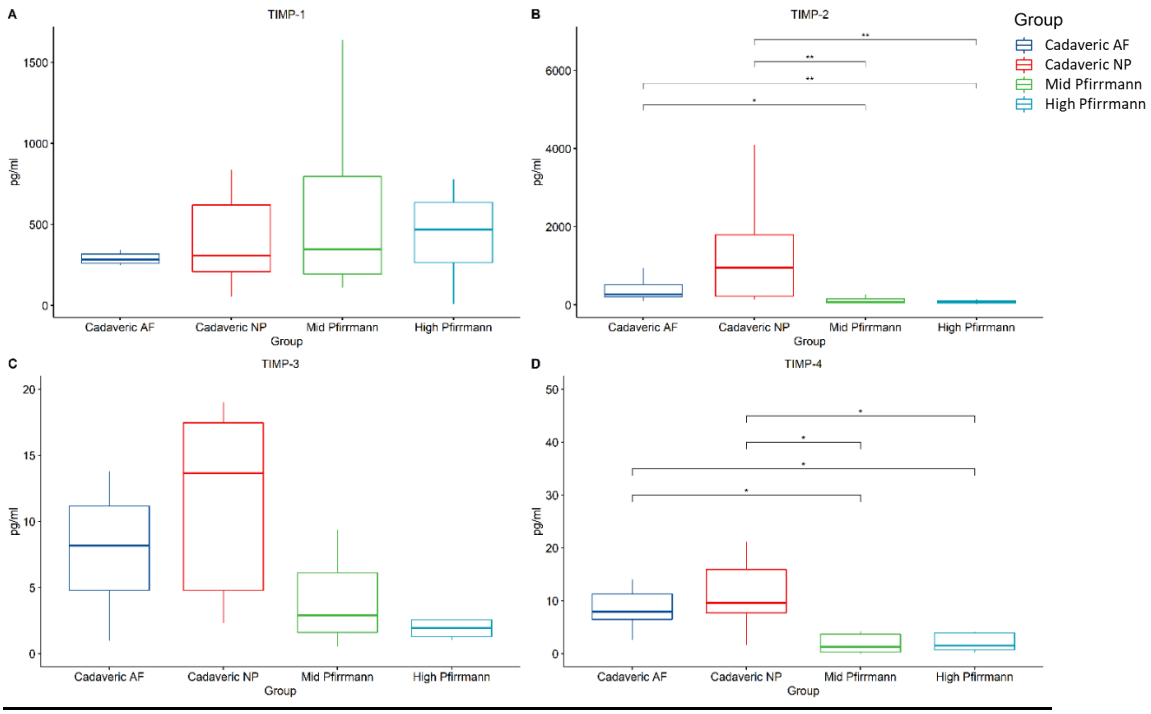

Figure 4: Boxplots for inhibitors of matric metalloproteinases production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) during ex vivo culture for 3 days.
*: p≤0.05. **:p ≤0.01.

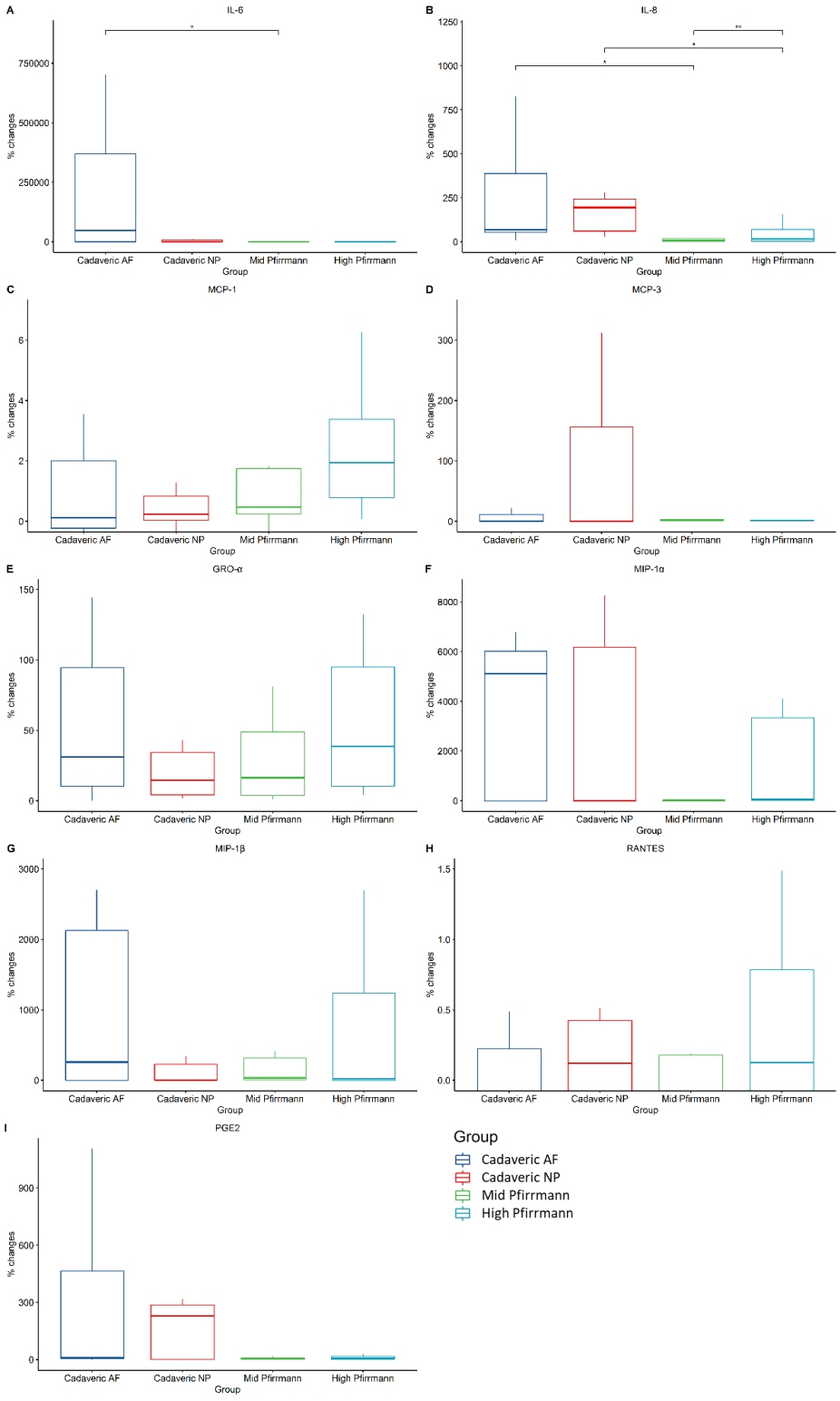

Figure 5: Boxplots for pro-inflammatory biomarker production % change by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) with cytokine stimulation during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01. ***: p ≤0.001.

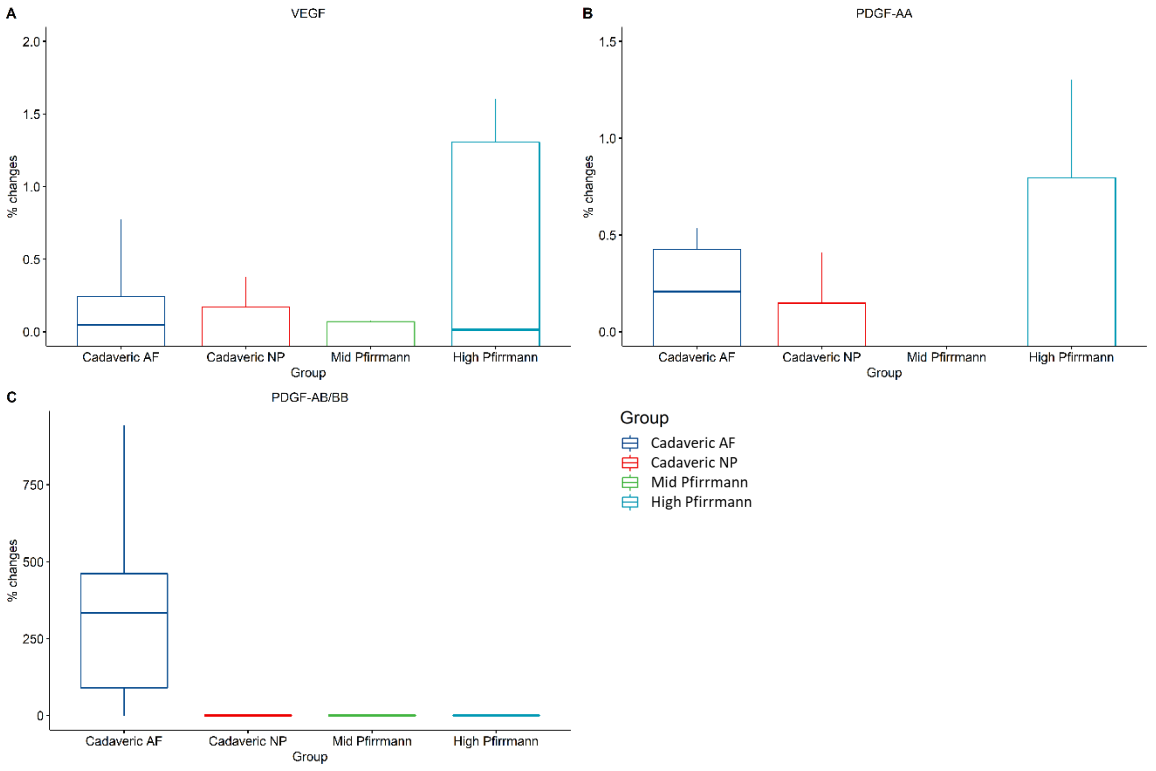

Figure 6: Boxplots for growth factor biomarker production % change by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) with cytokine stimulation during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01. ***: p ≤0.001.

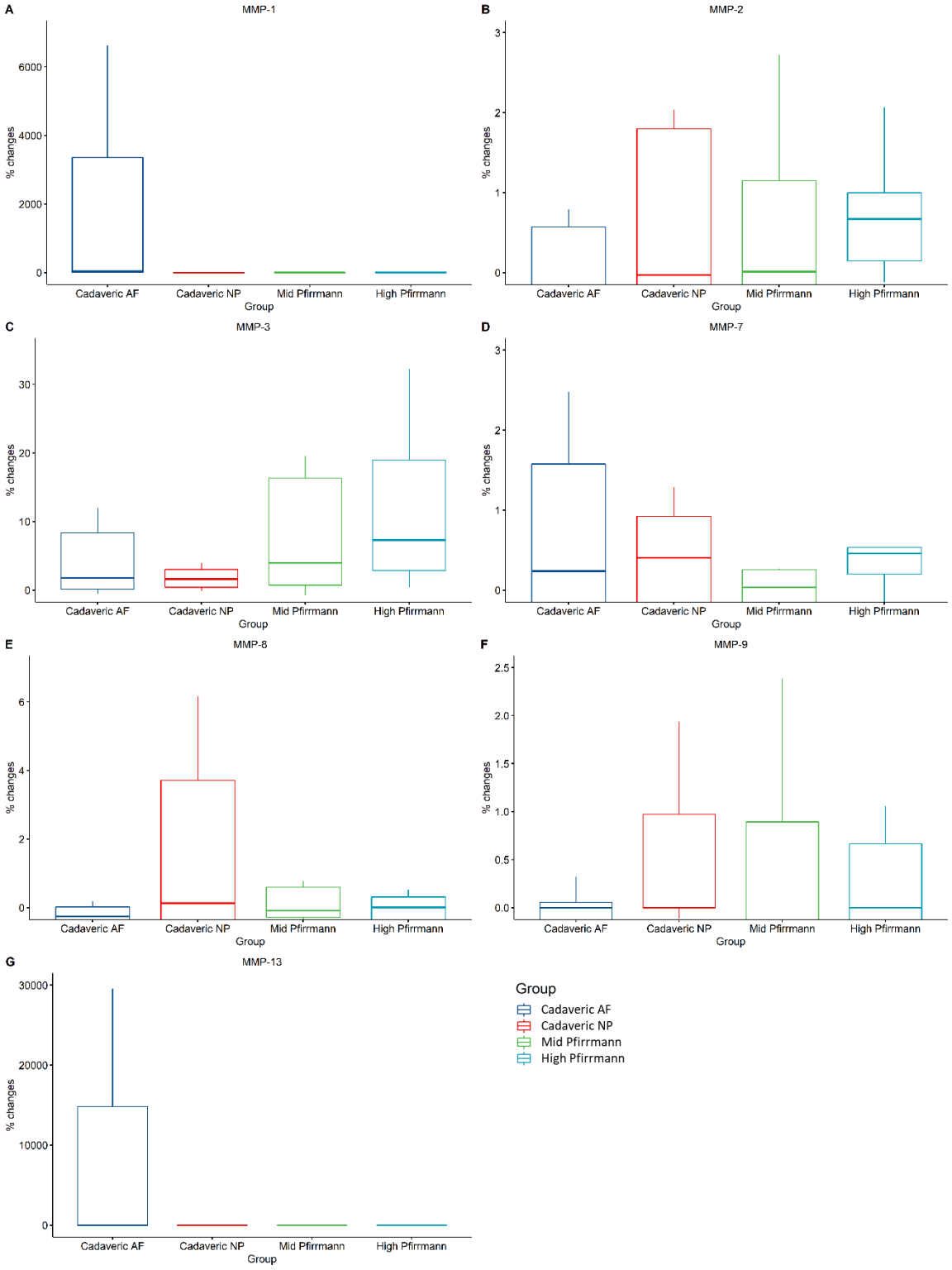

Figure 7: Boxplots for matrix metalloproteinases production % change by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) with cytokine stimulation during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01.

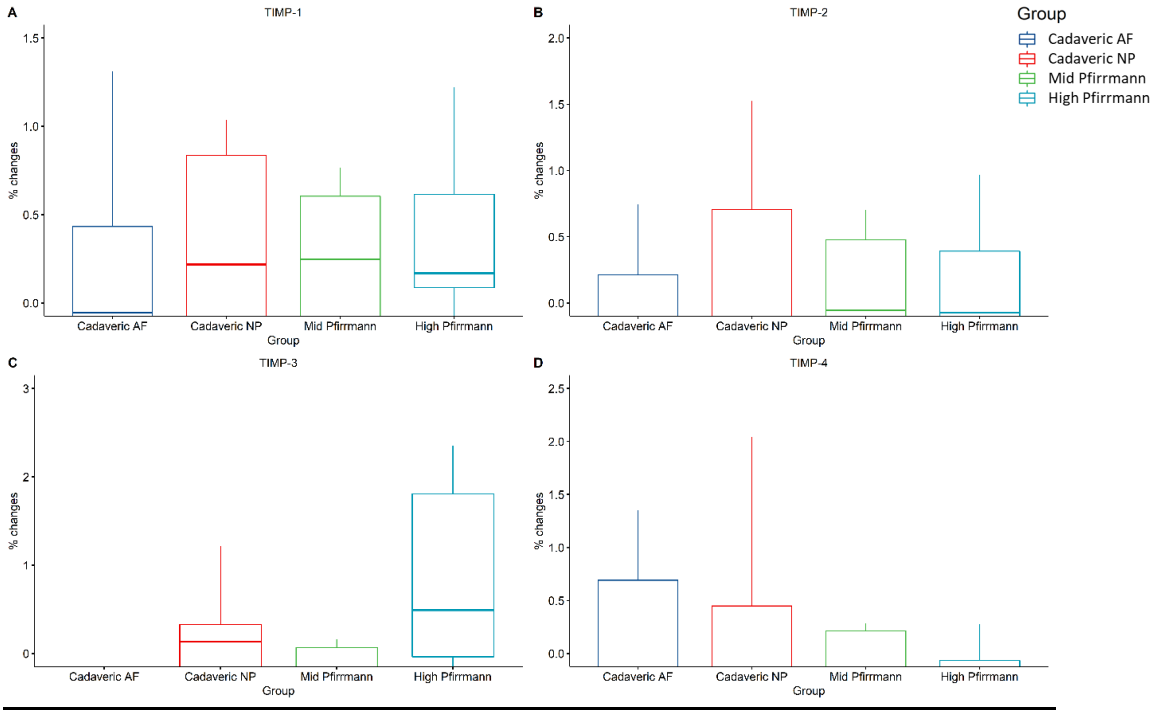

Figure 8: Boxplots for inhibitors of matric metalloproteinases production % change by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann) with cytokine stimulation during ex vivo culture for 3 days. *: p≤0.05. **:p ≤0.01.

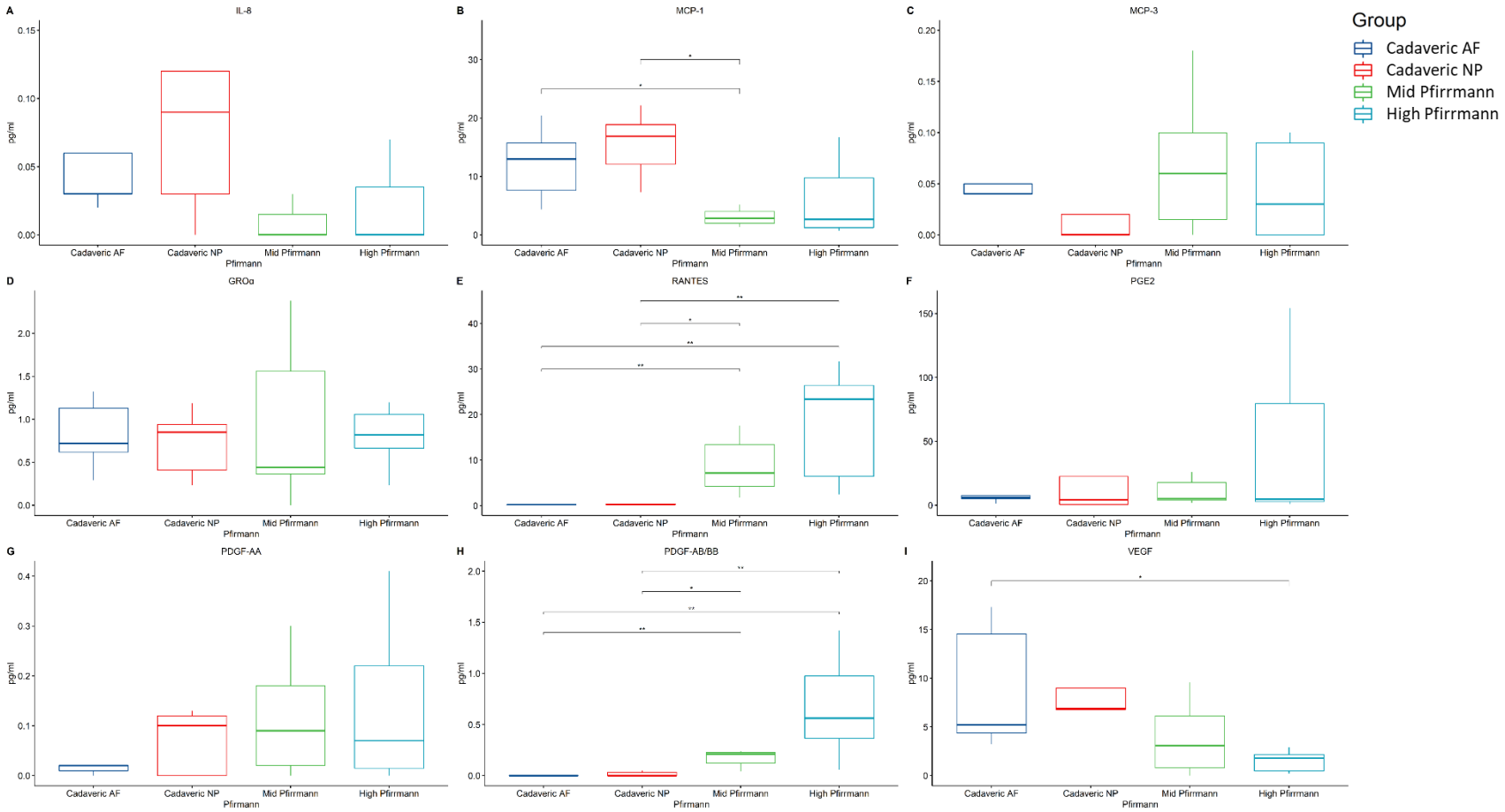

Figure 9: Boxplots for pro-inflammatory biomarker production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann). *: p≤0.05. **:p ≤0.01.

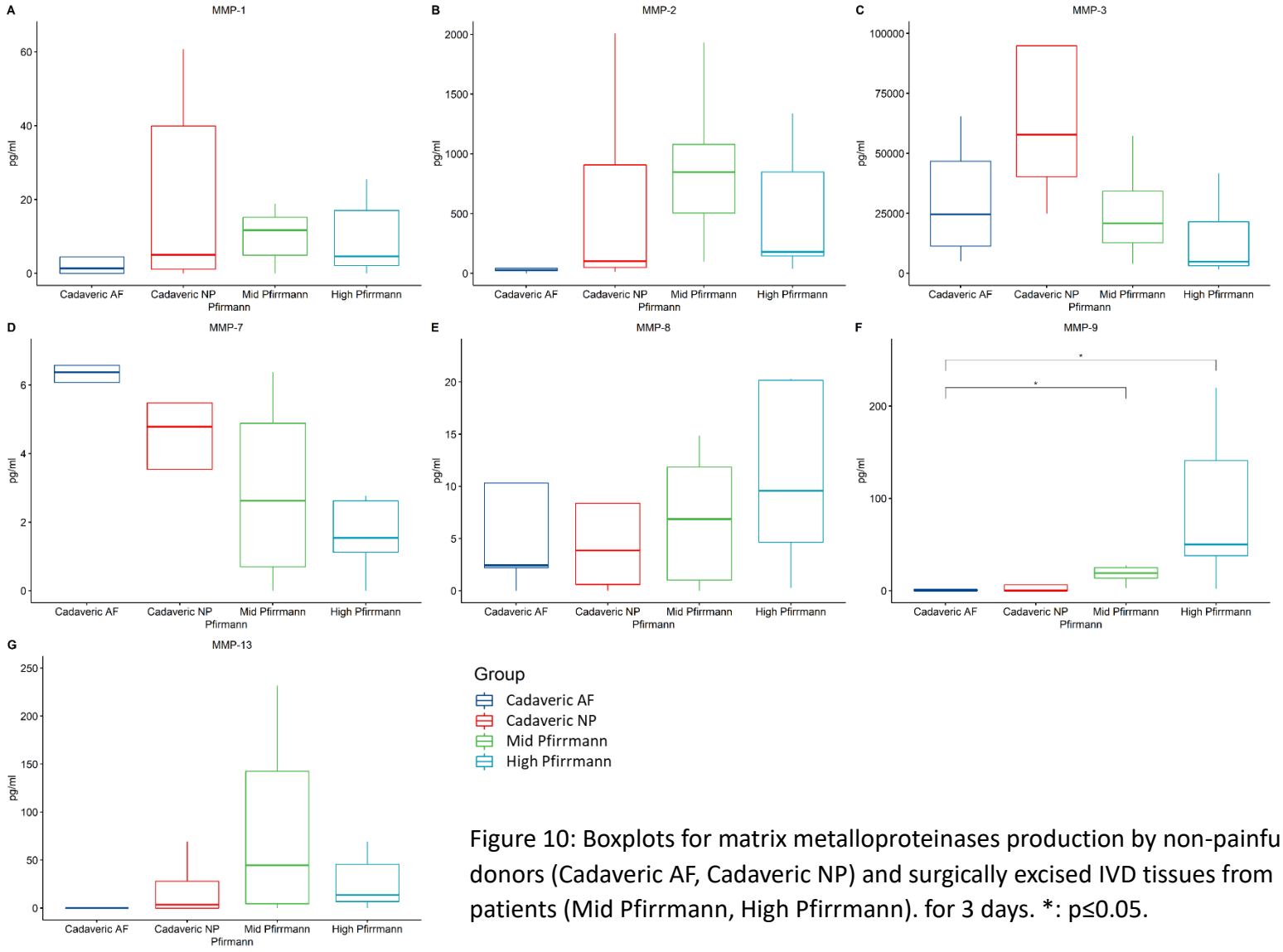

Figure 10: Boxplots for matrix metalloproteinases production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann). for 3 days. *: p≤0.05.

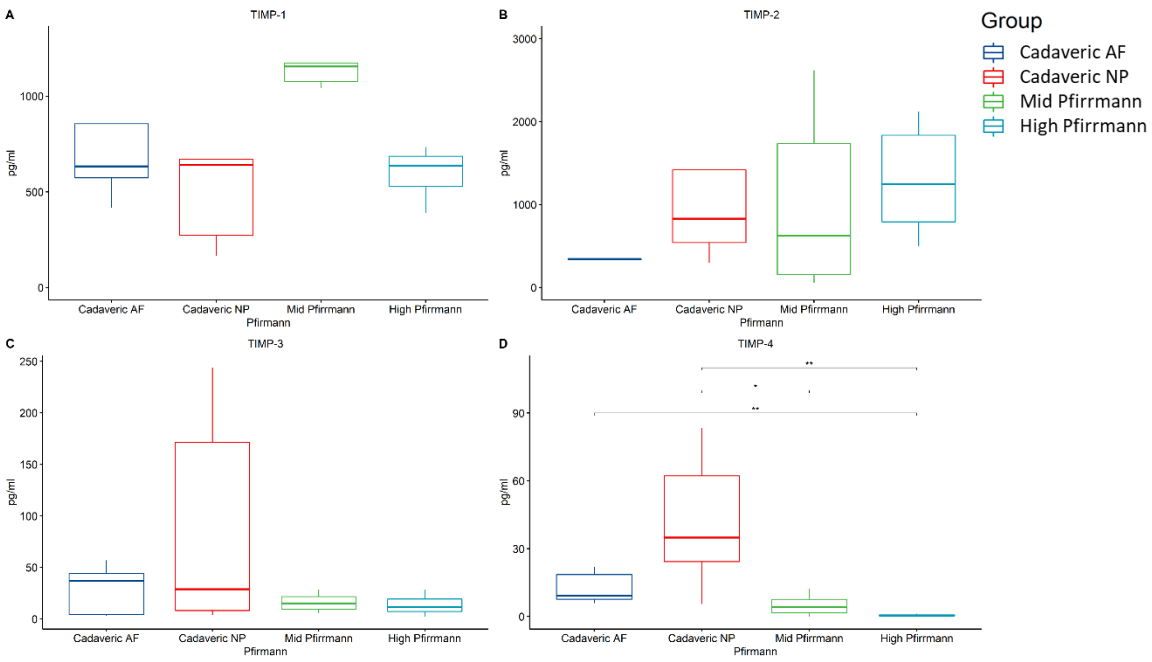

Figure 11: Boxplots for inhibitors of matrix metalloproteinases production by non-painful donors (Cadaveric AF, Cadaveric NP) and surgically excised IVD tissues from patients (Mid Pfirrmann, High Pfirrmann). *: p≤0.05. **:p ≤0.01.

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VITA

Dr. Naomi Lee was born in Seoul, South Korea on October 18, 1987 to Byung Hwan Lee and Hyun Pok Lee. She grew up in Ilsan, South Korea until the age of 15 then she spent six months in Cambridge, Great Britain. Dr. Lee moved to Irvine, California with her family where she attended University High School. She graduated from University of California, San Diego with her Bachelor of Science degree in Physiology/Neuroscience in 2011. After her undergraduate studies, she attended Colorado State University for her Master of Science in Environmental Health (Toxicology) and Doctorate of Veterinary Medicine. After her veterinary medicine training in 2016, Dr. Lee matched with Comparative Medicine Program at University of Missouri for her laboratory animal medicine residency training, which she completed in July 2019. In December 2017, she joined Thompson Laboratory for Regenerative Orthopaedics to pursue Doctor of Philosophy in Pathobiology under the guidance of Dr. James Cook. As for the plan after her PhD, Dr. Lee has accepted a faculty position as an Assistant Research Professor/Clinical Veterinarian at City of Hope in Duarte, California and hopes to contribute her training and grow her knowledge in ethical laboratory animal care, preclinical model developments, and biomedical research.